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REVIEWS

EXPERIMENTAL ARTICLES

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INFORMATION COMPUTER TECHNOLOGIES FOR USING IN BIOTECHNOLOGY: ELECTRONIC MEDICAL INFORMATION SYSTEMS

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The purpose of the study was to review mordern information systems elaborated for medicine and biotechnology. Classifications of modern medical information systems and detailed description of their different versions based on numerous publications are given. Electronic information system for the biochemical laboratory with individual patients' data protection was developed for the hospitals in Polissia region of Ukraine polluted due to Chornobyl accident is suggested.

Key words: bioinformatics, electronic medical information systems.

Information computer technologies are widely used in contemporary biotechnology, which needs more specialized electronic information systems. The importance of information technologies (IT) and computer technologies (CT) use in modern biotechnology is indisputable fact nowadays. Primary there were no elaborated electronic information systems (IS) with databases (DB) developed specifically for biotechnology — it was economically unjustified [1, 2]. Constructing technical information systems (tIS) with DB, IT professionals could use wide spectrum of mathematical methods [1–9]; they could take prototypes from physics and techniques, because tIS were elaborated primary for these spheres. Later such tISs were accepted from medicine, biology, chemistry and etc.; they had to be modernized and adapted for biotechnological tasks solution. Because biological sciences and technologies have their peculiarities, professionals need their own electronic IS with DB. So, for the construction of tIS for biotechnology we suggested to study the experience of medical tIS elaboration because of the following reasons. 1 — medicine is one of the nearest spheres for biotechnology that demonstrates the great variety and quality of IS because of good finding (people take care about their health ever); 2 — for biotechnologists (as well as other professionals) there were not important from what IS to get the data for their work, if only this data were. Indeed, for the research work the real scientific data are necessary, independently on the source of their origin (in medical tIS there are lots of necessary data); 3 — therefore, for the construction of similar tISs for biotechnology, one needs to know the prototypes from medical tISs to develop the better ones, similar and even more suitable systems; 4 — in our originally constructed tIS, described at the end of this article, a biochemical electronic laboratory is added. According to this example, one can add to tIS all necessary segments: for biotechnology, and other ones; 5 — biotechnological applications in medicine are very important; 6 — finally, it is very useful for engineers in biotechnology to know the different types of medical tISs as prototypes for the success of their work. To give to professionals in biotechnology the possibilities to invent new IS versions more effectively, we decided to observe some medical tIS prototypes.

Diversity and variability of medical information systems. According to abovementioned, the types of medical

information systems were numerical and diverse in their construction. They were the most numeric in medicine in comparison with biology due to a good funding in medicine and health protection through over the world. Mathematic models that we described in our previous articles as well as published by other authors also may be used for ISs functioning or to be simulated in result of their functioning [9–79]. A spectrum of mathematic methods is used for the newest biomedical ISs elaboration [1, 11, 74, 76–140]. Databases content described in this article was obtained from the results of biological and medical observations and experiments [10, 12–17, 22–44, 47–49, 61, 68, 71–74, 79–85, 90, 93, 94, 104, 107, 109, 110, 113, 120, 126–148]. Indeed, all such tISs are network-based and linked with databases today [1–11, 25–71, 90–109, 112–119]. In our numerical previous publications [1–6, 18–37, 61, 62, 64–66, 75, 126–148] we have investigated repeatedly the various aspects of below described phenomena and processes.

Medical tIS described below demonstrated a great variety; we elaborated and offer their classification. Information for this classification we found in different scientific and technical sources published since 2000. In our list there are contemporary electronic databases with access to the Internet with information in biology and medicine, designed during the last 25 years.

Despite the diversity of such systems (and, accordingly, publications), it was possible to distinguish certain well-defined types of ISs among them. It is necessary to emphasize that such classification in finished form did not exist in scientific and technical literature until 2008; so, it is original.

Thus, in medical IS with DB one could distinguish: medical ISs of general purpose, expert systems, electronic systems for working with images, electronic systems for working with medical documents, systems for scientific purposes, library medical systems, electronic educational systems in medicine, electronic medical databases

Placing these ISs types in hierarchy, we followed the principle: the more publications contain modern scientific and technical sources about this type of systems — respectively, the higher its name is in our list. It means that the developers payed the most attention to them; consequently, they were the most popular in practice (in modern hospitals, laboratories and etc.) Apparently, the most often published works fall into the section “Medical IS of general purpose”. This could be explained by the practical needs of clinical medicine, which requires the most such versions. When viewed this list from the top to the end, the number of publications corresponding to one specified type of the system decreases, and in the section “Electronic medical databases” falls to the lowest number of publications. This does not mean that electronic medical DB were not important in medical practice. Such a pattern can also be explained by the fact that the procedure for creating of such databases is standard, described in university textbooks, and perhaps the developers of such databases do not see them as

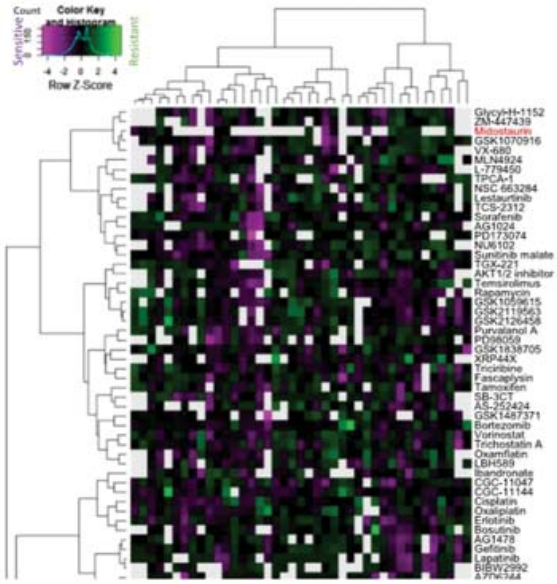


Fig. 1. **Use of informatics in medicine: two-dimensional hierarchical clustering of drugs based on similarity of anti-proliferation effect profiles on breast cancer cell lines [73]**

novelty, required for scientific publications. Our proposed examples of classification of the main IS types for biology and neurophysiology (as a branch at the junction of biology and medicine) were described in our previous publications [1]. Result of the use of medical "systems for scientific purposes" is illustrated on Fig.1. In result of contemporary informatics' methods use, two-dimensional hierarchical clustering of drugs based on similarity of anti-proliferation effect profiles on breast cancer cell lines was done as well as, in result, the conclusion about midostaurin action. Midostaurin suppressed the proliferation of TNBC cells among the breast cancer cell lines presumably through the inhibition of the Aurora kinase family. The precise study of midostaurin on cell growth would contribute to the development of the drug for the treatment of TNBC using computer technologies.

Some theoretical approaches for the construction of the largest groups of medical electronic tISs. As as it was noted, the most recent publications in the world according to our classification show that the most numerical ones are: "Medical IS of general purpose" and "Medical expert systems". Let's observe the information of these sections, which explains this fact.

Some theoretical data about the general-purpose medical information systems. Today the Internet is used widely in clinical practice, medical research and education. Examples of its applications were, for instance, telemedicine, the collection and accumulation of clinical and experimental data, the making of electronic collections of professional literature, analysis of DNA and protein sequences of patients and distant learning for some medical courses, and etc. All of these applications required the development of electronic databases. The first medical ISs appeared in the 1970-th, and their main features, according to their performed functions, became established until the mid-1990-th. The "traditional" core of medical ISs was: clinical decision-making system, clinical record system (including patient records) and clinical databases [112]. During the first years of the XXI century, medical ISs demonstrated a real upswing in their development due to the numerous new technologies that have emerged in those years. In [78, 112] it was shown that the modern Internet provides opportunities for patients, their families, physicians to communicate with each other, to study new medical information and to exchange it. For these purposes there were established the centers for patients; these centers were equipped with electronic ISs, for example, the "eHealth system". It was calculated that the services of such electronic ISs in the USA during 2002 used, according to various estimates, from 73 to 110 million users (estimates were made for Medline (1966–2002), CINAHL (1982–2002), Cochrane Controlled Trials Register (till 2002), PsychInfo (1967–2002), and some others). The virtual capabilities of such systems were needed, in the first order, for people with disabilities and for lying patients.

Medical expert systems. The Internet provides good opportunities for the construction of powerful expert medical systems. In such systems, opportunities for the obtaining of information (OI) can be done rather cheaply, it is the best way for exchanging by large information volumes, to obtain variable information and even controversial one, from medical experts from different fields of medicine and from remote geographic regions. Clinical decision support systems (CDSS) have become used in medical practice more in recent years. The first such system, which has been widely used since 1970, has become the medical expert system MYCIN. After it, a number of systems were created for provision of access to medical information, interpretation of diagnoses, and so on. During these systems development two important problems were solved: choosing of methods for efficient system construction and data usage from the DB. For this 2 groups of methods were used — *automatic OI* and OI receiving in manual mode (*manual OI*).

The method of *automatic OI* (also called "Knowledge Discovering" and "Data Mining" — "knowledge acquisition") was relatively new method. His most important step was to extract abstract rules from a large number of cases. The most used automated OI methods were neural networks, discriminant and cluster analyzes, linear programming, evolutionary algorithms, and others [1–37, 41–148]. However, these methods were not considered as perfect due to the extremely complex algorithms that were not well developed. For example, during the data searching from the large DB, some data may be incorrect or not enough correct, and this would influence on the output rules. As a result, the huge efforts and expert time could be spent in a wrong way.

Consequently, most of the modern medical bases of knowledge refer to the *manual OI*, although knowledge bases designed for this method were usually small, referring to

very specific and relatively narrow areas of medicine. Manual OIs were elaborated usually in close collaboration with medical experts and engineers, sometimes it took a lot of time; it was important that the medical diagnosis is a complex cognitive process that medical experts are sometimes unable to formalize. Manual OI were not available everywhere, therefore, not all users outside of the health center could use these systems.

The Internet can solve these problems better than traditional platforms, since 1 — the Internet is widely available; 2 — Web browsers provide a common multimedia interface; 3 — for expert systems developed software can be obtained from the Internet; 4 — there are protocols for the interaction support between such expert systems. Experts can communicate online in real time; they can eliminate duplication of information, and etc.

"*Medical ISs of general purpose*". Let's observe some samples of electronic medical ISs. Some authors considered that ISs occurred spontaneously in hospitals and these systems need to be modernized in accordance with present day requirements. It is necessary to develop the general concepts of such systems and their modernization [1].

In [122] the principles of Web-system He@lthCo-op functioning have been described. It was noted that the healthcare industry is characterized by the need of close cooperation and information exchange between many professionals working to improve the health of patients at different times and often remote from each other in space. Modern IT and CT allowed us to make the systems through which such tasks can be solved. The He@lthCo-op modular Internet system has been constructed and implemented into practice, which makes possible the joint work of medical staff and transmission of confidential, protected information about the patient to remote sites. The He@lthCo-op system permitted to collect, maintain, and easily access patient information, at any time and from anywhere where access to the Internet was available. This function was not easy for implementation, since such information is completely heterogeneous, has different formats, it included not only medical data, but also personal and administrative information.

One of the main requirements for working with such data was to protect patient information in accordance with the current Health Insurance Portability and Accountability Act (HIPAA) that suppose friendly attitude of the nursing staff to patient's and their data. Based on this viewpoint, various medical institutions have tried to establish procedures for conducting of their own standardized patient records, and subsequently to elaborate a complete electronic records' databases for patients. For such service it was necessary to construct united IS, which would be easy to use in different geographically distant medical institutions, to enter it outside of medical facilities if the patient moved to another country. He@lthCo-op system [122] solved the same problems. Internet had provided the ability to develop such systems with different scenarios, on different software and hardware platforms. At the same time till 2010, only a few clinical establishments had well-adapted Web-based ISs for electronic patient records, which makes it difficult for doctors to work with them if they are outside of this center. IS designed for the purpose of health care, should operate in environment with many different types of users, for example, a medical institution, technical staff, nurses, doctors and patients by themselves. All these persons should exchange heterogeneous multimodal medical data, documents, other information, operate through the system simultaneously or at different moments of time, within a single medical center or at different geographical locations, and there must be a system of feedback between patients, doctors and administrators. For such functions realization the Web-based systems and database-based systems were suitable; they are highly flexible, can easily transfer information to final user, can provide a friendly interface, have developed set of services and navigation tools. The system He@lthCo-op had exactly these characteristics (Fig. 2).

In other publication [124] an electronic IS called "eMAGS" (Medical Agent System) was described. According to performing functions it was similar to the previous He@lthCo-op system, but differs because it not only made medical records databases for patients, but also it was focused on the data streams exchange between different medical organs. The authors wrote that one of the main characteristics of modern medical ISs is the high level of cooperation between different medical institutions. The eMAGS system was an ontologically-based, multi-agent system that is designed to interact in a distributed medical environment without a certain boundary and traditional client server. The eMAGS was based on the messaging standard adopted today

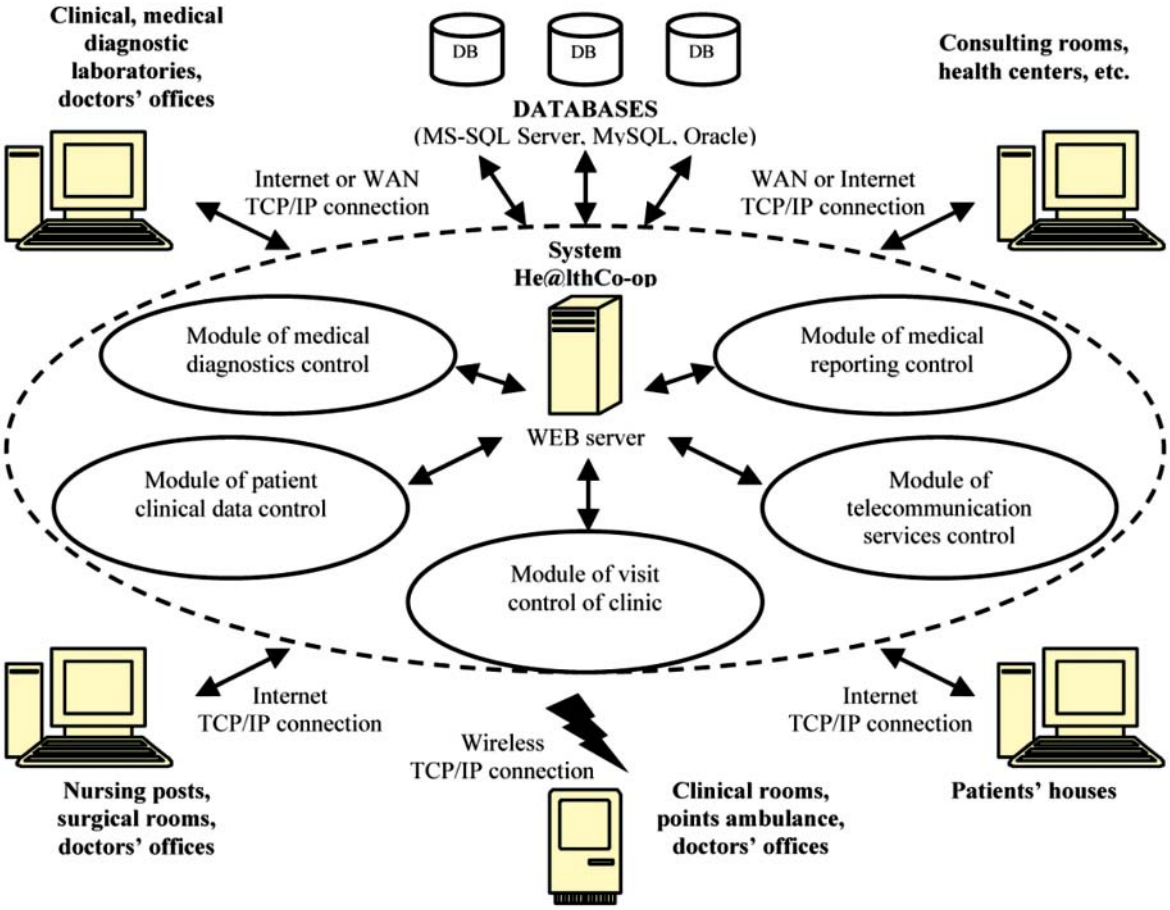


Fig. 2. **Communication scheme of medical system He@lthCo-op, which combined distributed database repositories and various points of medical care provision [122]**

in medicine and named HL7 (Health Level Seven) and facilitates the patient information transmission to healthcare organizations. Constructing this system, the authors took into account the fact that with the growth of even one medical center, the electronic service network and information transmission between its various components become more diversified and complicated with time. The health control was knowledge-intensive, divided into professional sub-domains, each of which may have its own dictionary, database and software; it could be multyplatformal, which significantly increases the complexity of this area. The authors paid attention to the fact that with such complexity increases also the fragmentation of information on patient detected by electronic system. The degree of fragmentation increased if the patient turns to the medical center periodically for a certain time. Recently, the peculiarity of medical ISs was that the user can contact with such system through mobile communication devices or through home monitors in the case of patients at home. All this required the consolidation of information from heterogeneous data resources and has been called *interoperability* between these resources.

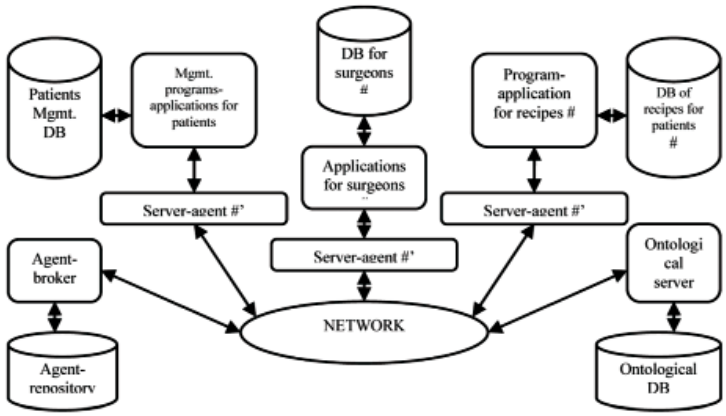
The situation of interoperability of medical ISs associated with the extraction of information from heterogeneous resources and work with them in real time. This situation was quite unique for ISs and it has no analogs in the industry, banking, insurance or other areas. With the quick growth of the Internet, the researchers and IT professionals have faced the problem of consolidating and managing of information in order to maximize the interaction and relevance of received information. Consequently, the key focus must be on integrating of the data from existing systems and making of user-accessible mechanisms for information sharing and distribution. The problem was that data from the disparate resources have different formats and they are incompatible often. The authors saw this problem solution in the use of federalized approach to databases, XML-

based integration, semantic meta-data-based integration of the data, in the creation and the use of a certain software samples, in the use of a single conceptual model or ontology, and in eGATE-type toolkits for transmission. For many problems solution, the popularity of ontologies had increased significantly — from the projects of academic data presentation to the commerce. Yes, commercial systems like CycSecure and EcoCyc have expanded the scope of ontologies. In their system eMAGS the authors could get flexible solutions through the use of multi-skilled mobile agents that provide active access, decryption, study and use of information presented in different medical ISs. eMAGS component HL7-RIM was the basic intermediary for data exchange between programs. eMAGS agents could be communicatively interconnected, since ontology establishes a single terminology for the domain (Fig. 3, *a, b*).

Medical expert systems. Using the above theoretical approaches, a medical expert system was developed [97]. The authors described their system which is based on three databases, client-server architecture and invented by developers type of information processing management. To facilitate the knowledge presentation, data in DB and to obtain knowledge from the Internet an 8-bit encoding scheme and a weighting system were proposed. The system has been tested already in clinic. The authors set the following purposes: 1 — to create a medical IS for information processing and management system for it to facilitate the elaboration and maintenance of medical knowledge bases; 2 — to maximize the information distribution and its following use by medical institutions and doctors; and 3 — to facilitate the process of decision making by medical expert systems. The authors described the method of control of Internet information processing, which they used to construct large databases of medical knowledge. The testing system was developed using Delphi 5.0 and Microsoft SQL Server 2000, it was available online for the testing during one year. The authors argued that their method and system made easier the operation by large volumes of medical knowledge.

Another Internet-based system was

a



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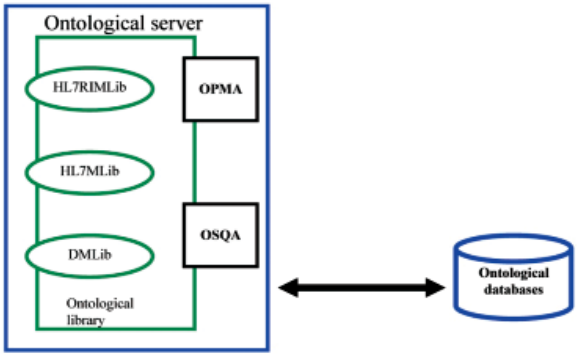

Fig. 3. **eMAGS medical system [124]:**
a — Architecture of eMAGS medical system. The essences of "agent" and "ontological" types were components of eMAGS system. Indications: # the greater number of such entities were supposed to be in medical institution; ' the essence of this type had a database and an interface for the user; *b* — Ontological server and its components

developed to select patients for clinical trials in oncology [103]. Large-scale clinical trials in the modern world are often multicentral, which means that they are based on different geographical points of the Earth, often in different countries. They are conducted to study effects of important potential medical preparations which could be recommended then for people treatment. For such a work it is necessary to provide the highest level of standardization, to record in databases numerous test results, and to fulfill a number of other specific requirements. Modern Internet technologies provided the opportunities for such work, although until the last days the patients' selections for tests carried out manually sometimes. Developers of expert system from the Moffitt Cancer Center, USA, described their expert system for patient selection [103]. The data about each patient were recorded to it, and if there are not enough data, the system offers additional tests. The system permitted the automating of selection process, the increasing the number of patient that can be selected (previously up to 60% of eligible patients were lost) with a significant reduction in cost of testing procedures. A user-friendly interface had been developed, which allows a healthcare professional to add test data and to make new selection criteria without the help of programmer. This system has been tested in oncological hospitals. This is extremely important because, according to statistics, only in the United States 550.000 people dies of cancer every year. For this sphere of medicine a large number of new medicals are developing and testing constantly. In case of successful results they come to patients immediately — thus for the newly developed medicals the shortest path from the laboratory to the patient was invented.

Electronic systems for the work with images. The following several ISs were designed for the work with images. There were some well-known methods for the work with images in medicine, for example, diagnosis of X-rays, ultrasound diagnostics, and etc. For techniques of images processing it was necessary to have few, sometimes few hundreds images even for one patient. So, a problem appeared concerning images recording and their ordering in DB with subsequent use.

An electronic medical system NORMA was elaborated in Genoa, Italy, for radiological center for the needs of radiotherapy [101]. It was designed jointly by the teams of physicists and radiologists in order to develop the most optimal schemes for the planning of treatment by radiotherapy methods. The system was based on databases that contained, together with standard patients data, numerous images of tumors and areas of patients' bodies where is a risk of their occurrence. NORMA provided new for its time interesting opportunities for patients to record in DB and to visualize a large number of images made for tumor diagnosis and related body areas of risk. There was a possibility to study, to analyze, and to discuss such images by physicians and radiology physicists simultaneously in different loci of the system. NORMA had a client-to-server architecture and it is platform-independent. Internet technologies that make it easy to use it for people without special computer knowledge were used, due the commentaries at each step that help to user to perform the next actions. The system was subdivided into server based and client sides on Java software applications. For implementation optimization the project also included, in addition to TCP/IP, another relevant protocol that organizes the data exchange and message control. Images for diagnoses were stored and removed from the appropriate DB or standard DB of DICOM (Digital Images and Communications in Medicine) through the connection DICOM-WWW, which allows to connect the normal Internet browsers used by NORMA system and DICOM software via the HTTP protocol. Browser requests were sent to the Web server connection via CGI (Common Gateway Interface). The DICOM software converted queries to DICOM messages and organizes the connection to the remote site of the DICOM Application system.

In [53] the authors described their developed web-based medical education system that simulates images. The system was elaborated for training students and doctors who work in design and processing of medical images. Using this system it was possible to train them for the work with X-ray, tomography, ultrasound and other images and documents. Internet technologies allowed ones to work with them online, in an interactive web — site environment. Some techniques for working with images, image processing algorithms and exercises for training in an interactive virtual laboratory were described in [53]. Each illustration has extended comments, including profound explanations of physics and math. For the work in the Internet a user-friendly interface was developed, trainings are held in MATLAB Web Server environment. Macromedia Director MX was used to develop an interactive animation

theory with graphical-oriented simulation. HTML and JavaScript were used to enable the user to apply these modules online in a web browser. The teaching quality grew due to the use of multiple choice questions, ability to analyze image data, and material submission according to module principles.

Another system for the work with images was elaborated for medical hospital in Shanghai, China [79]. The authors have developed a web — based system for interactive demonstration of electronic patient records, such as DICOM images, graphic images, report structure and therapeutic records for the hospital's internal network software and for the Internet. This system consisted on three main components, client-server architecture for patient data obtaining and authorization, and Internet-based system for data transmission. The system that visualized the data in the Internet includes multimedia display modules and remote control module for managing of software functions and for interacting with patient data. This system has been successfully tested twice during teleconsultations of patients with acute respiratory syndrome in the Shanghai Infectious Disease Hospital and Xinhua Hospital. During the consultations, doctors in area of infection control, and remote experts could interactively used this system to work with electronic images and patient records, which facilitated the correct diagnostics. The techniques developed by the authors provide new opportunities for making of images of patients' documents using Internet technologies and DICOM standards. This system could be used both in the intranet and in the Internet for tasks' solutions in telemedicine, teleconferencing and distance education.

Electronic systems for the work with medical documents. In publication from Brazil [51] it was upraised the problem of medical documents' processing. The availability of a huge number of medical documents in the modern Internet today is inconvenient for users, since it is difficult to find the right documents. Moreover, among them there are many documents with inaccurate and incorrect information, and documents without critical inspection of professionals. The authors suggested the MedISeek metadata model, which allows providing ones with medical visual information, including information on the properties, components, connections and image authorship. The model used web-architecture and support of International Classification of Diseases and Related Health Problems (so-called ICD-10). The derivative metadata model was integrated into each medical image and specifies the semantics. Thus, the relevant information can be obtained directly from each image; the data integrity is stored in the Internet. Previous experimental results indicated that authorized users of the system can describe, store and transmit medical images and related diagnostic information (Fig. 4).

Some principles of medical documents processing are given in [119]. Concepts of automatic recognition of professional notions in medical informatics, the search for corresponding notions in the text are important tasks, as well as the task of medical documents obtaining from the Internet. In this work, the authors presented the software called the "keyphrase identification program" (KIP) to identify the main concepts from medical documents. KIP combined two functions: the extraction of nouns from phrases and identification of key phrase. Then, for nouns from the selected phrases from medical documents the weight were given, which depends on how important they are to medical documents and on the specificity of their medical domain. Experimental results demonstrated that the proposed extractor of nouns from phrases is effective in identifying of noun phrases of medical documents that is why it is suitable for identifying of important medical conceptual terms.

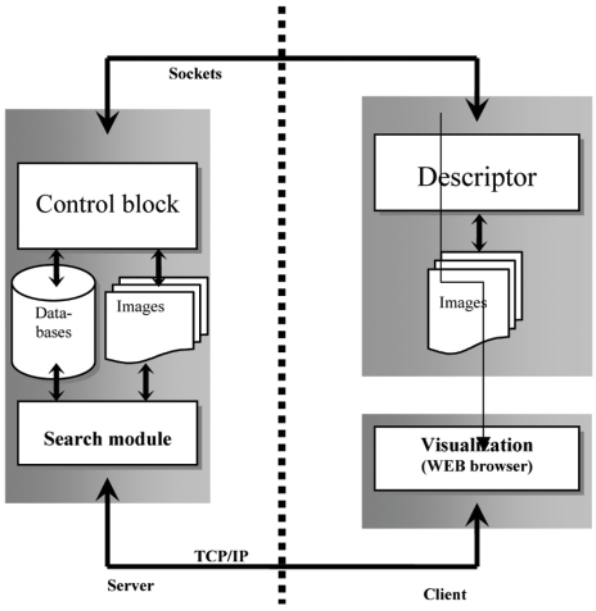

Fig. 4. **The scheme of MedISeek subsystems [51]**

The exchange of information streams in medical networks reveals the serious problem of medical vocabulary and related dictionaries standardization. The following electronic dictionary systems were elaborated for this problem solution [100]. During the past twenty years the WordNet system has been elaborated for the English-speaking countries; it is believed there is the most modern lexicon of modern English for its time. With the start of electronic medical information systems development, the MedicalWordNet electronic lexical system, which virtually overlapped the sets of WordNet vocabulary and medical terminology, began to be developed. The new MedicalWordNet repository contains 3 large collections: 1 — forms of words used in medicine and structured according to the Princeton WordNet; 2 — medical-important suggestions called medical facts that were united into MedicalFactNet; 3 — records formed on the basis of nonprofessional questions, which refer to this system, combined in MedicaLFactNet. In such a way the developers have formed a new type of medical resources, which is based on the database related to the medical domain. The sentences were generated from WordNet. There are 2 "sub-body" sentences referring to MedicalBeliefNet or MedicalFactNet. The first type had to be evaluated by users — nonprofessionals, the second one — by medical experts, and the possibility of this type of double assessment of the system the authors saw as their great achievement. The results of the developed system implementation were examined during a small pilot experiment; the widespread use of this system was expected.

Electronic teaching systems in medicine. The following system related to electronic teaching systems in medicine [104]. Japanese developers have designed a multimedia educational Internet system for medical lectures that has friendly characteristics and relatively low price. The system has been elaborated using the RealSystem package with TCP/IP network. Lecturers could demonstrate their lectures and presentations during conferences with video and audio over the Internet. Each slide from video or audio resources was projected onto a high quality screen. The system use demonstrated good results in process of distant teaching (Fig. 5).

The system was developed for Japan, where in 2003 all universities, institutes and main hospitals were connected by gigabit Ethernet network with high speed (up to 1024 Mb/s) and wide data band. After the systems have been upgraded from the point of view of compressing the data files to increase the streams' speed in the network, the transmission of high-quality video and images become possible via the Internet. It was noted that commercial "video-on-demand" system, which was elaborated earlier, could not broadcast only a variety of medical lectures and performances, but also to record images and synchronously with them to record audio files in user's library. If somebody needed to review the materials again, students or doctors could do it easily, as well as to study lectures from remote universities in a convenient place and time. However, the previously done "video-on-demand" system for 2003 was still quite expensive in order to install it in many universities in Japan. The newly elaborated system, based on the RealSystem and TCP/IP packages was less costable than previous ones; it was more convenient and easier in use, which makes it more suitable for wide spread use in universities and hospitals.

Electronic information system for monitoring of the population health in Ukraine. In our previous publications [1–6] we have already described how we developed some theoretical principles for the creation of technical electronic information systems (tIS) for biology and medicine, as well as the experience of practical developments of such systems. The next step was the development by Klyuchko O. M. the electronic technical system for environment monitoring [1], in particular the system called "EcoIS". Being a complex network system that permitted the improvement and relatively independent development of its segments, Klyuchko O.M. and Tsal-Tsalko V.I. at the next stage decided to expand the "EcoIS" system capabilities by adding a medical sector which is described below. This is especially important for the conditions of Ukrainian Polissia region, whose

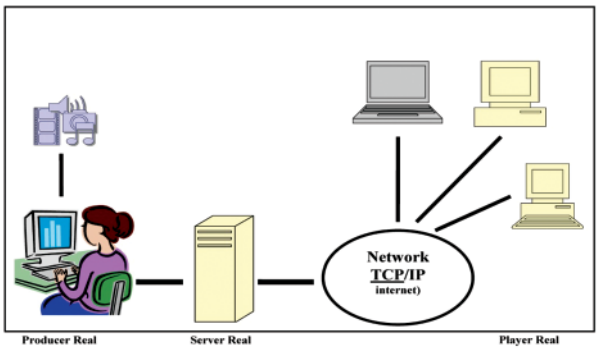

Fig. 5. **The scheme of multimedia system for information spreading RealSystem [104]**

environmental conditions have been worsened in many areas in result of Chornobyl accident pollution (1986) [1, 29]. Therefore, we have expanded the EcoIS functionality so, that it became possible now to monitor not only the presence of pollutants in the environment and the response to them invertebrate organisms, but also the monitoring of number of human health characteristics, including biochemical characteristics of organism functioning. Practice has shown that for health monitoring of the population living in adverse environmental conditions, it is necessary to organize and implement local IS diagnostic centers, electronic working places for individual doctors, formation of standard and at the same time flexible formats for diagnostic data recordings. At the next step it is necessary to unite subsequently such electronic working places in a network; initially — at the level of the clinical institution, and then at the higher level, up to the national one. The effectiveness of medical ISs depends in great degree on the maximal possible formalization of diagnostic information, which requires the active collaboration of engineers and doctors [1]. One application of IS functions was screening-filtering for a certain set of patients' diagnostic parameters during the mass screening and in the allocation of risk groups for a more complete survey. It did not require the high reliability of the primary diagnosis, since the selection threshold can be given with sufficient margin. For screening the fairly simple algorithms such as the type of tree of features or the calculation of some weighting metrics of input parameters could be used. More complex diagnostic programs — expert systems — were based on a certain knowledge base, which is formed by accumulation of experience in the application of other diagnostic methods. They used complex algorithms based on the analysis of links between features or based on neural networks models that can self-tune on some training sample, which makes them virtually universal. To obtain objective diagnostic information the texture analysis of tomograms or ultrasound images, automatic allocation of objects, definition of characteristics with subsequent identification and classification by images recognition systems could be used.

Such computer diagnostic systems could be used both together with IS with electronic disease history, as well as autonomously, for example, directly in diagnostic centers or in the reception rooms of medical specialists. Together with diagnostic equipment, the special applications that were optimized for narrow use may also be used — optimal visualization settings, making of slices or projections, 3D simulation, image matching, formatting of image groups for printing [1]. All this made free diagnostic physicians and auxiliary staff from the routine operations, it is greatly simplified, facilitated and accelerated their work. In recent years, such software products had been actively developed and implemented somewhere in Ukraine [1]. The task of fulfilled work was to develop the system segment for monitoring of number of medical characteristics of population health state, organism biochemical parameters, patient electronic medical card, and etc. for the regions of Ukrainian Polissia and to suggest an adequate technique for information protection in such system.

Development of the software complex "General medical database" in the "EcoIS". A medical IS with patients database (as well as healthy residents) we developed for the use in ecologically polluted areas of the Ukrainian Polissia (in particular, town Novohrad-Volynskyi and its surroundings) [145]. This IS we called "General Medical Database". During this IS development two important features of Polissya region were taken into account. The first one — is radioactive contamination as a result of Chornobyl accident, and in such areas it is extremely important to conduct long-term monitoring of both the ecological situation and the population health in order to reduce indicators of morbidity and mortality [29]. The second one — the region is characterized by the large forest areas, small density of population living in remote, hard-to-reach small settlements. In such conditions, it is problematic not only quick medical care, but also medical care by itself, because even simple communications between settlements are difficult. Modern IT technologies help to achieve significant success in such problems solution.

Our developed networked IS with databases for population medical care and monitoring of their health status was called "General medical database" within the greater our IS — "EcoIS" [145]. The following elements of this IS have been developed:

1 — medical databases;

2 — electronic medical card of examined person (or patient);

3 — electronic key for the protection of private individual health information (person or patient being examined), since such information is confidential.

Below the information on each element of this IS development is suggested.

Development of electronic medical database in the "EcoIS". An electronic medical database is an electronic analogue of patient's traditional medical card. This was a repository of records, each of which contains medical information: complaints, diagnosis, prescribed medicals, results of laboratory tests, medical indicators, and etc. [145]. Each entry can contain text information and tabular data, graphic images, as well as attached files of any type (spreadsheets, documents in.PDF format other). In addition, records may contain medical images in DICOM format (CT, MRI, ultrasound, etc.). Developed electronic medical database may be available in any hospital of Ukraine with available Internet; the data loss is prevented.

Design of algorithm and code for electronic medical database. At the beginning the program interface was developed; it includes about 20 forms. The structure of interaction between the forms is shown on Fig. 6.

"Form1" is a "Greeting" interface; it also has a function of entry to the main program with reading the data of electronic key and patient's identification number, if it is already recorded into the database. A code for reading of "doctor ID" from the electronic key was written also.

In Form2 there are several tabs according to the physician's access point to the information. For example, the doctor-radiologist can not see the history of the patient from infectious department. The first tab shows patient data. If the electronic key with the access level of "Registration", then the user can add or edit the personal data of the patient. Other doctors can see only the information without editing.

On the other tabs there are automated workplaces (AWPs) of defined physicians (other name is "electronic work places" — EWP). The doctor can open the history of disease in any moment from his AWP and review it. The search is possible according to the date of database entry.

The tab "Laboratory" is the entry to biochemical laboratory and to results of patient biochemical analysis. On this tab there are several buttons that cause appropriate forms to save or to view the results of patients' tests (Figs. 6–9).

Tables MySQL and interaction of C# with databases. Sometimes it is necessary to connect these different technologies. For example, to write an offline client for CMS [1], who works using MySQL, development of local database / program that uses it without any productivity limitations due to a free version of Microsoft SQL. Let's describe how the works on MySQL and.NET connection were done [145]. Navicat8.1 for MySQL was used to simplify the work with MySQL — there are free versions of Freeware. Of course, it could be completely replaced with MySQL Command Line Client.

It is necessary to install correctly the software on personal computer (PC). This is described in details on the MySQL site — put the studio, MySQL, and then put MySQL. NETConnector. After that, one need to create a project that can use MySQL Connector for the work with databases. We have to launch the studio, to create a new project — Windows. Forms, the language C #. After that we add Reference to the Mysql.Data component (right-click -> Add reference). Now the namespace Mysql.Data is available for us. From it we will use MySql.Data.MySqlClient — we have to add the corresponding directive "using".

Listing of program "Connection". The process how to write the code correctly for the connection with database has been demonstrated in listing "Connection". In it the words marked by "*italic*" were written in Ukrainian (fragment of code).

Listing of program "Connection":

```
// connection data
string MySQL_host = "localhost";
string MySQL_port = "3306";
string MySQL_uid = "root";
string MySQL_pw = "nopassword";
MySqlConnection Connection = new MySqlConnection("Data Source=" + MySQL_host + ";Port=" + MySQL_port + ";User Id=" + MySQL_uid + ";Password=" + MySQL_pw + ";");
MySqlCommand Query = new MySqlCommand();
Query.Connection = Connection;
try
{
Console.WriteLine ("Connection with the database server");
Connection.Open();
}
catch (MySqlException SSDB_Exception)
{
Console.WriteLine ("Check server settings!\n: " + SSDB_Exception.Message);
 return;
}
Console.WriteLine("OK");
```

To save information and for its subsequent search, it is necessary usually to design a

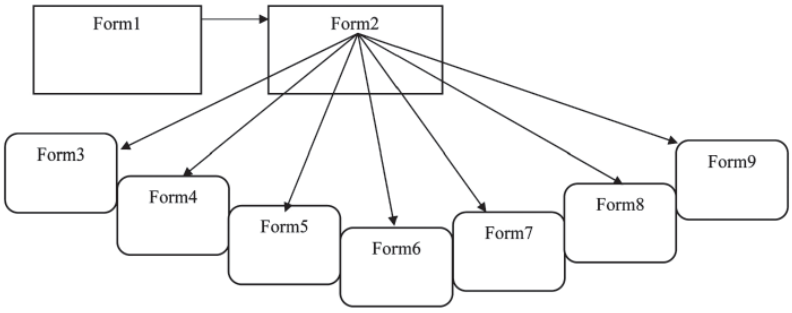

Fig. 6. **The structure of software complex "General medical database" [145]**

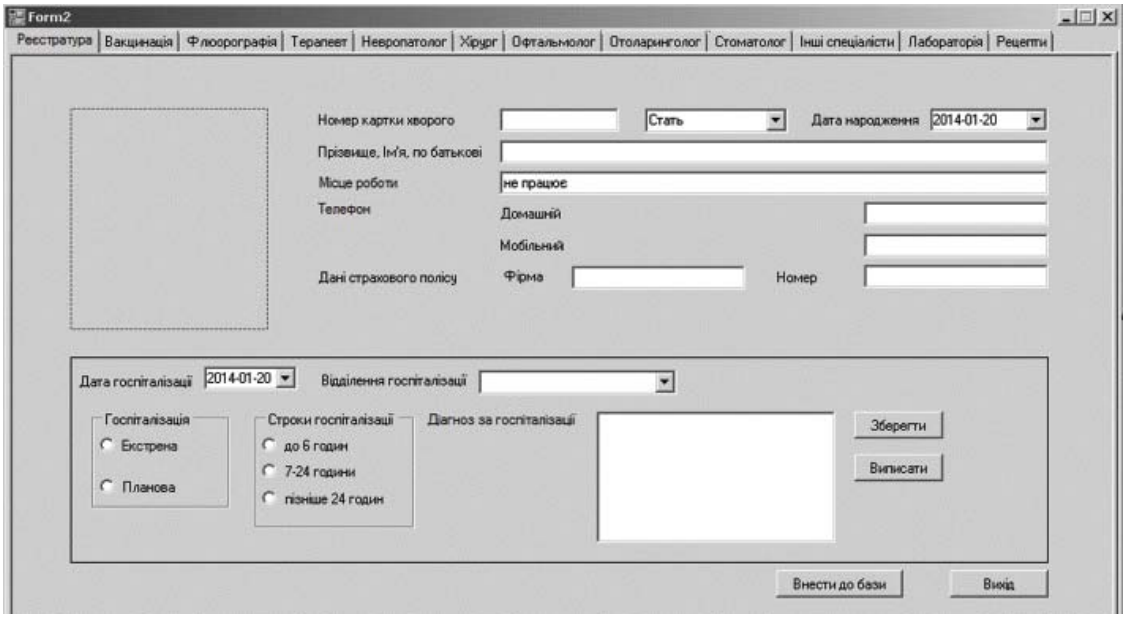

Fig. 7. **Form 2, the tab "Registration" [145].**
The original interface was designed in Ukrainian

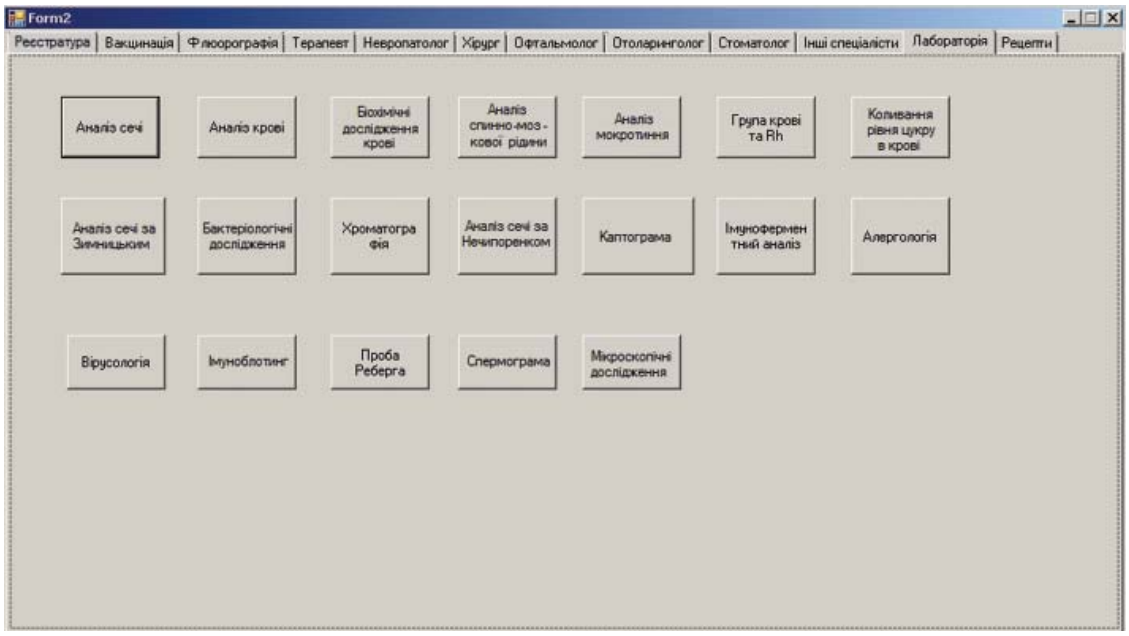

Fig. 8. **Form 2, the tab "Laboratory" (for biochemical laboratory) [145].**
The original interface was designed in Ukrainian

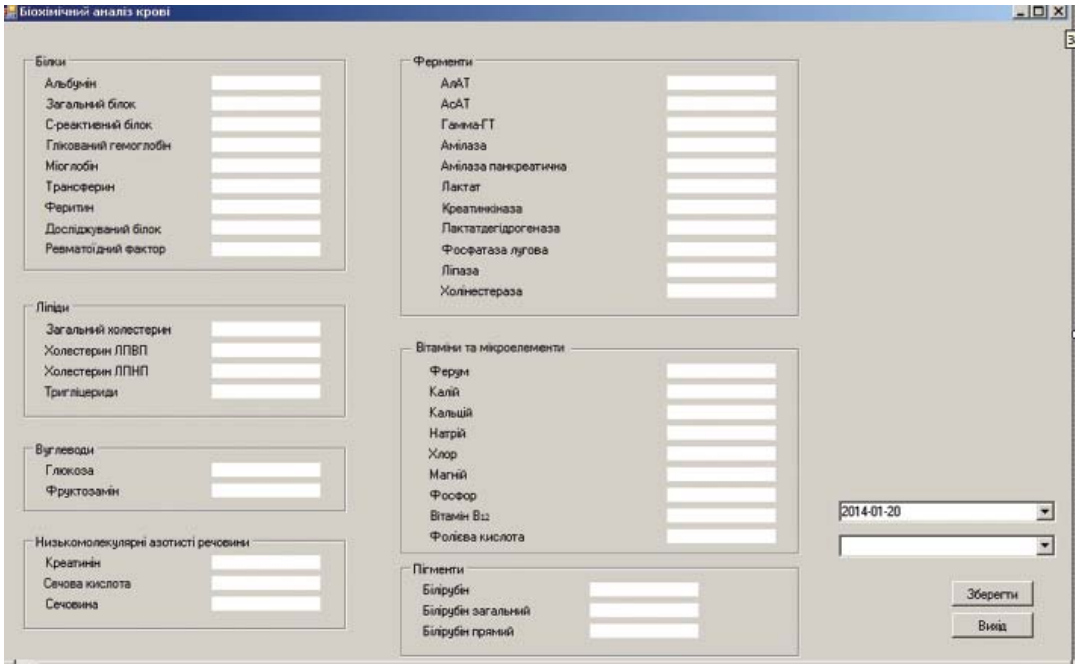

Fig. 9. **Form 6, the tab "Biochemical analysis of the blood" [145]**
The original interface was designed in Ukrainian

database in tables [1]. For this purpose MySQL was used. In the process of programming, the following tables were done: "Patient", "Doctor", "Hospital", "AccessLevel" and others (for each tab — program and form was done). The table "Patient" have been recorded the general information about patient (name, home address, telephone, other contact information, hospitalization time, referral department), as well as his ID, which permits to make a search in corresponding database according to the history of disease. In tab "Hospital" there are the data about hospital where works the doctor, who input patient data records into the database (contact information of admission and related physicians, etc.).

Construction of electronic key for the protection of medical data in "EcoIS". To protect the patient personal data in our IS, the method of program protection with the help of a hardware key was applied. Using this method, the confidential information about the health of examined person (the patient) was protected. The need to protect such confidential information arises particularly in the case of long-term monitoring of health indicators for the large numbers of people in regions with polluted environment (Figs. 10, 11).

Many specialized software packages use this method of hardware key. After the purchasing of the program, the author or distributor sends to user a physical device,

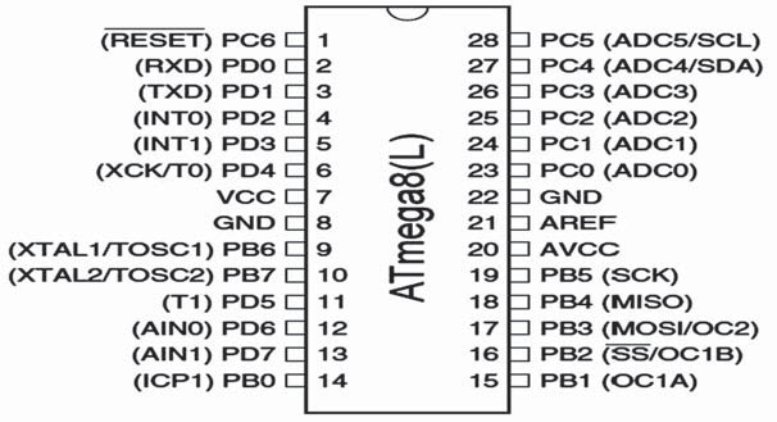

Fig. 10. **ATmega8 used for the hardware key construction [145]**

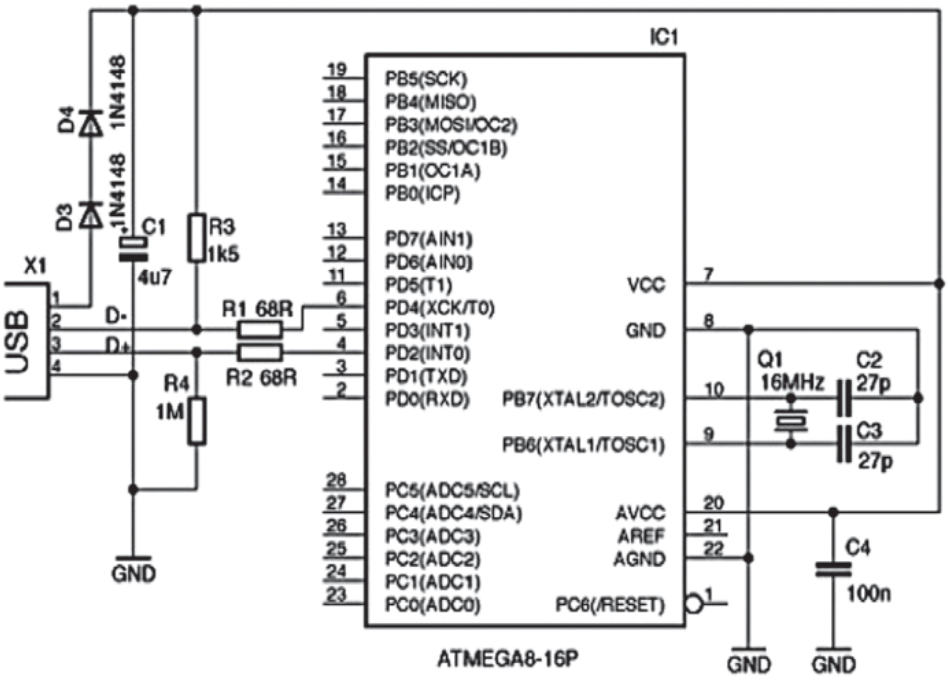

Fig. 11. **The principle scheme of hardware key [145]**

which is connected directly to the USB port — this is the hardware key. This is direct protection of programs from copying. The hardware key is a chip with some information that can not be reached (except as software), because it is impossible to look into this "black box". Most often, the hardware key does not have installed physical memory, but microcontroller that functions according to its inside program. So, a hardware key is a "black box" with unknown algorithm. This allows protecting software effectively against hacking.

To make the original electronic key we need a microcontroller and a circuit that implements the USB interface. We used Atmega microcontrollers. For them free software is available, and they have a low price in comparison with analogs. For the key the ATmega8 microcontroller was used because it does not include the internal USB function — we implement the USB function independently. We picked up the TQFP case.

Principal scheme of hardware key and code. Let's describe how to work with USB port, since operations with this port are not an easy task. We used the existing V-USB development, it was necessary only to make the circuit. This scheme is quite simple [145] and includes USB stick, microcontroller ATMEGA8, 2 diodes to reduce the voltage to the microcontroller since USB gives a voltage 5V. For proper microcontroller operation at 16 MHz on legs, the microcontroller should be fed 3.3 V. For key operation we needed 4 resistors and 3 capacitors (the cost is enough low). Also microprocessor has to be programmed using appropriate program. The listing below demonstrates how to transfer the data that are into the operative memory of the microcontroller (fragment of program code) [145].

Listing of data transfer program

```
uchar usbFunctionRead(uchar *data, uchar
len)
{
 if(len > bytesRemaining)
 len = bytesRemaining;
  eeprom_read_block(data, (uchar *)0 +
currentAddress, len);
 currentAddress += len;
 bytesRemaining -= len;
 return len;
}
```

In the main PC program the following code for information reading from microcontroller should be written:

```
MyUsbDevice = UsbDevice.
OpenUsbDevice(MyUsbFinder);
 if (MyUsbDevice == null) throw new
Exception("Device Not Found.");
 IUsbDevice wholeUsbDevice =
MyUsbDevice as IUsbDevice;
 if (!ReferenceEquals(wholeUsbDevice,
null))
```

```
{
wholeUsbDevice.SetConfiguration(1);
wholeUsbDevice.ClaimInterface(0);
}
  UsbEndpointReader reader =
MyUsbDevice.OpenEndpointReader(ReadEnd
pointID.Ep01);
byte[] readBuffer = new byte[1024];
while (ec == ErrorCode.None)
{
int bytesRead;
 ec = reader.Read(readBuffer, 5000, out
bytesRead);
  if (bytesRead == 0) throw new
Exception(string.Format("{0}:No more bytes!",
ec));
  Console.WriteLine("{0} bytes read",
bytesRead);
   Console.Write(Encoding.Default.
GetString(readBuffer, 0, bytesRead));
}
Console.WriteLine("\r\nDone!\r\n").
```

If there were no information from microcontroller within 5 second we considered that all information was accepted.

Thus, in present publication the examples of highly developed technical information systems with databases elaborated for medicine were observed. The data from their databases and other their abilities it is possible to use in biotechnology as well as to use this experience for the construction of new information systems in this branch. For the solution of this problem the methods of modern informatics and computer sciences were used. The use of such information computer systems can be applied for the exploring of complex unexplored objects (databases of biological cell receptors, and etc.) in situations where the use of traditional methodologies is either complicated or too expensive.

At the beginning of article the scheme with classification of modern medical information systems is presented as well as detailed description of medical information systems' different versions on the base of more than 150 scientific and technical sources examination. Electronic information system with biochemical laboratory and personal patients' data protection developed by the authors for hospital in Polyssia region of Ukraine polluted during Chernobyl accident is suggested as well. This software complex was called "General medical database"; it has been elaborated as a segment of our primary developed "EcoIS" system. To do this, we developed our own original DBMS-based program and its protection using a hardware key based on the AVR ATmega8 microcontroller [145].

The main details of the software complex "General medical database" are described in present article [145]. A block scheme of interactions between program forms is shown, and the connection of the C # language and MySQL database management system using MySQL Connector is explained. During the development of the electronic system for protection of the software complex "General medical database" in the "EcoIS" there were developed our own original programs on the basis of database management system (DBMS), protected with the help of a hardware key based on the microcontroller AVR ATmega8. The main stages of design of a hardware key used for the protection of personal medical data in the database were described. There were presented its electrical circuit and software with the help of AVR Studio programming environment. Also there were explained the linking of the electronic key with the software complex using the library LibUSBdotNET (the library has become standard in the programming languages C, C ++). All recipes issued by doctors can be stored in the developed databases, their falsification is impossible and, therefore, all relative information can be checked by pharmacists. Almost a dozen programs — applications were used to fulfill the project. Implementation of the main program was under the Windows 8 on the.NET Framework 4.5 platform. The developed program can be installed on professional PC and used for the connection with databases in remote locations from hospitals.

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ІНФОРМАЦІЙНО-КОМП'ЮТЕРНІ ТЕХНОЛОГІЇ ДЛЯ ВИКОРИСТАННЯ У БІОТЕХНОЛОГІЇ: ЕЛЕКТРОННІ МЕДИЧНІ ІНФОРМАЦІЙНІ СИСТЕМИ

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Мета роботи — дати огляд сучасних інформаційних систем з базами даних, розроблених для медицини та біотехнології. У статті наведено класифікацію сучасних медичних інформаційних систем та детальний опис їх різних версій. Подано також опис розробленої електронної інформаційної системи із захистом персональних даних, призначеної для використання в біохімічних лабораторіях лікарень в районі Полісся України, забрудненого внаслідок аварії на Чорнобильській АЕС.

Ключові слова: біоінформатика, електронні медичні інформаційні системи.

ИНФОРМАЦИОННО-КОМПЬЮТЕРНЫЕ ТЕХНОЛОГИИ ДЛЯ ИСПОЛЬЗОВАНИЯ В БИОТЕХНОЛОГИИ: ЭЛЕКТРОННЫЕ МЕДИЦИНСКИЕ ИНФОРМАЦИОННЫЕ СИСТЕМЫ

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Цель работы — дать обзор информационных систем с базами данных, разработанных для применения в медицине и биотехнологии. В статье приводится классификация современных медицинских информационных систем и детальное описание их различных версий. Дается также описание разработанной электронной информационной системы с защитой персональных данных, предназначенной для использования в биохимических лабораториях госпиталей в Полесском регионе Украины, загрязненном вследствие аварии на Чернобыльской АЭС.

Ключевые слова: биоинформатика, электронные медицинские информационные системы.

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BIOLOGICAL PROPERTIES AND MEDICAL APPLICATION OF DIPHTHERIA TOXIN DERIVATIVES

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The aim of the review was to analyze the literature data related to the application of a variety of diphtheria toxin derivatives. Although the studies interaction with sensitive and resistant mammalian cells have been held for a relatively long time, there are still some unresolved issues concerning the molecular mechanisms of diphtheria toxin functioning. Native diphtheria toxin and parts of its molecule which preserve toxicity are used as instruments in the newest biotechnological methods for specific cell subtype ablation in multicellular organisms. New recombinant derivatives of diphtheria toxin are periodically obtained in the laboratories throughout the world. Most of these analogs of DT are used in biological studies as the convenient tools for analysis of the functions of natural toxin. A non-toxic analog of diphtheria toxin, protein CRM197, is used in clinical practice as a component of vaccines and as an anticancer agent. Diphtheria toxin — based targeted toxin therapy is another perspective trend for cancer treatment. Therefore, studying of diphtheria toxin derivatives is of a great relevance for biotechnology and medicine.

Key words: cell ablation, CRM197, diphtheria toxin, immunogenicity, targeted toxin therapy, toxoid.

Over the past few decades, a lot of the new information related to diphtheria toxin (DT) have appeared. However, in the most cases, not the DT itself was in the spotlight, but rather its derivatives. There are many different derivatives of DT obtained in cells of a natural producent, such as *Corynebacterium diphtheriae*. However, for several reasons, considerable attention is paid to recombinant analogs of DT. For the first, natural DT possesses one of the lowest values of semi-lethal dose ($LD_{50\%}$) for sensitive cells among the other bacterial exotoxins [1]. The non-toxic DT mutants allow carrying out the research work in a much more safe and convenient way, as they do not pose a threat to laboratory personnel and do not require the implementation of multiple biosafety means. On the other hand, recombinant derivatives of DT are much easier to obtain in the laboratory than native toxin and its fragments. Moreover, nowadays the methods of genetic engineering allow altering the molecules of studied proteins in a desired way. The most commonly used recombinant DNA approaches include introduction of desired mutations and additional amino acid sequences like a fused fluorescent label or a specific affinity tag, deletion of undesired amino acids, construction of the chimeric molecules which combine the necessary functions, etc. Sometimes, in order to study the function of individual structural parts of the whole protein, it is necessary to obtain some certain separate parts of its molecule. The wide possibilities and convenience of the modern recombinant DNA technology led to the almost complete replacement of DT natural mutants and fragments obtained by proteolytic cleavage by corresponding recombinant products. Most of these derivatives are used in the biological studies of native toxin functions and interaction with cells.

Derivatives of DT are important tools for biomedical research, as well as for the most advanced biotechnological methods. For

example, a combination of the catalytic and translocation domain of DT is used for the creation of the targeted toxins, which are mainly used in cancer therapy. DT and its subunit A (SbA) are used for the specific ablation of the desired cell subtypes in multicellular organisms.

In medicine, the most common application of the nontoxic derivatives of DT — is production of vaccines. For example, formalinized diphtheria toxoid (anatoxin) is a standard component of acellular vaccines against diphtheria infection. Besides this straightforward application of diphtheria toxoid, the non-toxic point mutant of DT, protein CRM197 is used as a carrier in conjugate vaccines, as this derivative like the native DT is highly immunogenic. But an even more surprising application of CRM197 is the therapy of oncological diseases — recently, such a medication as BK-UM [2–4] has been successfully introduced in cancer therapy.

There are many other peculiar applications of DT derivatives, as well as outstanding questions relating to the biological functions of respective DT structural parts. The purpose of the present review was to summarize the current literary data on the variety of derivatives of DT molecule produced by the *C. diphtheriae* or either in heterologous systems, to analyze the main features, advantages and problems related to practical application of DT derivatives and provide a description of their current use in the fields of biology and medicine.

Structure and functions of the native DT molecule. DT is produced by the gram-positive cells of *C. diphtheriae* and some other *Corynebacterium* species [5]. It is known that the tox^+ gene which encodes DT [6] is not a native part of *Corynebacterium* genome. The *tox* locus is present in the genomes of several bacteriophages [1]. Most often, this gene is introduced in *C. diphtheriae* with a corynephage β during lysogenic transformation [7, 8]. It is interesting that the synthesis of this foreign to bacteria gene is regulated by the system of bacterial host cells in response to environmental iron concentrations [9, 10].

The precursor of DT [11], is synthesized on polyribosomes in the form of a single-chain polypeptide with the approximate M_r of 68 kDa. This precursor has a signal peptide on its N-terminus, that guides the toxin for cotranslational secretion in the extracellular environment by a bacterial Sec translocation system [12]. After the cleavage of the signal peptide during the process of translocon transfer, a mature DT is already formed (Fig. 1).

Fig. 1. **The elements of the secondary structure of secreted form of DT superimposed on the amino acid sequence in a single-letter code:**
PDB code — 1GSK, according to [15] by UCSF Chimera software: α-helices are highlighted by yellow, β-strands — by green, non-structured regions are not highlighted , the area of the hinge loop which was not visible on the electronic density maps is marked by a red frame

Mature DT is a single-chain protein of 535 amino acid residues with the SDS-PAGE-estimated M_r of 62 kDa (58.342 kDa according to the theoretical calculations based on the gene sequence). This toxin contains no unusual amino acids and no non-protein moieties [13]. DT belongs to the A-B group of bacterial exotoxins, because the molecule of DT is traditionally divided into two subunits: A and B (SbB). Among the toxins of this group, DT was a first characterized member [1]. Division of DT molecule on subunits emerged historically, because during the proteolysis under mild conditions and in the presence of a reducing agent, the original molecule of toxin breaks up into these two parts. According to SDS-PAGE, SbA possesses the M_r of 24 kDa and SbB — 38 kDa. It should be mentioned that unlike SbB, SbA is characterized by an increased thermostability [14].

At the level of the tertiary structure, DT consists of a C-terminal receptor-binding or R-domain (residues 385–535), a central translocation or T-domain (residues 201–384), and an N-terminal catalytic or C-domain (residues 1–191) [15]. SbA is represented only by the C-domain while SbB includes two domains: T- and R-. The fine structure of particular domains of DT molecule was investigated by X-ray diffraction in protein crystals (Fig. 1): the C-domain contains α-helices and β-strands, T-domain is entirely α-helical and R-domain is a flattened β-barrel with a jelly-roll-like topology, similar to that of the immunoglobulin variable domain [16].

DT contains four cysteine residues, which form two disulfide bonds: Cys186 – Cys201, and Cys461 – Cys471 [17]. The hinge loop that is formed by the disulfide bridge between Cys186 and Cys201 combines the C- and T-domains together. The second disulfide bond is located inside the R-domain.

DT binding to its receptor on a plasma membrane triggers internalization of DT::receptor complex through the clathrin-dependent endocytosis [18].

After DT binding to its receptor, transmembrane furin proteases at the surface of sensitive cells, cleave the peptide bonds that follows after residues Tyr190, Ala192 or Gln193 inside the hinge loop after DT binding. However, after such cleavage, C-domain is still remaining covalently tethered to B-subunit by a respective disulfide bond (the "nicked" or proteolytically cleaved toxin). For cytotoxicity, the mentioned disulfide bridge should be reduced to release the C-domain in the cell cytosol where it can implement its cytotoxic action. It is believed that this reduction occurs due to the glutathione GSH of cytosol [13].

When C-domain is released from the rest of the DT molecule, it is able to catalyze ADP-ribosylation of eukaryotic translation elongation factor 2 (eEF-2). The ADP-ribosyl group from NAD^+ is transferred to the diphthamide residue (post-translationally modified histidine which is found in eEF2). This leads to an almost complete arrest of protein synthesis and cell death. It should be mentioned here, that entire DT which was not cleaved and treated with a reduction agent, is incapable of ribosyltransferase activity in cell lysates [14]. SbA is toxic for cells only in the presence of SbB, which is required for the binding to DT receptor, consequent uptake into endosomes, and translocation of fragment A into the cytosol [19].

Molecular mechanism of SbA translocation through the lipid bilayer is still unknown but it is obvious that T-domain which forms pores in lipid bilayers [20,21] is crucial at this step.

Characterization of the DT receptor. DT receptor is the precursor of heparin-binding epidermal growth factor-like growth factor, proHB-EGF [22]. ProHB-EGF is a single-chain transmembrane glycoprotein of 208 amino acid residues [22, 23]. Significant amounts of this protein can be found on the surface of epithelial, endothelial, smooth muscle cells, fibroblasts, macrophages, etc [24]. ProHB-EGF contains heparin-binding, EGF-like, transmembrane and cytoplasmic domains [25].

R-domain of the DT binds to the EGF-like domain of proHB-EGF. Binding of DT to proHB-EGF is highly specific — the K_b of DT::HB-EGF interaction was estimated to be 10^{-8}–10^{-9} M [26, 27]. The presence of proHB-EGF on the cell surface causes cellular sensitivity to DT. Cells that do not express the proHB-EGF on plasma membrane are not sensitive to DT.

It is known that proHB-EGF forms complexes with some other membrane proteins, such as integrin α3β1, heparan sulfate-containing proteoglycans and CD9 [28,29]. Tetraspanin CD9 is known to sufficiently enhance the DT binding activity of proHB-EGF [30–32].

It was found that mice and rats can tolerate relatively high doses of DT that are enough to kill susceptible animals (dogs) which are much larger in size and weight and with no necrosis occurred at the seat of inoculation [33, 34]. In cell culture experiments it was demonstrated that DT dose which reduces the rate of protein

synthesis by 50% is 10^5–10^6 times bigger for murine L929 cells than for human HeLa and KB-S cells [35].

Not all the rodents possess resistance to DT. Chinese hamsters and especially guinea pigs are sensitive to DT. Information on the resistance of other members of the mammalian class, as well as on the proHB-EGF polymorphism in various taxonomical groups of mammals is limited.

The DT receptor from resistant and sensitive organisms possess a different primary structure due to the amino acid substitutions. It is obvious that differences in the amino acid sequence of proHB-EGF are the main reason for DT resistance in mammals, as murine cells which express human proHB-EGF also become highly sensitive [26]. However, there is no a definite opinion regarding how these differences in receptor structure alter the processes of DT binding and internalization by resistant cells compared to sensitive.

According to one point of view, the receptors of insensitive cells are unable to bind DT [26, 35–39] which is the only reason for DT resistance. As to another opinion, DT binds proHB-EGF from insensitive cells and internalized by endocytosis [40–42]. According to the authors who found the endocytosis of DT by cells of resistant organisms, unsusceptibility to DT is due to the lack of the SbA translocation in the cytosol of resistant cells that may be caused by several factors: a low binding constant of DT to the HB-EGF receptor under low pH of endosomes [43, 44], high activity of endosomal proteases [40], etc.

On the cell surface, proHB-EGF can undergo splitting by metalloproteases to form a soluble growth factor HB-EGF [45], which carries only heparin-binding and EGF-like domains (residues 106–147 of the proHB-EGF primary translation product with signal and pro-peptides [24]). HB-EGF is a natural ligand for the EGF receptor and HER4 [46]. Soluble HB-EGF is a potential mitogen and chemoattractant for various cell types, including smooth muscle cells, fibroblasts and keratinocytes [47, 48]. This factor is involved in many physiological and pathological processes, which include the eyelid closure [49], wound healing [50–52], retinoid skin hyperplasia [53], cardiac hypertrophy [54], hyperplasia of the smooth muscle cells [55], collecting duct morphogenesis [56], blastocyst implantation [57], pulmonary hypertension [58] and oncogenic transformation [59].

Since the binding of DT to its receptor is very effective and highly specific (with the affinity that is close to that of an antigen-antibody interaction), labeled DT derivatives are very perspective for detecting of this receptor in different biological samples and studies of internalization and intracellular transport of proBH-EGF [60].

Classification of DT derivatives. Now, when we have considered the structure of DT and its receptor, it's time to get closer to a variety of its derivatives, which have some differences compared to the original toxin. Derivatives may be ranked according to their structural similarity to the natural toxin and in order of decreasing of their M_r. Compounds with practically the same M_r may differ in the number of amino acid substitutions. Derivatives may be classified by the presence of additional amino acid sequences and tags that are absent in the native toxin.

Besides, all the derivatives can be divided according to some kind of their function: the presence or absence of toxicity, receptor binding, internalization, etc.

I suppose that it is also necessary to distinguish between the DT derivatives that were obtained in the cells of the natural producer *C. diphtheria* and those derivatives that were synthesized in the foreign host cells. Such a division can be useful for systematizing the historical information on the obtaining of certain recombinant derivatives of DT, as at first DT derivatives were produced exclusively in *C. diphtheria* strains and lately — in heterologous systems based on *Escherichia coli* and other producents.

Structure and functions of DT derivatives produced in C. diphtheria. According to [14], the lytic cycle of corynephage β — was induced in *C. diphtheriae C7(β)* strain by UV-light exposure and then nitrosoguanidine was added. The surviving phage particles produced in presence of the mutagen were plated on *C. diphtheriae C7(–)* cells, that does not contain prophage β. Lysogenised corynebacteria from turbid plaques were spotted on agar and their toxinogeny was firstly estimated by the rabbit intradermal test.

By this method, a number of non-toxigenic *C. diphtheriae* clones were found [14]. Obtained mutants produced the non-toxic proteins serologically related to DT. These toxoids were called "crossreacting materials" that contained single or multiple mutations in the tox^+ gene that resulted in deletions or substitutions of individual amino acids in the polypeptide chain of DT.

Among the obtained mutants, protein CRM197 [61] become the best-studied non-

toxic DT analog of same M_r. Substitution of Gly 52 to Glu in this toxoid leads to an almost complete loss of SbA activity, however, there are also some data that mild activity of mutated SbA in CRM197 is preserved [62–64]. Despite the presence of a single mutation, there is a strong evidence that CRM197 has significant functional differences compared to the native DT [65–69].

Another DT mutants — CRM176 and CRM228 with same M_r as that of the native toxin, as well as truncated CRM45 (M_r of 45 kDa) and CRM30 (30 kDa) were created together with CRM197.

CRM45 includes residues 1–386 [70] that appeared as a result of the "TAA" termination signal introduced by the (C to T) point mutation in the "CAA" codon for Gln387 which causes early termination at Thr386 and therefore — the C-terminal lost of 149 amino acid residues (M_r is 16.530 kDa). CRM30 appeared similarly — by a transition of a sense codon to a stop codon. The C-terminal residue of this CRM is probably Ala280 [71], however unknown exactly.

SbA of CRM228 has no transferase activity while the respective activity of CRM176 was approximately 2.6 times less than that of SbA from DT. Besides, CRM228 was also much less effective (10–15% of that of CRM197) in binding to the cell receptor, which indicates multiple mutations. The gene of CRM228 was sequenced [72] and 8 mutations in the mature form of CRM228 were revealed.

Among all the mutants described in work [14], CRMs 197 and 176 turned out to be the closest structural analogs of native DT, as they contain the single point substitutions — Gly to Gln at position 52 [70] for CRM197 and Gly to Asp at position 128 for CRM176. Mutation in CRM197 almost completely reduces its toxicity [14], thus, it became the most widely used and well studied non-toxic derivative of DT. However, a lot of another non-toxic CRMs were described in further works (Table 1) which contain substitutions in their C-domains and can be potentially used for the creation of another non-toxic single-point mutant by means of site-directed mutagenesis.

Another set of 11 CRMs was obtained by nitrosoguanidine mutagenesis of β-corynephage [73], among which CRM107 was shown to selectively kill cerebellar Purkinje neurons [74]. Besides, CRMs 102 and 103 were characterized, as they were used in the development of immunotoxins [75]. The sequences of the rest of the mentioned above proteins are unknown as the particular features of these mutants did not attract the attention of researchers.

Some other CRMs produced in *C. diphtheria* possess the unique and potentially valuable properties. For instance, CRM26 is even smaller than CRM30 and represents SbA with a little bit more truncated T-domain [76, 77]. CRM1001 which possess the transition of Cys471 to Tyr in R-domain was also produced in *C. diphtheria* [78, 79]. CRM1001 was shown to bind the proHB-EGF of the target cells as well as DT but is deficient in cell entry resulting in a reduced toxic effect [78].

Recombinant DT derivatives produced in the foreign host cells. Only DT derivatives from the *C. diphtheriae* cells were listed above. However, production of proteins in their natural producers can be rather inconvenient. Recombinant analogs produced in heterologous systems are much more easy to obtain in the laboratory. Therefore, a variety of recombinant forms of DT were created.

Native tox^+ gene of DT and some of its truncated forms were expressed in *E. coli* [80–82]. Perhaps, the creation of strains of *E. coli* with the native DT gene can be rather dangerous for humans and the environment. Moreover, it is noteworthy that due to the probability of a reverse mutation, production of the single-point full-length DT mutants can potentially provide the same threat.

The gene of CRM228 was inserted in *pKTHI637* vector and cloned in *Bacillus subtilis* cells for secretion in bacterial culturing media [83]. Two truncated forms of CRM228 which contain no R-domain were also described in [83], from which one form contained the C-terminal cysteine residue for conjugation of chemical linkage of targeting molecules. Thus, it was demonstrated that this expression system with *B. subtilis* host cells is completely suitable for the production of the full-length and truncated toxoids and possibly, the entire DT molecules. According to the opinion of the author of this review, production of DT derivatives in the culturing media is the most reasonable biotechnological solution, because folding of the proteins, in this case, can occur in the most correct way. However, there is a report that production of proteolytically split CRM197 by *B. subtilis* may occur [84].

Some studies are devoted to the production of recombinant CRM197 in the T7 RNA polymerase-based expression system and *E. coli* as a host cell [85]. In this case, recombinant CRM197 is accumulated in the cytoplasm and most frequently — in the inclusion bodies [86, 87]. In some cases, it was possible to obtain

Table 1. **The most important DT derivatives which were produced in *C. diphtheria* cells**

DT derivative	Structure alterations	Function alterations	References
CRM45	Deletion of the C-terminal portion Gln387 — Ser560	Loss of the receptor-binding activity, however weak cytotoxicity is preserved	[61, 70]
CRM30	Deletion of the unknown C-terminal portion, possibly Ala280 — Ser560	Loss of the receptor-binding activity, however weak cytotoxicity is preserved	[61]
CRM26	Deletion of the unknown C-terminal portion larger than in CRM30	Loss of the receptor-binding activity, however weak cytotoxicity is preserved	[76,77]
CRM228	Substitutions Gly79 to Asp, Glu162 to Lys, Ser197 to Gly, Lys200 to Ser, Asn389 to Phe, Gly431 to Ser, Asn507 to Asp and Lys528 to Ser in C- and R-domains	Loss of the SbA catalytic activity and receptor-binding activity	[61, 72]
CRM197	Substitution of Gly52 to Gln in the C-domain	Loss of SbA catalytic activity	[61, 70]
CRM176	Substitution of Gly128 to Asp in the C-domain	Partially reduced catalytic activity of native SbA	[61]
CRM107	Substitutions Leu390 to Phe and Ser525 to Phe in R-domain	Deficient binding to DT receptor, however selectively kills the Purkinje neurons, about 10 times less toxic to Vero and Jurkat cells than CRMs 102 and 103	[73 75]
CRM103	Substitution of Ser508 to Phe in R-domain	Retained full enzymatic activity but had defective receptor binding, weak toxicity	[75]
CRM102	Substitutions Pro308 to Ser and Ser508 to Phe in T- and R-domains	Retained full enzymatic activity but had defective receptor binding, weak toxicity	[75]
CRM1001	Substitution of Cys471 to Tyr in the R-domain resulter in the absence of a disulphide bond between Cys461 and Cys 461	Preserving the ability to bind DT receptor, but deficient in the internalization step of intoxication	[78, 79]

CRM197 protein in the soluble fraction of *E. coli* cell lysate [88, 89].

Recombinant SbB and SbA of DT — another well-studied DT derivatives. There are several studies in which for some reasons production of SbA [90, 91] or SbB [90, 92, 93] was established in *E. coli*.

R-domain is the part of DT molecule of the smallest M_r which preserves the ability to bind the DT receptor. An attempt was made to obtain a mutated R-domain, the binding of which to the DT receptor would have an enhanced affinity [94]. Besides, R-domain was cloned in *E. coli* for the purposes of enhancement of bioavailability of curcumin to cells [95]. Curcumin, a perspective for cancer treatment secondary metabolite of plant cells is poorly soluble in water, however, it's solubility can be effectively increased when it is adsorbed to protein and also R-domain. Cloning of R-domain was also described in [96] for characterization of its interaction with the DT receptor.

Of the particular interest are fluorescent derivatives of DT fused to some fluorescent proteins (EGFP, mCherry, etc.), which were described in works [60, 97]. Such labeled fragments of the toxin molecule can be successfully used to study binding of living cell receptors, the expression levels of DT receptor, as well as its internalization by endocytosis in cells [43, 44, 98].

The information about the most important DT derivatives produced in foreign host cells is summarized in Table 2.

Application of DT derivatives for studying the biological functions of native toxin. The most of DT recombinant derivatives with specific mutations and functional tags have been developed specifically to study the biological properties of the native toxin and the interaction of the eukaryotic cells with its

Table 2. **Fragments and analogs of DT molecule which were produced by cells of a foreign producents**

DT derivative	Host cells	Specific features	References
CRM197	*E. coli*	Non-toxic DT analog	[86–89]
	B. subtilis	Extracellular secretion, non-toxic	[84]
CRM228	*B. subtilis*	Extracellular secretion, non-toxic	[83]
Truncated forms of CRM228 with no R-domain (with and without C-terminal Cys residue)	*B. subtilis*	Extracellular secretion of the C- and T-domains combination for development of targeted toxins, non-toxic	[83]
SbA	*E. coli*	Preserves catalytic activity, non-toxic (as it unable to translocate across lipid bilayer by itself)	[90, 91]
SbB	*E. coli*	Preserves receptor-binding and pore-forming activities, non-toxic	[90, 92, 93]
T-domain	*E. coli*	Preserves pore-forming activity, non-toxic	[111, 116–121]
R-domain	*E. coli*	Preserves receptor-binding activity, non-toxic	[94–96]

molecules. For today all possible derivatives that have a certain defective function of the native toxin have been identified. For instance, a variety of mutations were introduced by site-directed mutagenesis into the recombinant derivatives of DT in order to study the biological functions of various amino acid residues. Among them, there should be noted mutations in the active site of C-domain [99–105] and T-domain [75, 106–115].

However, until now, the question regarding the mechanism of translocation of the subunit A DT to the cytosol through the lipid membrane remains unresolved. It is supposed that translocation of the polypeptide chain of the SbA moves through a protein-conducting channel, which is formed by a T-domain of DT. Recombinant T-domain and its pore-forming activity in lipid bilayers have been extensively studied in black lipid membranes [111, 116–121]. In classical works on DT conductivity, it was suggested that at least two T-domains participate in the formation of a single pore [20, 21]. However, recently appeared a message that just a single T-domain is completely sufficient for the formation of a typical DT channel in black lipid membranes [122].

Nonetheless, the most unclear thing about the SbA transport is not how the translocation channel is arranged itself, but what is the force that pulls the polypeptide chain through this channel. There are some findings that lethal and edema factors of anthrax toxin could be translocated by a proton–protein symport through the channel which is formed by protective antigen, the third component of this toxin [123]. It is natural to assume that the polypeptide chain of subunit A can also be transported by a similar mechanism. Similar ideas were already presented in [20, 124] and [125]. There are some findings that certain factors from the host cell can directly participate in the transport of SbA and possibly facilitate this process [126].

Specific cell ablation with DT and its catalytic domain. As it was already mentioned above, mice are resistant to the cytotoxic action of DT. Toxin-resistant animals survive when they are administered DT doses that lead to the death of cells in their organism that contain on their surface a receptor that is normally expressed only in sensitive species. This fact allowed the development a technique for specific ablation of cells in the body of transgenic mice using native DT — the toxin receptor-mediated cell knockout (TRECK) [127].

The first step of TRECK is generation of transgenic mice expressing human DT receptor under the control of a cell type-specific promoter. DT is injected into the transgenic mice at the desired time points to ablate those cells in which the promoter is active. One disadvantage of this method was that due to the high immunogenicity of DT, repeated injections which are necessary for complete cell ablation were ineffective. To solve this complication, the authors created a murine line with the immune tolerance against DT [127]. The receptor of DT deficient in epidermal growth factor-like biological activity but which preserves its ability of binding DT [128] was also created for this purpose to avoid

potential problems with DT receptor acting as a growth factor in mice.

Specific cell ablation in multicellular organisms serves mainly to study the functions of certain cell populations which express a specific marker that is non-expressed in other cell types in the body of laboratory animals. TRACK was used for generation of a murine model of type 1 diabetes [129], a similar conditional cell ablation was used by another collective of authors for depletion of dendritic cells [130, 131]. A large amount of work was done by this approach to study the *in vivo* functions of murine myeloid cells [132].

Expression of active SbA directly in the cytoplasm — is another strategy for specific cell ablation [133–137] which does not require application of native DT. The gene of the SbA in cells of the transgenic organisms can be inserted under the controllable promoter, so that gene expression can be induced by a certain factor [134, 137] (conditional cell ablation), or the promoter can be activated by itself during ontogenesis only in certain specific types of cells [133, 136, 138] (non-conditional, promotor-dependent ablation). The last approach is frequently used not only in animals but also in plant organisms to study the expression of certain genes in different plant cells [138].

Derivatives of DT as vaccine components

Formalin-treated DT is a component in combined pertussis-diphtheria-tetanus vaccines (DTaP and Tdap) [139]. DTaP is a vaccine that helps children younger than age 7 develop immunity. Tdap is a booster immunization given at age 11 that offers continued protection from those diseases for adolescents and adults.

The mechanism of formaldehyde detoxification is based on the reactivity of the carbonyl group regarding the primary amine groups on the protein (i.e. side chain of lysine and an N-terminal amino group of the polypeptide chain). During a reaction, a metilol intermediate is formed, which condenses with water to form a Schiff base. Then the Schiff base interacts mainly with a 5-position of the tyrosine ring to form stable covalent methylene bridges. In detoxification protocols for vaccine production, the resulting Schiff-base is stabilized by glycine or lysine [140]. Manufacturing of the anatoxin for vaccination, which for the first glance has a very simple principle, is a highly standardized multi-week and multi-stage process that is carefully regulated. Resulted anatoxin is tested in numerous assays to ensure that the toxicity has been completely neutralized. For more than 100 years, since the production of anatoxins for vaccination was incepted, the standard protocol for DT, tetanus and pertussis toxins inactivation did not change much [140]. Recombinant genetically inactivated DT, tetanus and pertussis toxins were proposed for development of the next-generation of DTaP and Tdap vaccines [141, 142].

Conjugate vaccines are created by covalently attaching a poor antigen to a strong antigen thereby eliciting a stronger immunological response to the poor antigen. The strong antigen to which the target poor antigens are conjugated is called the carrier [143]. Diphtheria anatoxin, tetanus toxoid, and CRM197 are also used as carriers in several widely used, routine childhood and adult conjugate vaccines against encapsulated bacteria such as *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, and *Neisseria meningitidis* [144, 145].

Derivatives of DT as anticancer agents

The ability of native DT to inhibit the growth of malignant cells in resistant to toxin mice has been already known for a relatively long time [146]. The non-toxic to DT-sensitive species CRM197 turned out to be promising in applying to humans. It has been demonstrated that this toxoid effectively inhibits the growth of human malignant cells *in vivo* in nude mice model [147–149] and increases the survival of patients with progressive cancer [4, 150, 151]. There is a lot of evidence that CRM197 is effective in suppressing the cancer of breast [149, 152, 153], oral cavity [154], stomach [155], immune cells [156] and ovaries [147, 148].

CRM197 was introduced into a medical practice for the treatment of human cancer as the main component of BK-UM medication [2–4]. Recombinant CRM197 was produced in *E. coli* which greatly facilitates obtaining of this protein for the manufacturing of diphtheria toxoid-based HB-EGF-targeted medications [86, 88, 89].

The effect of CRM197 on tumors is implemented by the interaction of this protein with soluble HB-EGF. It was demonstrated that proHB-EGF is often overexpressed in transformed cells and that HB-EGF promotes the development of a malignant phenotype. The gene of HB-EGF is considered to be strongly responsible for chemotherapy resistance [157] and oncogenic transformation [59]. Cell treatment with CRM197 leads to reduced malignant potential since when CRM197 is bound to HB-EGF is unable to interact with its cell receptor EGFR [155,157,158]. Nowadays,

it is generally accepted that the mechanism of the CRM197 antitumor action is blocking of the soluble HB-EGF.

However, as a medicine for intraperitoneal administration, CRM197 possess an essential disadvantage, as, like the native DT, it is also highly immunogenic to humans. The anticancer potential of less immunogenic than CRM197 toxin derivatives was not studied properly.

DT-based targeted toxin therapy

Monoclonal antibodies specific for tumor cell surface antigens or their Fv-fragments have been linked to toxins or toxin subunits to generate a new class of therapeutic drugs called immunotoxins. Most often antibodies and their Fv to clusters of differentiation proteins 3 (mostly to CD3ε) [159, 160], 19 [161, 162], and 22 [163, 164] are used as immunotoxin targets. The most known immunotoxin which is based on DT is Resimmune [165]. More information on DT-based immunotoxins could be found in works [161, 162, 166, 167]. Another noteworthy immunotoxin, Moxetumomab pasudotox, was developed based on *Pseudomonas aeruginosa* exotoxin A (PE) [164].

Not only antibodies can be used for targeted toxin therapy — different ligands of the overexpressed receptors in cancer cells, like growth factors, hormones, cytokines and some other specific molecules can be employed as well.

Sometimes, a complete DT molecule was combined with a targeting molecule [168,169]. Since only C- and T-domains are necessary for translocation of SbA, a variety of truncated fragments with no R-domain were obtained. At this point it should be noted that despite R-domain is absent in some DT derivatives, such fragments can still exhibit toxicity in certain cell cultures [170].

For substitution of R-domain instead of antibodies most commonly were used such factors as vascular endothelial growth factor [171, 172], α-melanocyte-stimulating hormone [173], interleukin-2 [174], interleukin-3 [175–177] and interleukine-13 [178], granulocyte-macrophage colony-stimulating factor [179–182], urokinase [183] and even transferrin [184]. In introduction to the medical practice, only interleukin-2 fused to the first 388 amino acids of DT (Denileukin diftitox or Ontak) was successful [185–188]. Interleukin-3 fused to the same DT fragment also demonstrated good results in clinical trials [189, 190], however, it was not introduced in cancer therapy.

DT derivatives, used to construct the targeted toxins are also should possess a high immunogenicity, as they contain the sufficient part of DT molecule.

Immunogenicity of DT derivatives. It remains unclear why DT possesses such strong immunogenic properties compared to other proteins. There is no detailed comparison of the immunogenicity of individual functional domains of the DT molecule, but attempts of such studies have been already done [191]. There are some not systematic data on immunogenicity of different fragments of DT [91, 192, 193] or on the immunodominant areas of DT surface [194], however, it is unknown exactly, which of functional domains is the most immunogenic.

Investigation of the immunogenicity of individual fragments of the DT is valuable for medicine since CRM197 and DT fragments without R-domain for targeted toxins are repeatedly administered in cancer therapy.

When immunogenic DT-based means administered repeatedly, they are fast eliminated from the bloodstream. Directed modification of DT [161] and PE [195–198] is carried out in order to reduce such immunogenicity.

Therefore, the search for DT derivatives that retain the most pronounced anti-tumor effects and possess the least immunogenicity is very perspective. Besides, it is also important to compare the immunogenicity between the variety of derivatives of other toxins (ricin, PE, etc.) used for targeted toxin therapy in order to find those that are the least immunogenic.

In a biological study, DT derivatives are used to investigate the function of respective components of the entire toxin molecule. The least understood question concerning DT functions is the translocation of SbA through the lipid bilayers. The phenomenon of the resistance of some mammalian species to DT has found a peculiar application for a specific ablation of certain cell types in multicellular organisms.

DT is excellent for use in vaccines, both anti-diphtheria and as a carrier protein for antigens of other pathogenic microorganisms. However, the use of DT in medicine is much broader.

Catalytically active SbA of DT complexed to the T-domain is used for the construction of recombinant means for targeted intoxication of cancer cells, like immunotoxins. The peculiarity of the anticancer effect of DT compared to other toxins of different origin is that its non-toxic derivatives, like CRM197, also exert the antitumor effect. Anticancer

properties of the non-toxic DT derivatives are explained by the involvement of DT receptor, which is inactivated by binding to a DT R-domain, in cancerogenesis and versatile range of other cell physiological functions. Therefore, in the anticancer therapy, it is necessary to use simultaneously both distinct functions of DT: toxic for directional cell elimination by targeted toxins and blocking of the soluble HB-EGF for reducing para- and autocrine activation of EGFR in malignant cells.

Thus, DT is suitable for developing on its basis the newest biomedical products and biotechnological application for specific cell elimination, because it has one of the highest toxicity among other toxins and it is easy to obtain its active recombinant forms. However, the main obstacle in application of DT derivatives for the purposes other than immune prophylaxis, like cancer therapy and specific cell ablation — is high immunogenicity. Thereover, the search for the least immunogenic recombinant derivatives of DT is of a high importance for biomedicine.

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БІОЛОГІЧНІ ВЛАСТИВОСТІ ТА МЕДИЧНЕ ЗАСТОСУВАННЯ ПОХІДНИХ ДИФТЕРІЙНОГО ТОКСИНУ

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Метою огляду був аналіз даних літератури, пов'язаних із практичним застосуванням різноманітних похідних дифтерійного токсину. Дослідження взаємодії дифтерійного токсину з чутливими і резистентними клітинами ссавців проводили вже протягом відносно тривалого часу, однак й дотепер існують деякі невирішені проблеми, що стосуються молекулярних механізмів його функціонування. Нативний дифтерійний токсин і частини його молекули, які зберігають токсичність, використовують як інструменти у новітніх біотехнологічних методах специфічного знищення підтипів клітин у багатоклітинних організмах. Нові рекомбінантні похідні дифтерійного токсину періодично отримують у лабораторіях у всьому світі. У біологічних дослідженнях аналоги дифтерійного токсину є зручними засобами для вивчення функцій природного токсину. Нетоксичний аналог дифтерійного токсину, протеїн CRM197, вже введено в клінічну практику як компонент вакцин і протипухлинний агент. Терапія спрямованими токсинами на основі дифтерійного токсину є потенційно перспективною для лікування раку, тому вивчення його похідних має велике значення для біотехнології та медицини.

Ключові слова: клітинна абляція, CRM197, дифтерійний токсин, імуногенність, терапія спрямованими токсинами, токсоїд.

БИОЛОГИЧЕСКИЕ СВОЙСТВА И МЕДИЦИНСКОЕ ПРИМЕНЕНИЕ ПРОИЗВОДНЫХ ДИФТЕРИЙНОГО ТОКСИНА

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Целью обзора был анализ данных литературы, связанных с практическим применением различных производных дифтерийного токсина. Исследования взаимодействия дифтерийного токсина с чувствительными и резистентными клетками млекопитающих проводили в течение относительно длительного времени, однако до сих пор существуют некоторые нерешенные проблемы, касающиеся молекулярных механизмов его функционирования. Нативный дифтерийный токсин и части его молекулы, которые сохраняют токсичность, используют в качестве инструментов в новейших биотехнологических методах специфического уничтожения подтипов клеток в многоклеточных организмах. Новые рекомбинантные производные дифтерийного токсина периодически получают в лабораториях по всему миру. В биологических исследованиях аналоги дифтерийного токсина представляют собой удобные средства для изучения функций природного токсина. Нетоксичный аналог дифтерийного токсина, протеин CRM197, уже введен в клиническую практику как компонент вакцин и противоопухолевый агент. Терапия направленными токсинами на основе дифтерийного токсина является потенциально перспективной для лечения рака, поэтому изучение его производных имеет большое значение для биотехнолоии и медицины.

Ключевые слова: клеточная абляция, CRM197, дифтерийный токсин, иммуногенность, терапия направленными токсинами, токсоид.

EXPERIMENTAL ARTICLES

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APPLICATION OF BIOFILMS IN REMOVAL OF HEAVY METALS FROM WASTE WATER UNDER STATIC CONDITION

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The aim of the research was to apply biofilms as a model in ecotoxicology to remove selected heavy metals (Cd, Cu, Cr, Zn and Pb) from the wastewater under a static conditions. Biofilms were grown in three graded concentrations of the metal leachates (0.625, 0.417 and 0.250%), harvested after 1, 2 and 3 weeks and analyzed for heavy metals. Mean accumulations peaked on Day 21, and of Cd ranged from 0.000 to 0.040 (mean = 0.00837 ± 0.002), Cu from 0.000 to 0.212 (meam = 0.03929 ± 0.012), Cr from 0.000 to 0.500 (mean = 0.05821 ± 0.021), Zn from 0.000 to 1.456 (mean = 0.31833 ± 0.109) and Pb from 0.000 to 0.099 (mean = 0.02129 ± 0.006) mg/g in resultant biofilm formations. Accumulation of the metals increased significantly with time [F(205.59) > Fcrit(3.95)] at the 95% confidence interval. Those of Pb was significantly higher in the 0.625% leachate mixture than control (Sig F = 0.034) at $P < 0.05$, even as those of Cd and Cu were slightly higher in the concentrations than control. Biofilm model removed small amounts of metals from waste water stream in static condition.

Key words: heavy metals, biofilms, bioaccumulation, waste water, static condition.

Heavy metals such as Cu, Fe, Mn, Mo, Zn and Ni can play a role as micronutrients, even though others such as Hg, Pb, Cd, Cu, Ni and Co can be toxic to humans [1]. Due to the discharge of large amounts of metal-contaminated waste waters in recent times, heavy metal pollution of aquatic systems has become one of the most serious environmental problems of concern. Industries bearing such heavy metals as Cd, Cr, Cu, Ni, Pb and Zn are the most hazardous among the chemical-intensive industries because these metals have high toxicity and solubility in the aquatic environment [2]. Heavy metals can be absorbed by living organisms once they enter the food chain, and large concentrations of them may accumulate in aquatic biotopes, as well as human body due to their recalcitrance in the environment [3]. When their concentrations are beyond tolerable levels, they can cause serious health disorders like gastro-intestinal effects, chronic renal disease [4] and central nervous system disorders [5]. Morphological and behavioural abnormalities in fish such as alteration in their sensory reception, reduced responses to normal olfactory function (such as feeding, mating, selection or homing), reduction in swimming performance, gills purge, and ventilation could also result. Other higher organisms may suffer learning impairment, loss of equilibrium that can lapse into paralysis, loss of reproductive efficiency, and irregular metamorphosis as symptoms of toxic exposure to metals [6].

Decontamination of heavy metals from waste water has been a challenge for a while in that, most of the heavy metal salts are soluble in water and form aqueous solution and so, cannot easily be separated using ordinary physical means. However, several different conventional treatment processes are commonly employed to remove heavy metals from industrial waste water before their discharge into the environment [7]. These methods include chemical precipitation, ion exchange, electrochemical treatment,

membrane technologies, and adsorption on activated carbon among others. Each of these methods has significant disadvantages. For instance, chemical precipitation and electrochemical treatments are ineffective, especially when metal ion concentration in aqueous solution is lower than 50mg/l [8]. Moreover, such treatments produce large amounts of sludge that are not environmentally friendly and need to be treated with great difficulties. Ion exchange membrane technologies and activated carbon adsorption processes are extremely expensive [9].

Therefore, there is a need for new, novel, efficient, eco-friendly and cost effective approaches in the treatment, minimization or even elimination of heavy metals in the environment. In this sense, biological alternatives such as the utilization of biofilms have shown promising results even when the metals are present in very low concentrations. Accordingly, the application of these microorganisms in the removal of heavy metals from waste water has been effective and widely recommended [10].

Biofilms are consortia of microbial cell that are attached on solid surfaces or wet environment [11]. In most natural environments, microbes are commonly found in close association with surfaces and interfaces in the form of multicellular aggregates glued together with the slime they secrete [12]. They occur nearly in every moist environment where sufficient nutrient flow is available and surface attachment can be achieved. Biofilms can be formed by a single bacteria cell species, although they can also consist of many species of bacteria, fungi, algae and protozoa [13]. Approximately 97% of the biofilm matrix is either water, which is bound to the capsules of microbial cells or solvent, the physical properties of which (such as viscosity) are determined by the solutes dissolved in it [14]. The formation of Extracellular Polymeric Substances (EPS) enhances the ability of cell to adhere to surfaces with the presence of flagella, pili, fimbriae, or glycocalyx [15]. The diffusion processes that occur within the biofilm matrix are dependent on the water binding capacity and mobility of the biofilm.

Heavy metals uptake by these microbial biomass is a new eco-compatible and economically feasible application that has been develop to remove heavy metals from waste water [16], and studies have shown that interaction of microbial substance with heavy metals reduced heavy metal ion concentrations in solution [17, 3]. This bioremediation option is based on the high metal binding capacity of biological agents, which remove heavy metals from waste water or contaminated sites with high efficiency. Research has revealed that they act as metal biosorbent as they have metal-sequestering properties [18]. Biofilms can decompose or transform hazardous substances into less toxic metabolites or degrade them to nontoxic end products. They can also survive in contaminated habitats because they are metabolically able to exploit contaminants as potential energy sources [19, 20]. In biological treatment or removal of heavy metals, microorganisms with biological activity such as algae, bacteria, fungi and yeast can be used in their naturally occurring forms.

The efficient removal of heavy metals from waste water is dependent on several factors, including sludge concentration, the solubility of metal ions, pH, the metallic concentration and waste water pollution load [21]. However this study was focused on effective removal of heavy metals from static waste water using biofilms; a bioremediation technology that is very important especially in developing countries such as Nigeria where waste water discharge regulations are flouted and treatment does not have top priority due to high cost of treatment facilities.

Meylan et al. [17] and Ogbuagu et al. [3] have conducted experiments on metal accumulation in algal biofilm in lotic streams and observed that biofilms are efficient model for the removal of metals in solution. However, reports on the application of this biological technique in static environments which mimic industrial effluent reservoirs are lacking. It is in this regard that the current study was conducted to investigate possible biosorption of metal contaminants in static set-up.

Materials and Methods

Preparation of metals leachates

Ten grams of soil sample collected from a waste dumpsite that had been in use for over 15 years, situated along Owerri-Aba Road in Owerri was mixed with 1000 ml of surface water sourced from Otamiri River and thoroughly stirred to attain homogeneity. The resulting solution was decanted into 1 litre plastic container as stock solution.

Establishment of leachate concentrations.

Serial concentrations of 75, 50 and 30 ml of the stock solution were made up to 12,000 ml with water sourced from the Otamiri River in 3 different 30 litres aquaria. The aquaria were labeled as Bexp A, Bexp B and Bexp C,

representing the 75ml (0.625%), 50 ml (0.417%) and 30 ml (0.250%) stock leachate-water mixtures respectively. There was also a 4th aquarium designated as Bexp control which served as a control and contained 100% diluent water only. The mixtures were stirred properly.

Shortly after preparations, samples were collected from each of the aquarium in 30 ml sterile plastic bottles, fixed with two drops of concentrated HNO_3, and sent to the laboratory as soon as possible for analysis of heavy metals.

Growth of Biofilms. The biofilms were formed from waste water leachates and were made up of same consortia of bacteria, fungi, etc that have already been established by earlier researchers, as stated in the Introduction of this article.

Biofilms were allowed to grow and investigated under relatively natural conditions in microcosms. The microcosms consisted of sterile plastic containers housing serially arranged sterile glass slides according to the method of Meylan et al. [17] and Ogbuagu et al. [3]. Three replicate microcosms were installed in each aquarium.

Harvest of Biofilms

Serial harvests were made after 1, 2 and 3 weeks from the date of installation. At each time, temperatures and pH of water in the aquaria were taken *in situ*. Biofilms were scraped off the surfaces of glass slides and introduced into sterile sample bottles that had been pre-rinsed with distilled water. The samples were then fixed with 2 drops of conc. HNO_3 for laboratory analysis.

Laboratory Analysis

The metals (Pb, Cd, Cu, Cr and Zn) in the stock solution and biofilm samples were determined using Atomic Absorption Spectrophotometer (AAS) (Varian 600 Spectra AA) after digestion and in keeping with the method of Karvelas et al. [22]. Centrifugation of the biofilm samples was completed during a 30 minutes period at 400 rpm and at 4 °C. A nitrate cellulose filter (0.45µm diameter) was used to filter the content prior to completion of a digestion procedure. Heated mixture of conc. HNO_3 was used for digestion, and the digestion mixture was prepared with 6 ml of 65% HNO_3 and 2 ml of 30% H_2O_2. After centrifugation, distilled water was added to make sample up to 20 ml. The mixture was used for quantification of the metals. Analytical blanks were run in the same way as the samples and concentrations were determined using standards prepared in the acid matrix. The concentration represented the dissolved metals in solution while those collected on the filter paper were digested with aqua regia before AAS analysis. The heavy metals concentrations in solution and filter paper were considered to be the total heavy metal concentrations in biofilm samples, and expressed in mg/g.

Statistical Analysis

The SPSS© V.22.0 and MS Excel© statistical softwares were used to analyze data. The student's *t*-test of significant variation was used to compare heavy metal biosorptions in biofilm formations, while the one way ANOVA and Duncan Multiple Range tests were used to establish homogeneity in mean variance and mean separations of biosorptions respectively at $P < 0.05$. Variation plots were used to represent accumulations of the metals in graded biofilm formations.

Results and Discussion

Water temperature and pH

Water temperature ranged between 27.4 and 35.5 °C in the four aquaria. In Bexp A, Bexp B and Bexp C experimental setups, it ranged from 27.5–35.5 (31.0±4.03), 27.4–35.4 (31.1±4.03) and 27.8–35.3 (31.3±3.78) °C respectively. However, in the Bexp Control setup, it ranged from 27.9–35.4 (31.4±3.77) °C. pH ranged from 4.30–6.45 (5.42±0.06) in the aquaria; with mean values of 6.40±0.01 (Bexp A), 5.21±0.02 (Bexp B), 5.10±0.01 (Bexp C), and 4.33±0.01 (Bexp Control).

Biosorption of heavy metals in biofilms

In the replicates of the 0.625% (Bexp A) biofilm formations, mean accumulations of Cd were 0.0183±0.011 and 0.01±0.008 mg/g, of Cu were 0.098±0.058 and 0.08933±0.051 mg/g, and of Cr were 0.044±0.024 and 0.18533±0.111 mg/g. Mean accumulations of Zn and Pb in the same biofilms were 0.420±0.390 & 0.521±0.386 and 0.0587±0.025 & 0.0400±0.025 mg/g respectively. In Bexp B (0.417%) biofilm formations, mean accumulations of Cd, Cu, Cr, Zn and Pb were 0.0097±0.005 & 0.0103±0.004, 0.0297±0.028 & 0.0277±0.026, 0.0563±0.045 & 0.0477±0.035, 0.463±0.044 & 0.4503±0.042 and Pb 0.020±0.013 & 0.021±0.015 mg/g in the replicates.

In the 0.250% (Bexp C) biofilm formations, mean accumulations of Cd were 0.006±0.004 and 0.012±0.008, Cu 0.050±0.038 and 0.0183±0.007, Cr 0.064±0.026 and 0.067±0.025, Zn 0.340±0.033 and 0.350±0.033, and Pb 0.015±0.008 and 0.015

±0.008 mg/g in the replicates. However, in the control aquarium, mean accumulations of Cd were 0.00067±0.0003 and 0.000±0.000, Cu 0.00067±0.0003 and 0.00033 ±0.0002, Pb 0.00067±0.0003 and 0.00033±0.0002, Cr 0.00033±0.0002 and 0.00033±0.0002, Zn 0.00067±0.0003 and 0.00033±0.0002 mg/g.

Comparison of metal accumulations in biofilms

The one way ANOVA test revealed that the accumulation of Pb was significantly different in the graded biofilm formations (Sig. F = 0.034; $P < 0.05$). A post-hoc Duncan Multiple Range Test revealed that accumulations of Cd and Cu differed significantly between the Bexp A and control biofilm formations at the 95% confidence limit (Table). The accumulation of Pb in the Bexp A biofilms also differed significantly from those of the Bexp C and Bexp Control setups.

Values with same superscript along same rows are not significantly different at P<0.05, Bexp A=0.625% leachate mixture, Bexp B=0.417% leachate mixture, Bexp C=0.250% leachate mixture and Bexp Control= 0.000% concentration.

Effect of time on accumulations of heavy metals in biofilms

Bexp A leachate mixture

On Day 7, mean accumulations of Cd, Cu, Cr, Zn and Pb were 0.0045±0.0005, 0.0200±0.001, 0.0005±0.0003, 0.022±0.001 and 0.0081±0.001 mg/g respectively in the biofilm formations (Fig. 1). On Day 14, mean accumulations of the respective metals were 0.008±0.002, 0.056±0.009, 0.0695±0.015, 0.0625±0.026 and 0.048±0.034 mg/g in the biofilm formations (Fig. 2).

On Day 21 mean accumulations of the metals were 0.030±0.001, 0.206±0.006, 0.274±0.226, 1.328±0.128 and 0.092±0.007 mg/g respectively in the biofilm formations (Fig. 3).

Bexp B leachate mixture

On Day 7, mean accumulations of the metals (Cd, Cu, Cr, Zn and Pb) were 0.005±0.001, 0.0005±0.0002, 0.000±0.000, 0.016±0.004 and 0.0005±0.0003 mg/g respectively (Fig. 1). On Day 14, mean accumulations of the metals were 0.006±0.001, 0.0025±0.0005, 0.0255±0.0025, 0.030±0.001 and 0.013±0.001 mg/g respectively in the biofilm formations (Fig. 2). On Day 21, mean accumulations of the respective metals were 0.019±0.001, 0.083±0.003, 0.1305±0.0155, 1.324±0.024 and 0.048±0.003 mg/g (Fig. 3).

Bexp C leachate mixture

Accumulations also varied in the 0.250% leachate mixture. On Day 7, mean accumulations of Cd, Cu, Cr, Zn and Pb were 0.002±0.001, 0.055±0.002, 0.0325±0.004, 0.0085±0.005 and 0.0005±0.0002 mg/g respectively (Fig. 1). Mean accumulations of the metals were 0.003±0.001, 0.0195±0.003, 0.0495±0.0005, 0.0235±0.004 and 0.017±0.003 mg/g respectively on Day 14 in the biofilm formations (Fig. 2). However, on Day 21, mean accumulations of the metals were 0.022±0.007, 0.0775±0.048, 0.1155±0.001, 1.004±0.004 and 0.028±0.003 mg/g (Fig. 3).

Bexp Control leachate mixture

On Day 7, mean accumulations of Cd, Cu, Cr, Zn and Pb were 0.0005±0.0003, 0.001±0.000, 0.000±0.000, 0.0005±0.004 and 0.0001±0.000 mg/g respectively (Fig. 1). On Day 14, mean accumulations of the respective

Table. **Mean separation of accumulation of heavy metals in biofilms formed in graded leachate mixtures using Duncan Multiple Range Test ($P < 0.05$)**

	Graded concentrations			
Heavy metals	Bexp A	Bexp B	Bexp C	Bexp Control
Cd	0.014167^{a}	0.010000^{ab}	0.009000^{ab}	0.000400^{b}
Cu	0.093833^{a}	0.028667^{ab}	0.034167^{ab}	0.000600^{b}
Cr	0.114667^{b}	0.052000^{b}	0.065833^{b}	0.000400^{b}
Zn	0.470833^{b}	0.456667^{b}	0.345333^{b}	0.000600^{b}
Pb	0.049333^{a}	0.020333^{ab}	0.015000^{b}	0.000600^{b}

Values with same superscript along same rows are not significantly different at $P < 0.05$, Bexp A=0.625% leachate mixture, Bexp B=0.417% leachate mixture, Bexp C=0.250% leachate mixture and Bexp Control= 0.000% concentration.

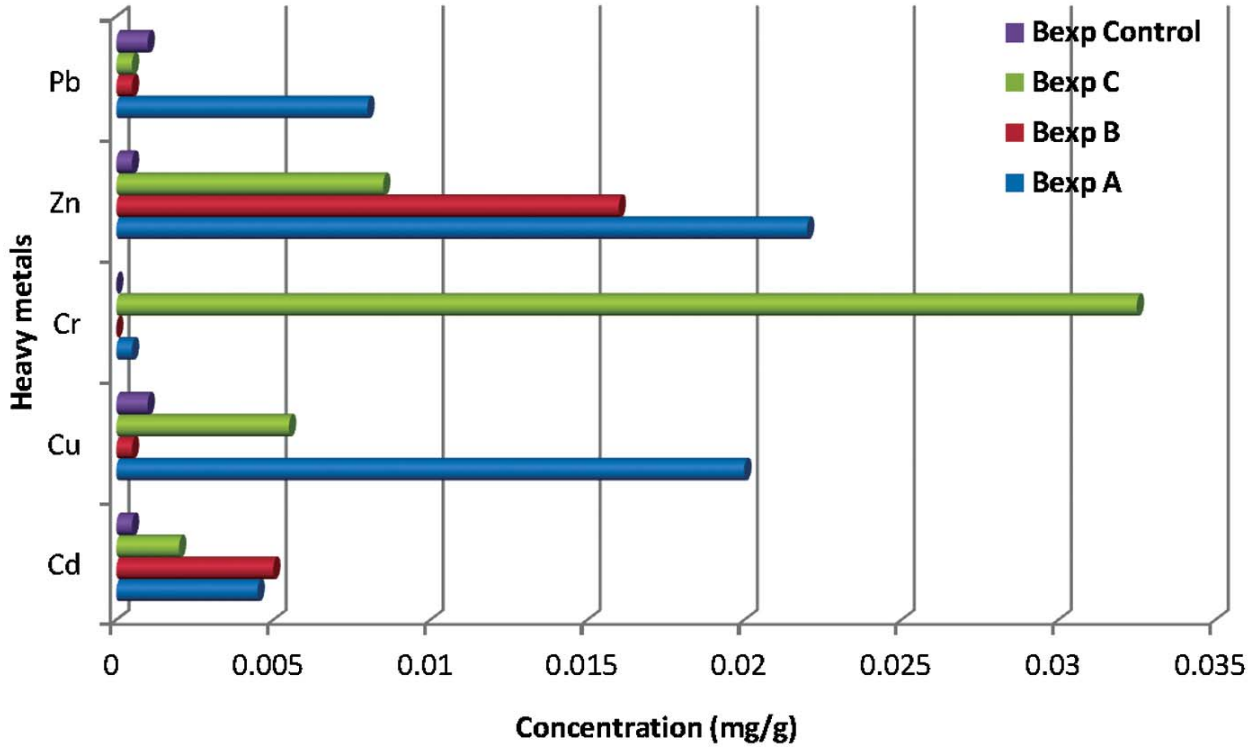

Fig. 1. **Mean accumulation of metals in biofilms formed in graded leachate mixture after 7 days**

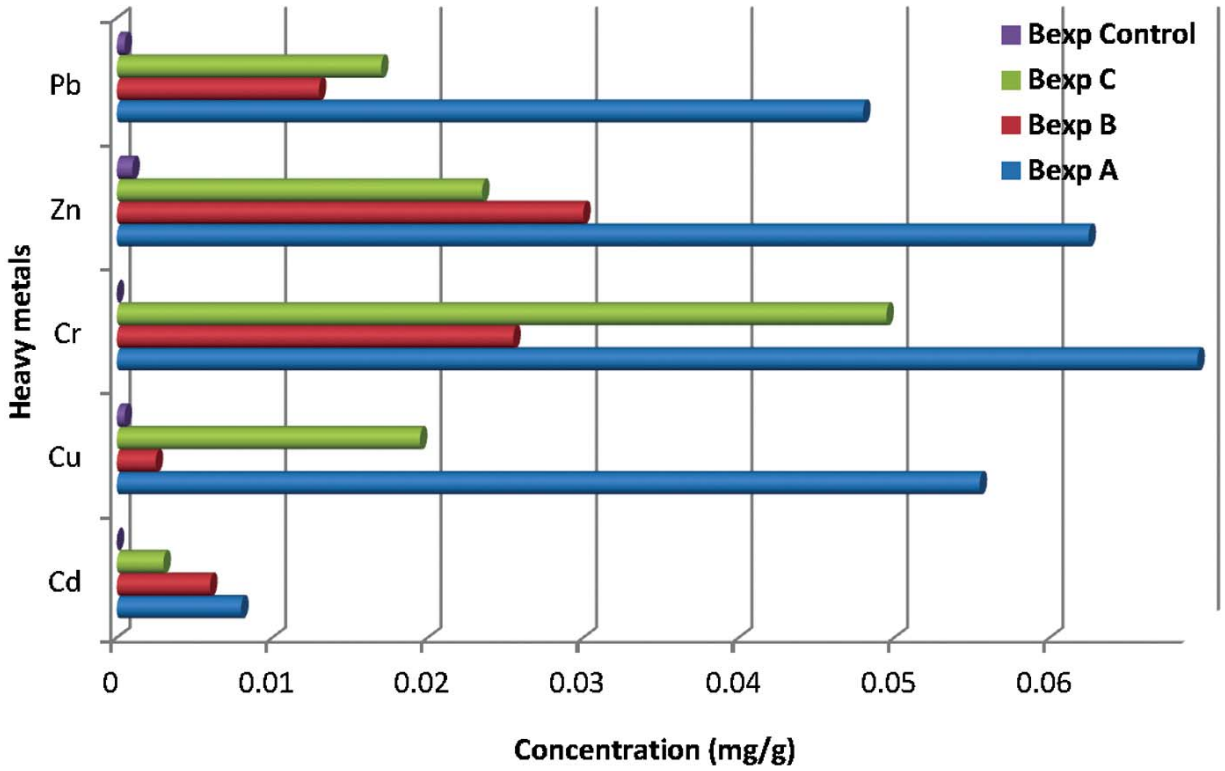

Fig. 2. **Mean accumulation of heavy metals in biofilms formed in graded leachate mixture after 14 days**

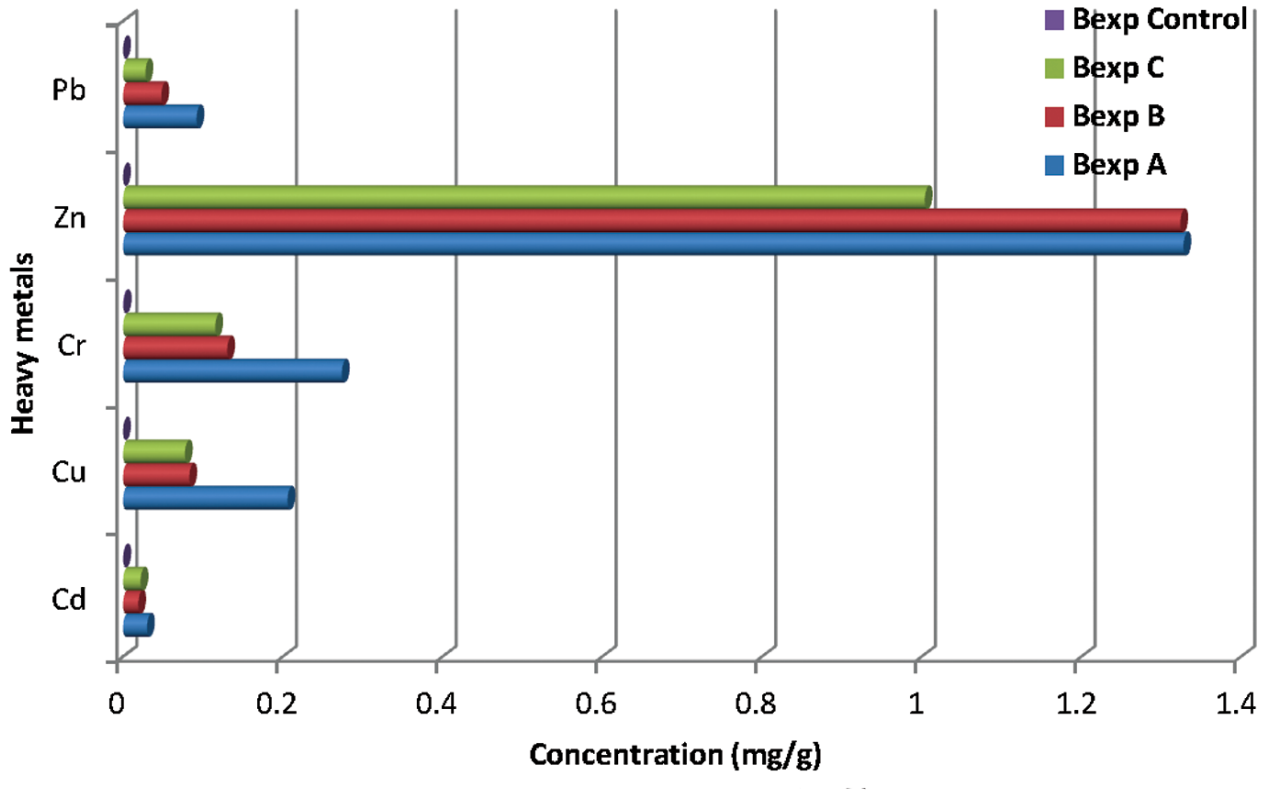

Fig. 3. **Mean accumulation of heavy metals in biofilms formed in graded leachate mixture after 21 days**

metals were 0.000±0.000, 0.005±0.001, 0.000±0.000, and 0.001±0.000 and 0.0005±0.0003 mg/g in the biofilm formations (Fig. 2). On Day 21, mean accumulations of the metals were 0.0005±0.0002, 0.000±0.000, 0.000±0.000, 0.000±0.000 and 0.000±0.000 mg/g (Fig. 3).

The ANOVA test of homogeneity revealed that accumulations of the metals differed significantly over the 21 days experimental period [F(205.59)>Fcrit (3.95); $P < 0.05$].

The mere observation of accumulation of some trace metals in this work confirmed that biofilm models can offer some solution in the removal of heavy metals from waste water streams even in static conditions. However, the rate and amount of accumulations were less than those observed in lotic aquatic environments by Doering and Uehlinger [23] in the Tagliamento River in Europe, Ogbuagu et al. [3] in Otamiri River in Nigeria and Meylan et al. [17] in the Furtback, Canton of Zurich. This technique therefore holds promises for effective, inexpensive and eco-friendly metal bioremediation technology for the removal of recalcitrant contaminants such as the persistent organic pollutants (including heavy metals) from complex industrial effluents, and hence can offer pollution free environment if optimized. Less biofilm formations were observed in this work and it could be attributed to absence of renewal and replenishment of biomass which are usually associated with lotic, but lacking in static conditions.

The observed significantly higher accumulations of Cd, Cu and Pb in the Bexp A than Control biofilms reflect bioavailability of the trace element in the treatment mixture. However, Cd, Cu and Pb were more readily removed from the leachate mixtures than Zn and Cr. This is similar to the observation of Azizi et al. [24], that Cu, among other metals was more readily removed from waste water stream. This research [24] presents the results of an evaluation of the removal of selective heavy metals (Cd, Cu, Ni and Zn) from waste water through a Modified Packed Bed Biofilms Reactor (PBBR).

The graded leachate mixtures were also associated with different pH levels, but their resultant biosorption trend did not support the observation of UNEP GEMS

[25] that heavy metals are usually more bioavailable in acidic media, and so would get more biosorbed. Rather, it appeared to be in consonance with the observation of Huang et al. [26] that pH had no significant effects on heavy metals (Cr, Ni, Cu, Zn, Cd, Pb) release in Huangpu River sediments, East China. The reason for this non-release could be attributed to metal speciation. In that work, Huang et al. [26] observed that even when available, the exchangeable fraction of the metals was only about 0.06 to 2.63% of all the metals in the sediment, while the residual fraction, which is the most stable one, accounted for about 51.50 to 86.45% of all the metals in the sediment. These reasons may have contributed to the low release flux of the metals studied.

Time played a vital role in the uptake of the metals in that development of biofilms took some time and at early stages, there were little or no accumulations of the metals. This was because biofilm communities require time to establish themselves hence, microbial dose or concentration also determined rate of metal uptake in the aquaria. However, data on the accumulation of Cr and Cu especially on Days 7 and 14 days does not seem to indicate the existence of a dose-dependent effect. This could reflect the early presence of little or no biofilm formations on those days to cause accumulation.

Heavy metal removal efficiency in this study was dependent on the presence of microorganisms as well as concentration of metal ions in solution. Deibel and Schoeni [13] had documented that biofilms can consist of many species of bacteria, fungi, algae and protozoa, though their composition in the current study was not determined. Accordingly, Chipasa [21] observed that biosorption became apparent as higher metal concentrations stimulated increased microbial activity, and so, increased removals from leachate mixture. This again is related to bioavailability of the trace elements. However, this was partially in consonance with the observations of Piccirillo and Pereira [27] and Karvelas et al. [22] wherein heavy metal removal efficiency was also dictated by the influence of microorganism but became apparent at lower metal concentrations. They rather observed that higher concentrations of metals reduced their removal from waste water stream, and so explained that this could be due to reduced activity of microorganisms in the system due to higher concentration of metals inducing stress on the microorganisms by reducing their action towards the metals. This in turn would negatively affect the functioning of biological treatment process.

The following observations were made in the study:

1. Biofilm model removed some heavy metals, especially Pb, Cd and Cu in static condition.
2. Less biofilm formations and bioaccumulation of metals were observed in static than reference lotic conditions.
3. Removal of metals was dose dependent in the biofilms; and
4. Removal of metals was also time dependent.

This study revealed that the application of biofilms can offer some solutions in the removal of heavy metals from waste waters in static condition.

Recommendations

Based on these findings, it is recommended that biosorptive method of metal removal from effluent streams in static condition should be encouraged and optimized as a more attractive and economic alternative in environmental solutions. Further kinetic studies on the relationship between metals accumulation in biofilms and time should be carried out.

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ЗАСТОСУВАННЯ БІОПЛІВОК ДЛЯ ВИДАЛЕННЯ ВАЖКИХ МЕТАЛІВ ЗІ СТІЧНИХ ВОД ЗА СТАТИЧНИХ УМОВ

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Метою дослідження було використання біоплівок як екотоксикологічної моделі для видалення відібраних важких металів (Cd, Cu, Cr, Zn і Pb) зі стічних вод за статичних умов. Біоплівки вирощували в трьох відградуйованих концентраціях фільтратів металів (0,625, 0,417 і 0,250%), збирали через 1, 2 і 3 тижні й аналізували на вміст важких металів. Середні значення піків в утворених біоплівках досягли максимуму на 21-й день: Cd — від 0,000 до 0,040 (середнє значення = 0,00837 ± 0,002), Cu — від 0,000 до 0,122 (середнє значення = 0,03929 ± 0,012), Cr — від 0,000 до 0,500 (середнє значення = 0,05821 ± 0,021), Zn — від 0,000 до 1,456 (середнє значення = 0,31833 ± 0,109) і Pb — від 0,000 до 0,099 (середнє значення = 0,02129 ± 0,006) мг/м. Накопичення металів істотно збільшилось із часом [F (205,59) > Fcrit (3,95)] за 95%-го довірчого інтервалу. Значення для Pb були значно вищі в 0,625%-й вилуговуваній суміші фільтрату, ніж контроль (Sig F = 0,034) за $P < 0,05$, тимчасом як для Cd і Cu — трохи вищі, ніж контрольні. Застосування біоплівки призвело до невеликого видалення важких металів зі стічних вод за статичних умов.

Ключові слова: важкі метали, біоплівки, біоакумуляція, стічні води, статичні умови.

ПРИМЕНЕНИЕ БИОПЛЕНОК ДЛЯ УДАЛЕНИЯ ТЯЖЕЛЫХ МЕТАЛЛОВ ИЗ СТОЧНЫХ ВОД В СТАТИЧЕСКИХ УСЛОВИЯХ

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Целью исследования было использование биопленок в качестве экотоксикологичекой модели для удаления отобранных тяжелых металлов (Cd, Cu, Cr, Zn и Pb) из сточных вод в статических условиях. Биопленки выращивали в трех градуированных концентрациях фильтратов металлов (0,625, 0,417 и 0,250%), собирали через 1, 2 и 3 недели и анализировали на содержание тяжелых металлов. Средние значения пиков в образовавшихся биопленках достигли максимума на 21-й день: Cd — от 0,000 до 0,040 (среднее значение = 0,00837 ± 0,002), Cu —от 0,000 до 0,122 (среднее значение = 0,03929 ± 0,012), Cr — от 0,000 до 0,500 (среднее значение 0,05821 ± 0,021), Zn — от 0,000 до 1,456 (среднее значение = 0,31833 ± 0,109) и Pb — от 0,000 до 0,099 (среднее значение = 0,02129 ± 0,006) мг/г. Накопление металлов существенно увеличилось со временем [F (205,59) > Fcrit (3,95)] при 95%-м доверительном интервале. Значения для Pb были значительно выше в 0,625%-й выщелачивающей смеси фильтрата, чем контроль (Sig F = 0,034) при $P < 0,05$, в то время как для Cd и Cu несколько превышали контрольные. Применение биопленки привело к небольшому удалению тяжелых металлов из сточных вод в статических условиях.

Ключевые слова: тяжелые металлы, биопленки, биоаккумуляция, сточные воды, статические условия.

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BIOSYNTHESIS OF VOLATILES BY *Pleurotus ostreatus* (Jacq.:Fr.) Kumm. MUSHROOMS ON SUBSTRATES ENRICHED WITH VEGETABLE OILS

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The purpose of the study was to analyze the ways and intensity of synthesis of volatile flavor compounds by *Pleurotus ostreatus* (Jacq.:Fr.) Kumm. mushrooms in the process of intensive cultivation on sunflower husk and barley straw with the addition of vegetable oils (sunflower and corn) as a potential source of unsaturated fatty acids. Methods of sensory profile analysis and ultraviolet spectroscopy were used. Sensory profile analysis of dried samples of fruit bodies showed an increase in the intensity of mushroom, meat and grassy notes of flavor on substrates with the addition of vegetable oils in a concentration of 1% and 5% of the weight of the substrate. For the strain IBK-551 marked increase in the intensity of sweet and floral attributes of the aroma on both substrates with the addition of corn oil. UV spectroscopy of hexane extracts of dried samples of fruit bodies revealed maxima of light absorption in the range of 200–210 nm and 260–300 nm. There was a difference in intensity of light absorption of samples of different strains cultivated on substrates with the addition of vegetable oils.

Key words: *Pleurotus ostreatus,* volatile aroma compounds, sunflower oil, corn oil, sensory profile analysis, UV spectroscopy.

In many countries, mushrooms and dishes from them are considered to be delicacies due to their specific aroma, which is provided by hundreds of volatiles such as octane derivatives and octens, lower terpenes, benzaldehyde, sulfur compounds, and others. Fatty C8-compounds such as 1-octanol, 3-octanol, 3-octanone, 1-octen-3-ol, 2-octen-1-ol, 1-octen-3-one are formed in lipoxygenase pathway from polyunsaturated fatty acids.

Mushrooms have long been used as food or food flavoring materials because of their unique and flavor. Fresh and preserved mushrooms are consumed in a lot of countries as a delicacy, particularly for their specific aroma and texture [1].

Consumers highly appreciate the characteristic flavor of mushrooms, which consists of hundreds of odourous compounds such as derivatives of octane and octenes, lower terpenes, derivatives of benzaldehyde, sulphur compounds and others [1]. Volatile aroma substances of edible mushrooms may be divided into three groups: carbon-eight (C8) derivatives impart the "characteristic mushroom flavor", terpenoid volatiles and sulfur-containing odour compounds [2].

Eight-carbon volatile compounds are a key contributor to mushroom flavor and have been described by many authors in fungi [3]. 1-Octanol, 3-octanol, 3-octanone, 1-octen-3-ol, 2-octen-1-ol, and 1-octen-3-one are the main of them. They account for 44,3–97,6% of the total volatile fraction. [4].

The aliphatic alcohol 1-octen-3-ol (first called "matsutake alcohol") is the principal compound contributing to the unique mushroom aroma and flavor [3].

Isoprenoids (also known as terpenoids) belong to a group of secondary metabolites that are synthesized in mushrooms. Twenty-four isoprenoids have recently been reported in fruiting bodies of the white *Tuber magnatum*. Limonene and cedrol were the most abundant of them [5]. Aromadendrene, alpha-farnesene and other terpenoid compounds were detected in *T. borchii* fruit body [6].

Sulfur volatile organic compounds (S-VOCs) are key contributors to truffle aroma. The diversity of sulfur volatiles in truffles is large, ranging from relatively small compounds, such as dimethyl mono- (DMS), di- (DMDS) and tri- (DMTS) sulfides, which are produced by most truffle species, to complex S-volatiles such as 2-methyl-4,5-dihydrothiophene, characteristic of the white truffle *T. borchii*, and bis(methylthio) methane, characteristic of the white truffle *T. magnatum*. The latter species contain further 27 sulfur volatiles [5].

The characteristic "sulfurous" note of Shiitake mushroom is composed from S-compounds. They include the straight chain compounds dimethyl disulfide, dimethyl trisulfide, 1-(methylthio)dimethyl disulfide and cyclic compounds lenthionine (1,2,3,5,6-pentathiepane, $C_2H_4S_5$), 1,2,4-trithiolane ($C_2H_4S_3$), 1,2,4,5-tetrathiane ($C_3H_6S_4$) and 1,2,3,4,5,6-hexathiepane (CH_2S_6) [7].

Eight-carbon volatiles are enzymatically formed by oxygenation of polyunsaturated fatty acids (PUFAs). This reaction is catalyzed by lipoxygenases (LOXs), thus starting the so-called lipoxygenase (LOX) pathway [8]. Metabolites originating from these pathways are collectively named oxylipins [9].

The purpose of the study was to analyze possible ways of synthesis of volatile flavor compounds in mushrooms and to determine the intensity of this synthesis by *Pl. ostreatus* (the oyster mushroom) in the process of intensive cultivation on substrates with the addition of vegetable oils as a potential source of unsaturated fatty acids, which are the main substrates for the synthesis of aliphatic aroma compounds.

Sunflower (*Helianthus annuus* L.) and corn (*Zea mays* L.) oils as vegetable oils were used in this work. Sunflower oil contains up to 90% unsaturated fatty acids (linoleic and oleic) and up to 10% saturated fatty acids (palmitic and stearic) [10]. Quantitatively the predominant components of the fatty acid composition of corn oil are palmitic, oleinic and linoleic acids [11].

The composition of fatty acids of sunflower [12] and corn [13] oils is given in Table 1.

Materials and Methods

Mushroom strains. Three strains of the edible mushroom *Pleurotus ostreatus* (Jacq.:Fr.) Kumm.: IBK-549, IBK-551 and IBK-1535 from the mushroom collection of the Kholodny Institute of Botany of the National Academy of Sciences of Ukraine were objects of the study [14]. This mushroom belongs to the *Pleurotaceae* family of *Agaricales* of the *Agaricomycetes* class of the *Basidiomycota*, regnum *Fungi*.

Solid-phase cultivation. The substrate for the production of fruiting bodies was the agricultural waste: sunflower husk and barley straw. As additives to substrates, sunflower and corn oils were used at a concentration of 1% and 5% of the weight of the wet substrate. The sunflower oil for unrefined cold pressing of the first spin of the first grade [12] and the corn oil refined deodorized grade P [13] were used in the research.

Preparation and sterilization of substrates were carried out according to commonly accepted methods [15]. The substrate was evaporated for 2 hours, $CaCO_3$ was added in an amount of 1% to the mass of the substrate and sterilized twice autoclaving at 121 °C for 30 minutes with an interval of 24 hours. Straw was pre-minced to a size of 2–3 cm. The cooled substrate was inoculated with *Pl. ostreatus* mycelium in an amount of 5% by weight of the substrate. Seeding mycelium was obtained on barley grain. Cultivation was carried out in glass jars at 26–28 °C and 70–80% humidity to the full mycelial overgrowth of the substrate. The weight of the wet substrate on the basis of sunflower husk in one jar was 150 g, and on the basis of barley straw — 110 g. Containers with the substrate were transferred to a growth room with a temperature of 15–16 °C, humidity of 80–90% and 8-hour photoperiod after full growth of the substrate by mycelium. The 1st and 2nd flushes were harvested. Mushrooms were dried at 40–45 °C in a dry oven for 24–48 hours.

Growing strains of *Pl. ostreatus* on the substrates without the addition of oils was used as a control experiment.

Table 1. **Fatty acid composition of vegetable oils**

Kind of oil	Mass fraction of fatty acid,% to the sum of fatty acids				
	Palmitic $C_{16:0}$	Stearic $C_{18:0}$	Oleic $C_{18:1}$	Linoleic $C_{18:2}$	Linolenic $C_{18:3}$
Sunflower oil	3.0–10.0	1.0–10.0	14.0–35.0	50.0–75.0	<1.0
Corn oil	9.0–14.0	0.5–4.0	24.0–42.0	34.0–62.0	<2.0

During the cultivation process, the following growth parameters of the *Pl. ostreatus* mycelium were determined: the time of the mycelial development on the substrate, the time of primordia formation, the number of formed bunches per 100 g of wet substrate, and the yield of the first and second flushes of fruitage per 100 g of wet substrate. Cultural and morphological mushroom features were studied in order to establish a relationship between them and the synthesis of aroma-forming substances.

Sensory profile analysis. The sensory profile of the aroma of dried mushroom samples was studied according to [16].

The panel consisted of 5 experts trained for organoleptic analysis. First, the characteristic attributes of the aroma were determined, and then the intensity of each of them on a 5-point scale: 0 — not present; 1 — just recognizable or threshold; 2 — weak; 3 — moderate; 4 — strong; 5 — very strong. The studied samples were evaluated three times.

The organoleptic evaluation of different strains of dried mushrooms, collected at the same stages of maturation, was carried out in specially prepared, well-ventilated rooms at the Department of Biotechnology of the Ukrainian State University of Chemical Technology.

Microsoft Office Excel 2007 software was used to construct the aroma profiles of dried mushroom samples.

Spectrophotometric analysis. For a spectrophotometric study, the dried fruiting bodies of the first flush were crushed on an electric mill to a powder. 1 g of the obtained material was placed in the extractor, then 100 cm^3 of solvent were added (the hydromodule was 1:100). Hexane was used as a solvent. Extraction was carried out at boiling point (69 °C) of the solvent for 30 minutes. The extracts were cooled in a fume hood, filtered through a paper filter on a Buchner funnel and transferred quantitatively into a volumetric flask of 250 cm^3. Then the solvent volume was adjusted to the mark. Absorption spectra were recorded using a spectrophotometer SF-2000 in the 200–350 nm wavelength range. Pure hexane was used as a comparative solution.

Statistical analysis. The obtained data were processed statistically using one-way analysis of variance [17]. All samples were carried out in triplicate. Values are presented as means ± standard error of the mean. Differences at $P \leq 0{,}05$ were considered to be significant.

Results and Discussion

Possible ways of synthesis of volatile flavor compounds in mushrooms.

Taking into account the data of other authors and our analysis, the most probable way of synthesis of 1-octen-3-ol is presented in Figure 1.

Fatty acids are precursors in the reaction of eight-carbon volatile synthesis and are key components in a variety of lipids [3].

Lipoxygenases (linoleate: oxygen oxidoreductase, EC 1.13.11.12; LOXs) are

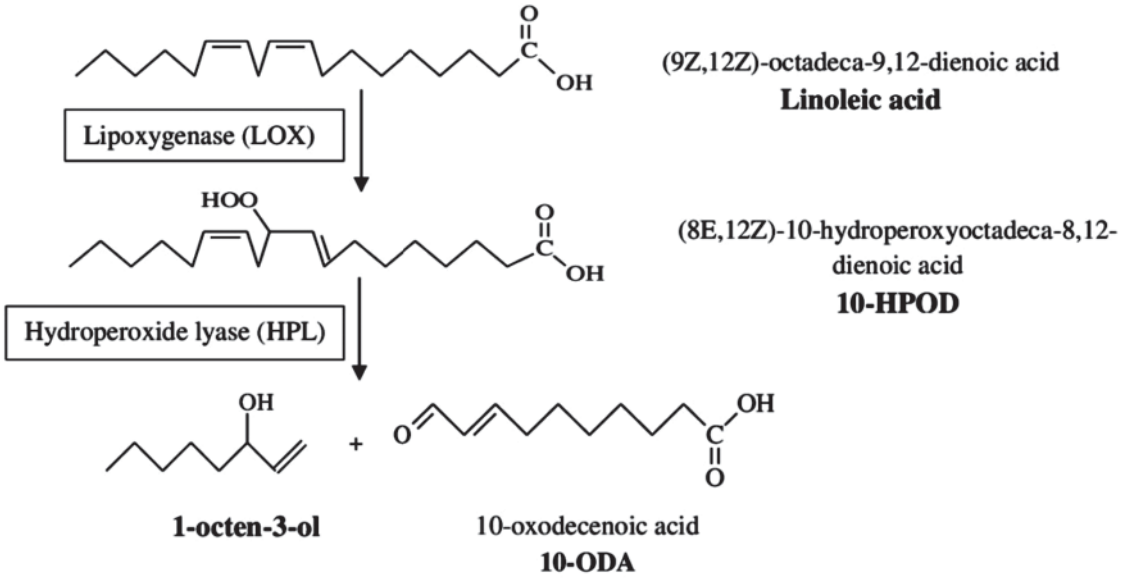


Fig. 1. **Lipoxygenase pathway of biosynthesis of 1-octen-3-ol:** (based on [3, 19, 21–23] with our modifications)

a family of enzymes found ubiquitously in plants and mammals, but have also been detected in coral, algae, fungi, yeast and a number of bacteria. In general LOXs are non-heme iron-containing dioxygenases [9]. This group of enzymes catalyzes the insertion of molecular oxygen into polyunsaturated fatty acids containing a (1Z,4Z)-pentadiene system, such as linoleic acid, linolenic acid, and arachidonic acid, yielding the corresponding hydroperoxides [18].

Although free fatty acids is the preferred substrate of LOX, it was recently reported that fatty acids acylated to phospholipids are slowly oxygenated by lipoxygenases [19].

A lot of researches conducted to study the structure and properties of plant and animal lipoxygenases.

Oxygen insertion into PUFAs by LOX is regio- and stereo-specific, and this specificity is used as the decisive criterion for LOX classification. In plants, linoleic acid and linolenic acid are the primary substrates of LOX, because they are the most abundant fatty acids. Plant LOXs are classified as 9- or 13-LOXs with respect to their positional specificity of linoleic acid oxygenation. Animal LOXs are classified as 5-, 8-, 9-, 11-, 12-, or 15-LOXs with respect to their positional specificity of the oxygenation of arachidonic acid, the predominant substrate of animal LOXs. Furthermore, LOXs are classified as S-or R-LOXs on the basis of the chirality of their hydroperoxide products [18].

Fungal LOXs, similar to plant LOXs, act mainly on C_{18} fatty acids. Linoleic acid (18:2, $\Delta^{9,12}$) is the most abundant in widely appreciated cultivated mushrooms, followed by palmitic and stearic acids. Most fungi convert C_{18} fatty acids into 9-hydroperoxy or 13-hydroperoxy fatty acids (i.e. they contain 9-LOX and 13-LOX, respectively). Interestingly, 10-hydroperoxy and 12-hydroperoxy fatty acids can also be formed as possible products of fungal polyunsaturated fatty acid metabolism [20].

Products formed by LOXs can be converted by a hydroperoxide lyase (HPL), an allene oxide synthase (AOS), a peroxygenase or a reductase.

Hydroperoxide lyases are said to perform either homolytic or heterolytic cleavage of hydroperoxides, yielding different short-chain volatiles, depending on their cleavage mode. The first cleavage mechanism, homolytic, involves the cleavage of the hydroperoxide between the carbon bearing the hydroperoxide group and the saturated carbon. This mechanism has been observed in algae and mushrooms. The second mechanism is called heterolytic. It is found in most plants: the enzyme cleaves the hydroperoxide between the carbon bearing the hydroperoxide group and the unsaturated carbon [3].

Wurzenberger and Grosch showed that after incubation of the 9-, 10-, 12- and 13-hydroperoxide isomers of linoleic with a protein fraction of mushroom *Psalliota bispora* only 10-hydroperoxide isomer was cleaved to 1-octen-3-ol and 10-oxo-*trans*-8-decenoic acid [21].

Results of Akakabe et al. indicated a stereochemical correlation between (R)-1-octen-3-ol and (S)-10-hydroperoxy-(8E,12Z)-8,12-octadecadienoic acid [(S)-10-HPODE] with homogenates of *Lentinula edodes* and *Tricholoma matsutake* [22].

Assaf et al. showed that 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13-HPOD) and 10-oxo-*trans*-8-decenoic acid (10-oxo-acid) were found to be the major nonvolatile metabolites of *Pl. pulmonarius* submerged culture, associated with the enzymatic cleavage of linoleic acid to 1-octen-3-ol. But despite its accumulation, 13-HPOD was found not to be the precursor of 1-octen-3-ol. These results suggest the involvement of two different lipoxygenases in 1-octen-3-ol and 13-HPOD formation [23].

But besides 1-octen-3-ol, other aliphatic saturated and unsaturated C_6-C_{10} compounds, which are likely to be formed with the participation of other lipoxygenases and hydroperoxidilases, are responsible for the formation of the characteristic aroma of mushrooms. Probably there are several metabolic pathways of the synthesis of volatile aroma compounds in fungal cells.

Terpenoids constitute the most abundant and structurally diverse group of plant secondary metabolites [24] that is why the metabolic pathways for the formation of volatile aroma compounds of terpenoid nature are sufficiently studied and highlighted in the literature on the example of plant organisms. The scheme of biosynthesis of terpenoid compounds is shown in Fig. 2.

Terpenoids are derived from the universal C_5 precursor isopentyl diphosphate (IPP) and its allylic isomer dimethylallyldiphosphate (DMAPP), which in higher plants are generated from two independent pathways located in separate intracellular compartments. In cytosol, IPP is derived from the mevalonic acid (MVA) pathway that starts with the condensation of acetyl-CoA. In plastids, IPP is formed from pyruvate and glyceraldehydes-3-

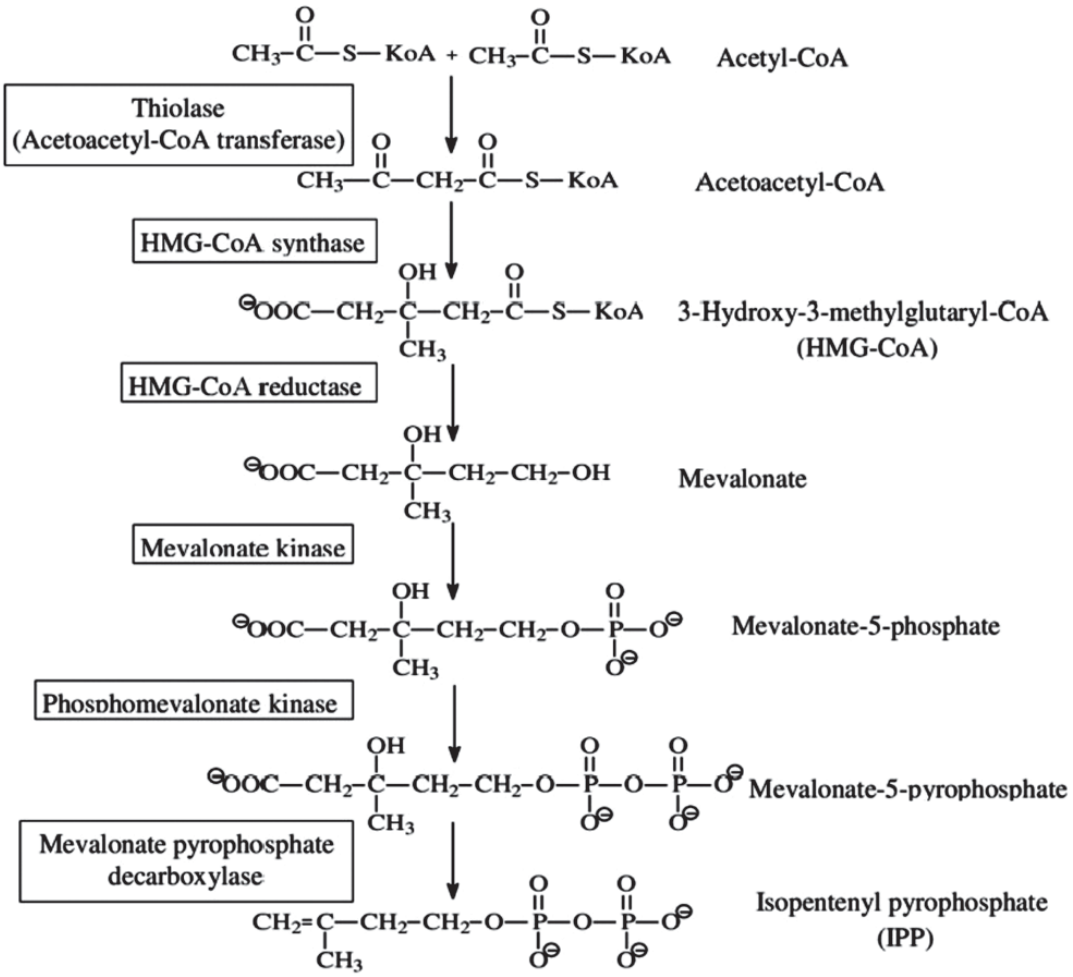


Fig. 2. **Mevalonate pathway of biosynthesis of terpenoids:**
based on [24–26] with our modifications

phosphate. This MVA-independent pathway, also called MEP pathway after the key intermediate methylerythritol phosphate (MEP), was discovered [24–26].

Two main S-VOC biosynthetic pathways (Fig. 3), both relying on L-methionine (Met) catabolism, have been investigated on bacteria and ascomycetes: the one-step conversion of L-methionine to metanethiol (MTL) by methionine lyase (a typical bacterial rather than yeast enzyme) or by cystathionine lyase; and a two-step pathway, initiated by L-methionine transamination to 4-methylthio-2-oxobutyric acid, which is then converted to 3-(methylthio)propanal (also known as methional) via decarboxylation, with the ultimate formation of MTL [5].

Lenthionine (1,2,3,5,6-pentathiepane), a cyclic sulfur compound found in *L. edodes*, is derived from a γ-L-glutamyl-cysteine sulfoxide precursor (lentinic acid) in a two-step enzymatic reaction. Lentinic acid is first activated by the removal of its γ-glutamyl moiety catalyzed by γ-glutamyl transpeptidase (GGT) producing a L-cysteine sulfoxide derivative, which then undergoes α, β-elimination catalyzed by cysteine sulfoxide lyase, resulting in a highly reactive sulfenic acid intermediate. The sulfenic acid is then rapidly condensed to form thiosulfinate, and the thiosulfinate is often further transformed into other sulfur compounds including lenthionine (Fig. 4) [28].

The analysis of possible ways of synthesis of volatile flavor compounds in mushrooms showed that the precursors of this synthesis are organic acids, including those that are part of vegetable oils.

Culture and morphological characteristics of fungal growth depending on the type of investigated substrate

The term of overgrowing of the substrate by mycelium was on the studied substrates

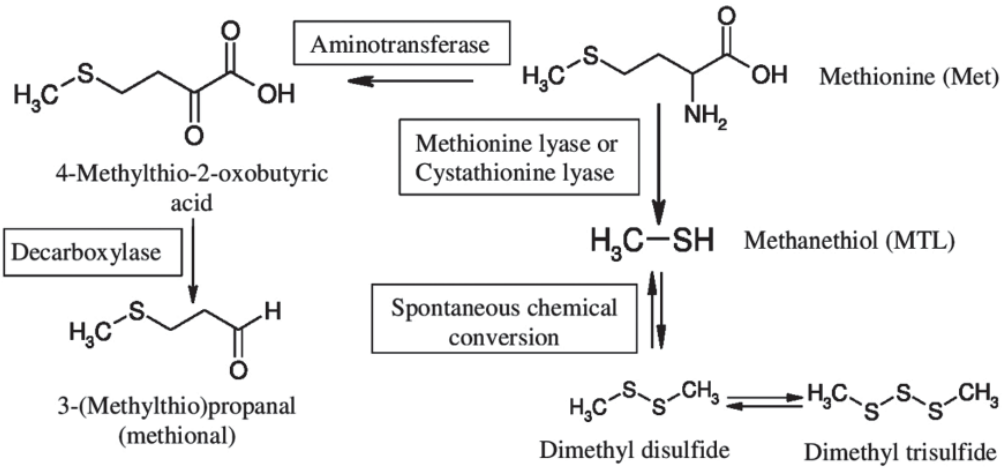


Fig. 3. **Biosynthetic pathways for the synthesis of sulfur volatiles:** based on [5, 27] with our modifications

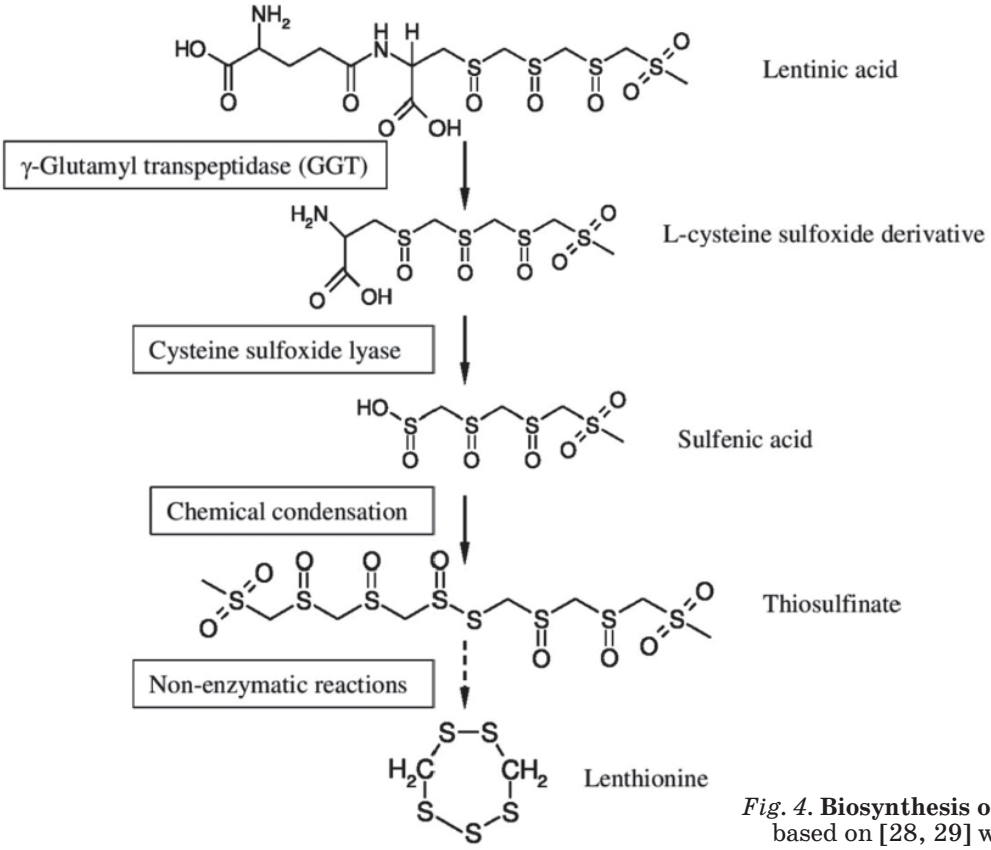


Fig. 4. **Biosynthesis of cyclic sulfur volatiles:** based on [28, 29] with our modifications

from 6 to 7 days, that is, there was no significant difference between this growth rate in different experimental variants compared with the control. According to the morphological features, *Pl. ostreatus* mycelia of all examined strains were white, fluffy, and denser in sunflower husk.

Pl. ostreatus fruit bodies cultivated on substrates with the addition of sunflower oil (A) and corn oil (B) are shown in Fig. 5.

Fruit bodies, obtained on different substrate variants within the mushroom strain, did not differ morphologically.

The growth parameters of *Pl. ostreatus* IBK-549, IBK-551 and IBK-1535 on different substrates are given in Table 2.

The period of primordial emergence varied depending on the mushroom strain from 18 to 30 days and did not differ significantly in various variants of substrates within the strain

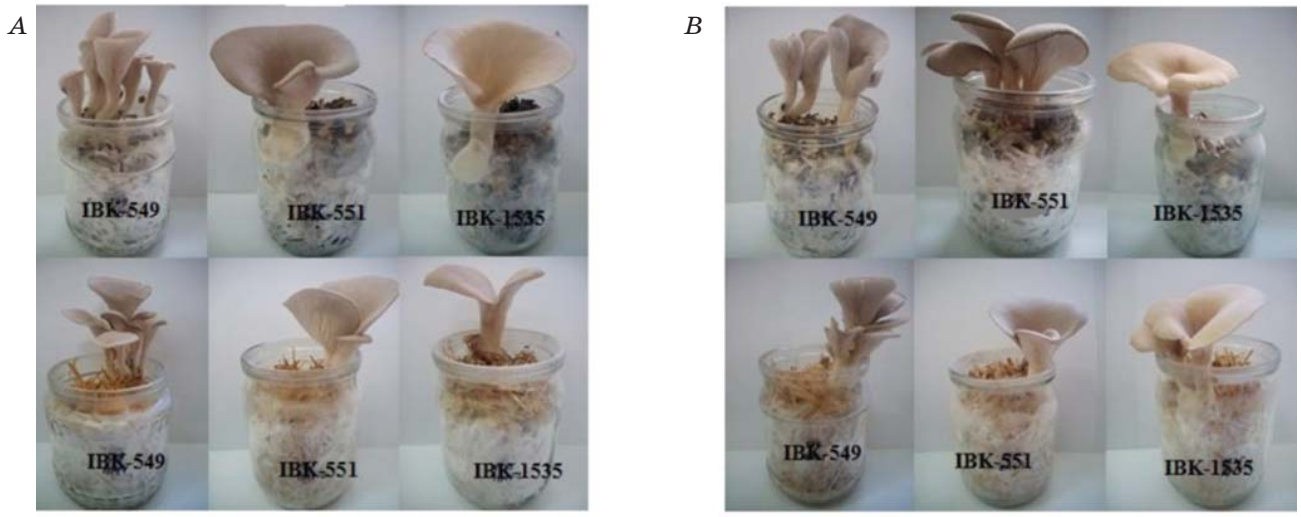


Fig. 5. **Fruiting bodies of various strains of *Pleurotus ostreatus* (Jacq.:Fr.) Kumm. cultivated on substrates with:** the addition of sunflower oil (*A*) and corn oil (*B*) (the upper line — the substrate is sunflower husk; the lower line — the substrate is barley straw)

Table 2. **The growth parameters of *Pleurotus ostreatus* strains on various substrates**

Substrate version	Time of primordial emergence. day	First flush of fruitage. day	Number of mushroom bunches per 100 g of substrate. pcs	Mushroom yield of the 1st flush. g/100 g	Mushroom yield of the 2nd flush. g/100 g	Time of primordial emergence. day	First flush of fruitage. day	Number of mushroom bunches per 100 g of substrate. pcs	Mushroom yield of the 1st flush. g/100 g	Mushroom yield of the 2nd flush. g/100 g
	Sunflower husk					Barley straw				
	Pl. ostreatus. strain IBK–549									
SO 1%	18	25	11.6±1.4	10.8±0.4*	3.8±0.3	17–18	25	12.7±1.1	13.0±0.4	5.6±1.0
SO 5%	18–20	25	10.4±0.7	10.9±0.4*	4.2±0.5	18	25–27	15.5±2.3	14.9±0.9	5.5±0.5
CO 1%	19–20	25	17.1±1.0	12.7±0.5*	3.4±0.4	16–17	24–25	18.5±1.0	13.9±0.7	5.6±0.8
CO 5%	20	25	15.1±2.0	10.5±0.3*	3.9±0.4	18–19	24–28	15.2±0.4	12.4±0.5	9.1±1.1*
Control	18–20	25–27	12.2±0.7	9.2±0.4	3.4±0.2	17	25–27	14.8±1.6	13.0±0.6	4.5±0.2
	Pl. ostreatus. strain IBK–551									
SO 1%	18–20	28	13.8±1.8	10.2±0.2*	3.7±0.3	18–20	28–29	13.0±2.1	13.1±0.4*	4.7±0.2
SO 5%	20–22	28–32	11.6±2.0	11.9±0.6*	3.4±0.1	20–22	27–29	15.8±2.6	13.2±0.4*	5.9±1.2
CO 1%	19–21	25	14.2±1.0*	10.8±0.4*	3.5±0.1	19–20	28	14.8±0.4	15.4±1.2*	4.8±0.4
CO 5%	21–23	25–29	8.9±0.7	11.4±0.5*	4.0±0.4	23	30	19.7±1.0	16.8±1.8*	6.1±0.4*
Control	20–22	27–28	10.4±0.5	9.0±0.4	3.1±0.3	22–23	29	19.7±2.6	11.6±0.3	4.4±0.3
	Pl. ostreatus. strain IBK–1535									
SO 1%	22	29	11.1±0.3*	9.7±0.3*	3.3±0.1	22	32–36	16.7±1.0*	14.9±1.6	4.9±0.4
SO 5%	25–26	32–36	14.9±1.5*	10.6±0.5*	3.5±0.4	22	29–36	14.5±1.3*	14.2±0.5	5.4±1.1
CO 1%	30	39	11.8±0.3*	11.3±0.4*	4.1±0.4	25	30–34	18.5±0.4*	12.8±0.5	5.3±0.3
CO 5%	25–29	30–36	8.4±0.7	10.8±0.1*	4.0±0.4	25–26	33–36	20.6±2.7*	17.1±0.8*	4.9±0.3
Control	24–28	30–36	8.4±0.3	7.8±0.5	3.5±0.4	22–24	30–34	8.8±0.7	11.4±1.5	5.4±0.4

Note: * — $P < 0.05$ with compared to control ; SO — sunflower oil; CO — corn oil.

of control. The primordia were formed first by the strain IBK-549, 2–3 days later by IBK-551, and 4-5 days later by IBK-1535.

The examined strains had significant differences by fruitage time. Fruit bodies were most rapidly formed by strain IBK-549, 2–3 days later by the strain IBK-551 and 4–8 days later by IBK-1535. Also, there was no significant effect of additives to the substrate on the terms of fruiting.

There was an increase in the formation of bunches on sunflower husk with the addition of corn oil at a concentration of 1% for all strains. The strain IBK-1535 produced 1.3–2.3 times more bunches on both substrates with additives of vegetable oils in both concentrations as compared to control.

Yield of the first flush was higher for all strains grown on sunflower husk with both vegetable oils. On barley straw the increase of yield in the first flush was observed only for the strain IBK-551.

Analysis of literary data on the application of oils in the cultivation of mushrooms has shown that the addition of soybean oil at a concentration of 3 g/l in submerged cultivation of *Pl. mutilis* contributed to an increase of mushroom biomass yield and an increase in the synthesis of fatty acids [27–30].

The stimulating effect on the growth of mycelium *A. bisporus* was also revealed in agar medium with sesame oil additives at 1% concentration [31].

Profile analysis of the fungal aroma

During the sensory analysis by the panel, the following attributes of the aroma of dried mushroom samples were determined: mushroom, sweet, woody, herbaceous, sour, fish, meat, earthy, floral, and putrescent.

The results of sensory analysis of dried samples of different strains of *Pl. ostreatus* are presented in circle plots at Fig. 6.

From the provided data it is evident that the aroma profile of mushroom samples varied depending on the substrate, the strain of mushroom and additives. For all strains with both additives there was an increase in the intensity of mushroom notes in 1.2–1.5 times.

Samples of all strains grown on sunflower husk had a 1.4–2.4 times higher intensity of herbaceous notes and in samples obtained on barley straw, the intensity of the herbaceous attribute was higher in 1.5–2.4 times when only corn oil was added to the substrate.

As for meat notes, their higher intensity (in 1.4–1.8 times) was noted in comparison with the control in samples of dried mushrooms of all strains cultivated on barley straw with additives of both oils. The higher intensity of meat notes was recorded for the strain IBK-551 (in 1.3–1.5 times) on sunflower husk with the addition of both oils, and for strain IBK-549 (in 1.5 times) with the additive of sunflower oil.

There was an increase in the intensity of sweet (in 1.4 times) and floral (in 1.8–2.6 times) notes for the strain IBK-551 and earthy (in 1.3–1.8 times) for strains IBK-549 and IBK-1535 on both substrates with the addition of corn oil.

For some samples there was a slight increase in the sour and putrescent characteristics of the aroma on the substrates with additives of vegetable oils. And the nature and strength of woody and fish notes were almost unchanged compared to control for any of the samples.

Statistical processing of the sensory analysis data showed that the standard error does not exceed ±1 point, indicating the statistical homogeneity of the set of expert assessments [32].

Ultraviolet spectroscopy

The registered UV absorption spectra of hexane mushroom extracts are presented in Figures 7 and 8.

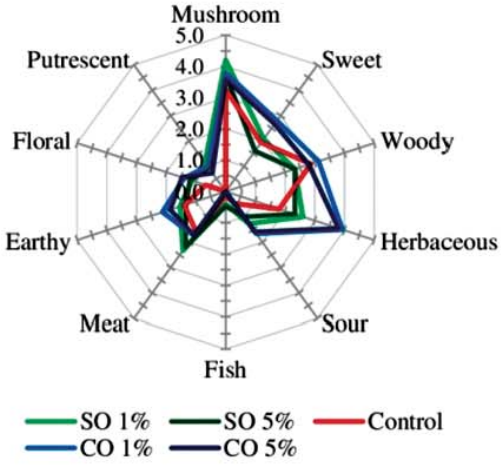
Hexane extracts of dried samples of *Pl. ostreatus* fruit bodies had light absorption maxima in ranges of 204–210 nm and 250–290 nm. Such spectral properties are characteristic of solutions of unsaturated compounds with unbound double bonds, saturated and unsaturated aldehydes and ketones, as well as derivatives of benzene [33]. As it was found in previous studies, the solution of 1-octen-3-ol in hexane has a maximum absorption at $\lambda = 207$ nm [34].

A 1.2–1.4 times higher light absorption intensity was observed throughout the studied range of wavelengths for samples of strain IBK-549 obtained on sunflower husk, with the addition of both oils at a concentration of 1%, and in the range of 250–290 nm only for the strain IBK-551.

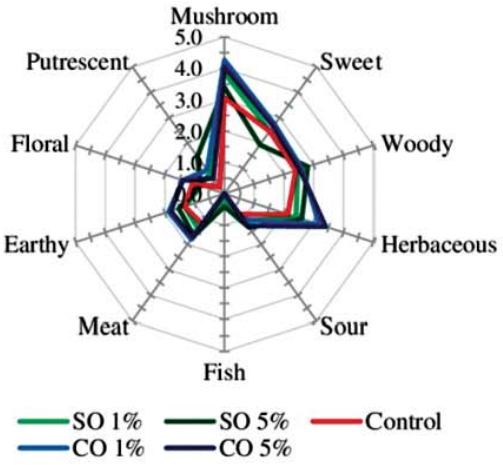
Mushroom extracts of the strain IBK-1535 cultivated on husk with sunflower and corn oil additives in both concentrations also showed an increase in light absorption compared to control. In addition, it is higher in 1.4–1.6 times as at 207 nm (the maximum is typical for 1-octen-3-ol), and 1.5–2.5 times higher in the range of 250–300 nm (the maxima inherent in aldehydes and ketones).

A similar dependence was observed for the strain IBK-1535, cultivated on barley straw with additives of oils. And also for the strain IBK-549, whose extracts revealed more intense (in 1.2 times) light-absorption maxima

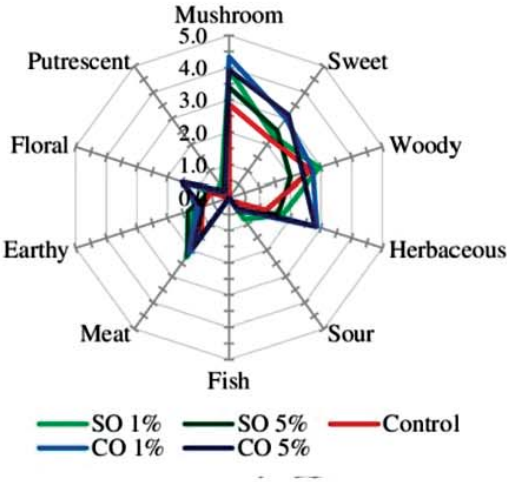
***Pl. ostreatus*, strain IBK-549**
(substrate is sunflower husk)



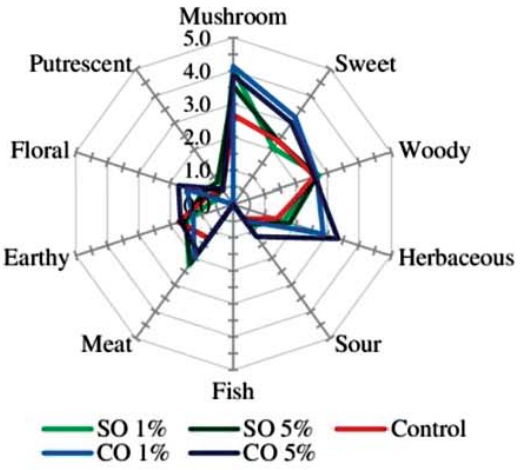
***Pl. ostreatus*, strain IBK-549**
(substrate is sunflower husk)



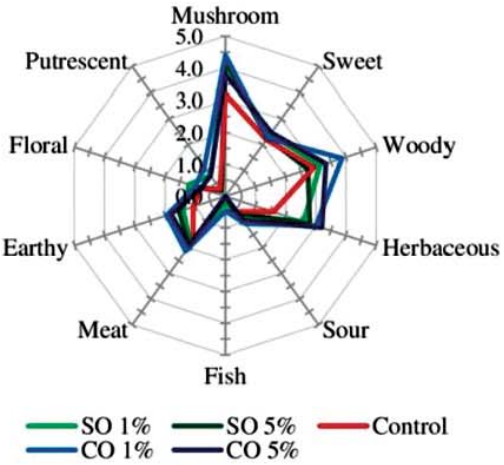
***Pl. ostreatus*, strain IBK-551**
(substrate is sunflower husk)



***Pl. ostreatus*, strain IBK-551**
(substrate is sunflower husk)



***Pl. ostreatus*, strain IBK-1535**
(substrate is sunflower husk)



***Pl. ostreatus*, strain IBK-1535**
(substrate is sunflower husk)

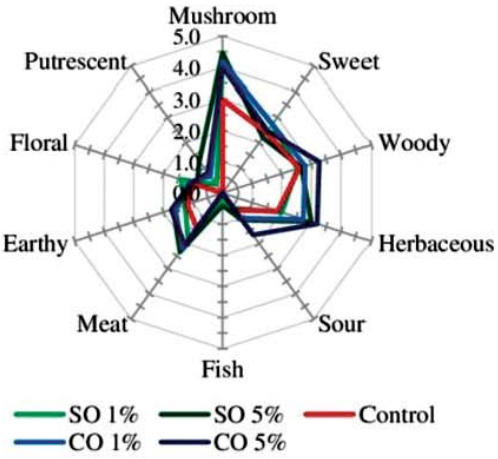


Fig. 6. **Sensory profile of aroma of dried samples of *Pleurotus ostreatus* strains**

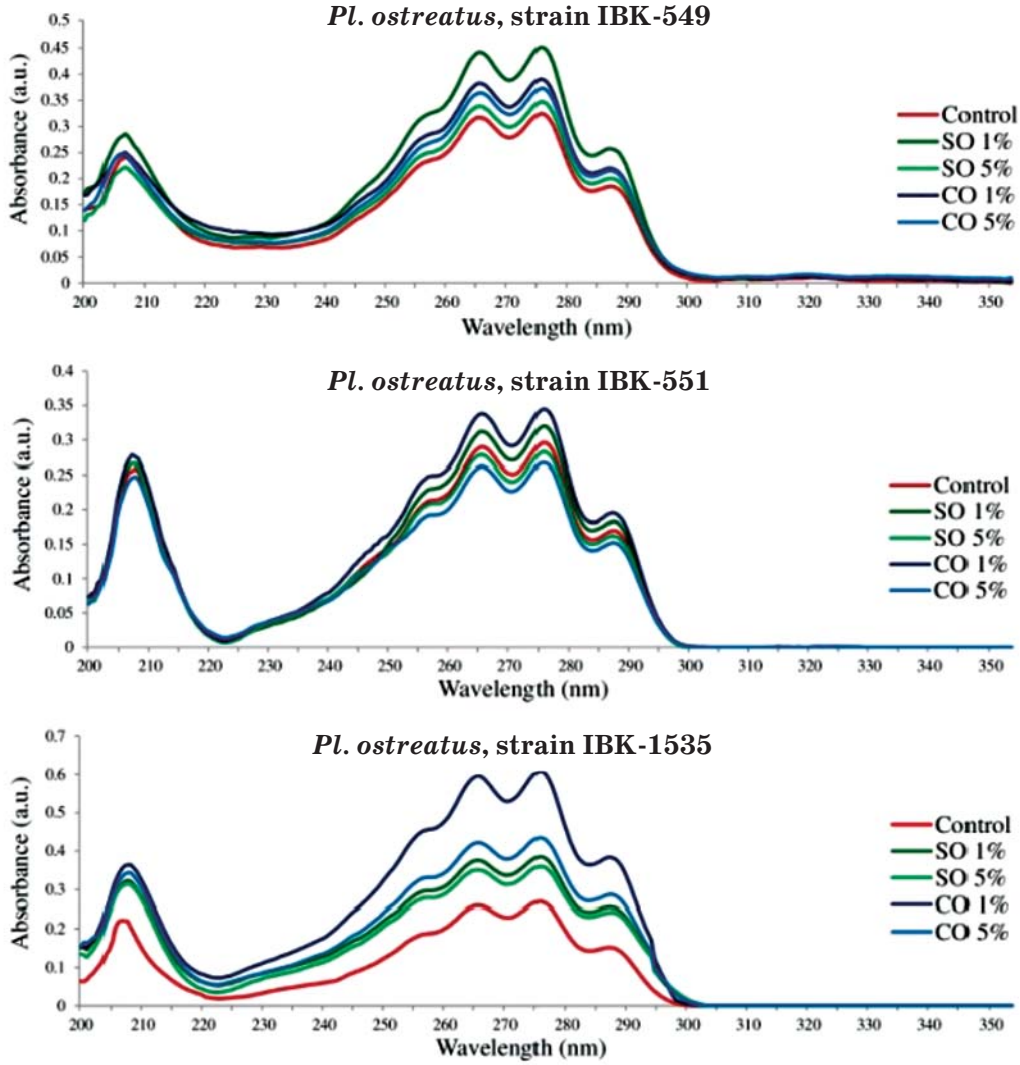


Fig. 7. **UV spectra of hexane extracts of *Pleurotus ostreatus* strains:** the substrate is sunflower husk

for strains grown on straw with both oils at a concentration of 1%.

The intensity of light absorption was 1.1–1.2 times higher only in the range of 260–290 nm for samples of the strain IBK-551, collected from substrates with additives of both oils at a concentration of 1%.

Comparison of the intensity of light absorption of various strains of *Pl. ostreatus* showed the highest level for the strain IBK-549 on both substrates compared to control.

It should also be noted that the ratio of the intensity of the light-absorption maxima at λ = 207 nm (typical for 1-octen-3-ol) and at λ = 260–280 nm (typical for aldehydes, ketones and benzene derivatives) for different substrates and strains was dissimilar. Investigated extracts of the strain IBK-549 cultivated on sunflower husk had in 2,3 times higher optical density at 260–280 nm than at near ultraviolet light. And for samples of this strain grown on barley straw, the intensity of the maxima in these ranges is almost the same. Both other strains showed almost the same ratio of intensity of light absorption on both ranges.

Thus, as a result of the study, it was found that the addition of vegetable oils to the substrate, as precursors of the synthesis of flavor compounds by the *Pl. ostreatus* strains, promotes the formation of aroma compounds by mushrooms during solid phase cultivation.

The sensory profile analysis of dried samples of the fruit bodies obtained on substrates with the addition of sunflower and corn oils at concentrations of 1% and 5% showed an increase in the intensity of mushroom, meat and herbaceous notes of

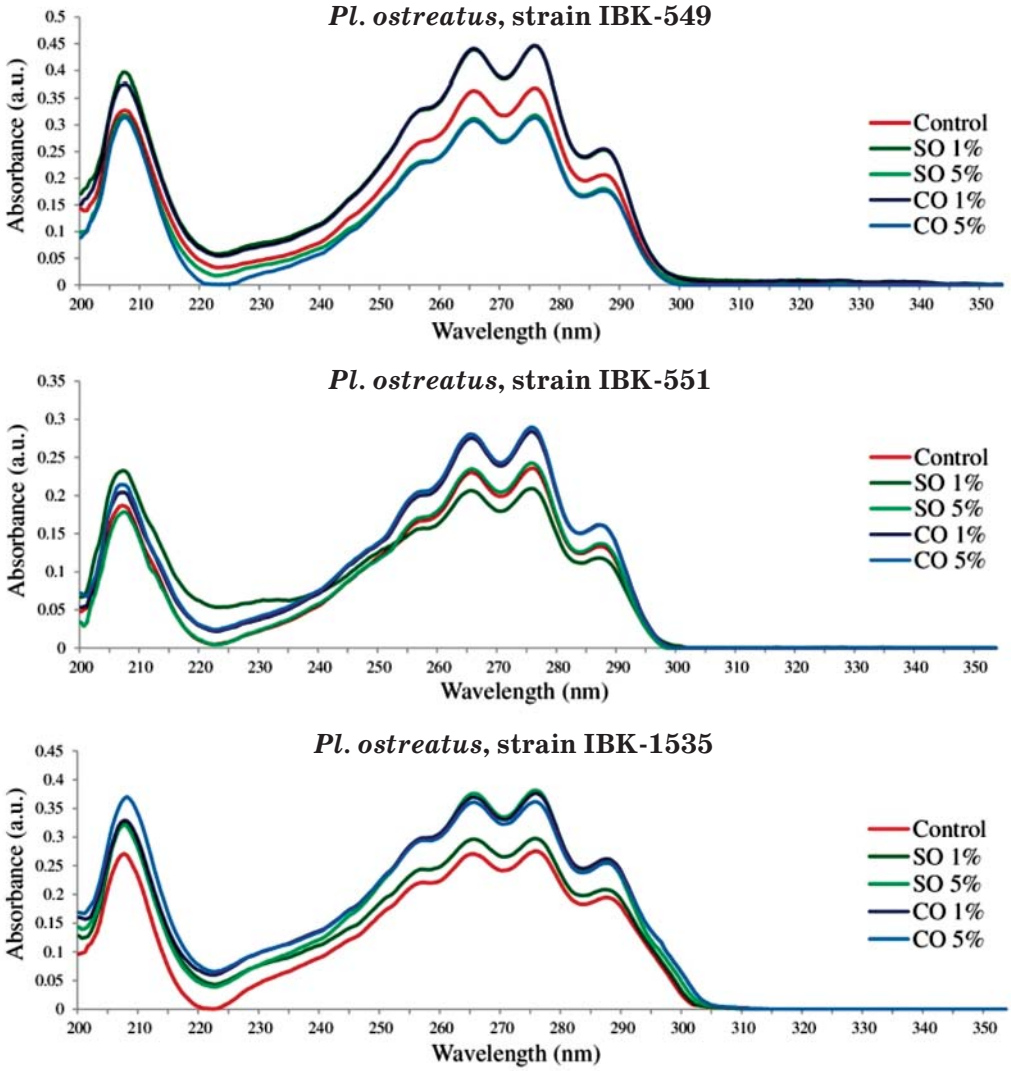


Fig. 8. **UV spectra of hexane extracts of *Pleurotus ostreatus* strains:** the substrate is barley straw

aroma. Spectrophotometric study of hexane extracts of dried fruit bodies of studied strains showed an increase in the intensity of light absorption of samples cultivated on substrates with the addition of vegetable oils compared to control.

The results of the study can be used in mushroom cultivation to increase the organoleptic quality of *Pl. ostreatus* fruit bodies through enriching the composition of lignocellulose substrates with vegetable oils.

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БІОСИНТЕЗ ЛЕТКИХ СПОЛУК ГРИБАМИ *Pleurotus ostreatus* (Jacq.:Fr.) Kumm. НА СУБСТРАТАХ, ЗБАГАЧЕНИХ РОСЛИННИМИ ОЛІЯМИ

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Метою дослідження було проведення аналізу можливих шляхів синтезу запашних сполук грибами *Pleurotus ostreatus* (Jacq.: Fr.) Kumm. за культивування на соняшниковому лушпинні та соломі ячменю з додаванням рослинних олій (соняшникової та кукурудзяної) як джерела ненасичених жирних кислот. Сенсорний профільний аналіз висушених зразків плодових тіл показав підвищення інтенсивності грибних, м'ясних та трав'янистих нот запаху на субстратах з додаванням рослинних олій у концентрації 1% і 5%. Для штаму ІВК-551 відзначено зростання інтенсивності солодких і квіткових складових запаху на обох субстратах із додаванням кукурудзяної олії. УФ-спектроскопія гексанових екстрактів висушених зразків плодових тіл виявила максимуми світлопоглинання у діапазоні 200–210 нм та 260–300 нм. Спостерігали неоднакове збільшення інтенсивності світлопоглинання зразків різних штамів, культивованих на субстратах із додаванням рослинних олій.

Ключові слова: *Pleurotus ostreatus*, леткі запашні сполуки, соняшникова олія, кукурудзяна олія, сенсорний профільний аналіз, УФ-спектроскопія.

БИОСИНТЕЗ ЛЕТУЧИХ СОЕДИНЕНИЙ ГРИБАМИ *Pleurotus ostreatus* (Jacq.:Fr.) Kumm. НА СУБСТРАТАХ, ОБОГАЩЕННЫХ РАСТИТЕЛЬНЫМИ МАСЛАМИ

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Целью исследования было проанализировать возможные пути синтеза летучих душистых соединений *Pleurotus ostreatus* (Jacq.: Fr.) Kumm. при культивировании на подсолнечной лузге и соломе ячменя с добавлением растительных масел (подсолнечного и кукурузного) в качестве источника ненасыщенных жирных кислот. Сенсорный профильный анализ высушенных образцов плодовых тел показал повышение интенсивности грибных, мясных и травянистых нот запаха на субстратах с добавлением растительных масел в концентрации 1% и 5%. Для штамма ІВК-551 отмечен рост интенсивности сладких и цветочных составляющих запаха на обоих субстратах с добавлением кукурузного масла. УФ-спектроскопия гексановых экстрактов высушенных образцов плодовых тел обнаружила максимумы светопоглощения в диапазоне 200–210 нм и 260–300 нм. Наблюдалось неодинаковое увеличение интенсивности светопоглощения образцов различных штаммов, культивируемых на субстратах с добавлением растительных масел.

Ключевые слова: *Pleurotus ostreatus*, летучие душистые соединения, подсолнечное масло, кукурузное масло, сенсорный профильный анализ, УФ-спектроскопия.

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Ganoderma SPECIES EXTRACTS: ANTIOXIDANT ACTIVITY AND CHROMATOGRAPHY

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Research aimed to isolate biologically active compounds from mushrooms fruiting bodies of *Ganoderma lucidum*, *Ganoderma adspersum* and *Ganoderma applanatum* and to estimate their antioxidant activities. Various techniques were used to isolate biologically active compounds. Antioxidant properties were estimated with spectrophotometricaly measuring free radical scavenging activity. High performance liquid chromatography was applied to analyze the isolated extracts. Half maximal inhibitory concentration (IC_{50}) was 8.25±0.88 μg/ml and 1.70±1.13 μg/ml for *G. applanatum* and *G. adspersum*, respectively. However, petroleum ether and chloroform extracts of *G. lucidum* demonstrated highler antioxidant activity with an IC_{50} about 33.66 ± 3.69 μg/ml. Chromatograms of components of acetone and methanol extracts of *G. lucidum* were recorded. The main outcome of such chromatograms is the possibility to detect the presence of active components in various mushroom species without the usage of expensive standards.

Key words: Ganoderma species mushrooms, antioxidant activity, high performance liquid chromatography.

Various mushrooms species are the focus of researchers' interest. Hitherto, lectins, polysaccharides, polysaccharide-peptides, polysaccharide-protein complexes, lanostane-type triterpenoids, phenolics and flavonoids were isolated from some mushroom species [1]. Furthermore, various biological activities such as antioxidant, antibacterial, antifungal [2, 3], antitumor [4], anti-inflammatory [5], cytotoxic [6] and anti-cholinesterase [7] activities of the isolated compounds and/or complexes were investigated. In recent years, more variety of mushrooms were isolated and identified, and the number of mushrooms being cultivated for food or medicinal purposes were increasing rapidly.

Chemicals isolated from mushrooms have significant biological activity that may cause noticeable curative effects on human health and therefore could be used in medicine [8]. Hence, the comparison of mushroom components is highly desirable for the creation of drugs. Chromatography techniques are widely applied to separate biologically active components in extracts of mushrooms at first [1]. Then, chromatograms of such extracts could be compared to each other and peaks with identical retention times could be identified. Such approach would help to identify the extracts that possess biological activity and ease of their future purification on the way of drug formulation.

The aim of present research is developing of effective methods for preparation of samples containing biologically active compounds. Preliminary sample preparation was performed using solid-liquid, ultrasonic and Soxhlet extractions [9, 10]. Then, antioxidant activity and chromatograms were estimated for all extracts.

Materials and Methods

G. lucidum, G. adspersum, and G. applanatum fruiting bodies were obtained from Mula, Turkey (Table 1). Petroleum ether, methanol, chloroform and acetone of analytical and gradient grade were supplied by Merck.

G. lucidum (120 g), *G. adspersum* (385 g), and *G. applanatum* (1200 g) material were collected from Koycegiz, Mula, dried in the air and crushed into small particle (2–6 mm).

Solid-liquid extraction. Bioactive compounds of *G. lucidum* (50 g), *G. adspersum* (175 g) and *G. applanatum* (400 g) were extracted with a mixture of petroleum ether and chloroform (4:1, v/v, 400 ml, 1 l, 2 l respectively). For the extraction, all biological materials should be covered with these solvents. Then bioactive compounds were sequentially extracted with acetone (1 l), methanol (1 l), and water (1 l) at 25 °C. Each extraction experiment was performed until the solvent became colorless.

Ultrasonic extraction. Bioactive compounds of *G. lucidum* (10 g), *G. adspersum* (10 g) and *G. applanatum* (10 g) were extracted with a mixture of petroleum ether and chloroform (4:1, v/v, 100 ml) at 25 °C for 20 min in triplicates. Then, they were sequentially extracted with acetone (100 ml), methanol (100 ml) and water (100 ml).

Soxhlet extraction. Bioactive compounds of *G. lucidum* (50 g), *G. adspersum* (50 g), and *G. applanatum* (50 g) were extracted in a Soxhlet apparatus with a mixture of petroleum ether and chloroform (4:1, v/v, 1 l) for 4 h. Then, mushroom materials were sequentially extracted with acetone (1 l), methanol (1 l), and water (1 l).

Each extraction experiment was performed until the solvent became colorless. Sediments were filtered by means of filter paper. Filtrates were concentrated under vacuum (V = 0.5 ml) using a rotary evaporator and dried in the air. The extracts collected under different techniques started above were subjected to *in vitro* tests to confirm their antioxidant activities.

1. *Extraction of polysaccharides. Solid-liquid extraction*. After methanol extraction, mushroom materials were collected and extracted with distilled hot water. The polysaccharide extracts were obtained by hot water extraction and precipitation with ethanol.

Mushrooms material was extracted with 500–1000 ml of distilled hot water at +80 °C (until samples became cold). The crude hot water extracts were filtered and finally concentrated under vacuum (V = 50–100 ml) using a rotary evaporator. Then 200–400 ml of ethanol was added to concentrated hot water extracts. Polysaccharides were precipitated overnight at +4 °C. The precipitated polysaccharides were collected after centrifugation (N ve NF800) at 3100 × g for 2 min, and extraction yield was calculated.

Ultrasonic extraction. After methanol extraction, 10 g of material were extracted tree times with 100 ml of distilled water at 80 °C (until samples became cold) for 20 min with ultrasonication. Hot water extracts were filtered and combined (V = 300 ml). Finally, concentrated under vacuum (V = 30 ml) using a rotary evaporator. 120 ml of ethanol was added to concentrated hot water extracts and polysaccharides were precipitated overnight at +4 °C. The precipitated polysaccharides were collected after centrifugation (N ve NF800) at

Table 1. **Characteristics of mushroom species collecting through 2014**

Number	Mushroom species	Tree type	Region of collection	Time of collection
1	*G. lucidum*	Sweetgum	Mula, Fethiye	September
2	*G. adspersum*	Sweetgum	Mula, Fethiye	September
3	*G. applanatum*	Mulberry	Mula, Koycheiz	September
4	*G. lucidum*	Sweetgum	Mula, Koycheiz	September
5	*G. adspersum*	Walnut	Izmir, Balchova	October
6	*G. adspersum*	Peach	Mula, Ula	October
7	*G. adspersum*	Plum	Mula, Fethiye	September
8	*G. lucidum*	Sweetgum	Mula, Marmaris	November
9	*G. adspersum*	Sweetgum	Mula, Marmaris	November
10	*G. lucidum*	Sweetgum	Mula, Ula	November
11	*G. adspersum*	Mulberry	Mula, Koycheiz	November
12	*G. lucidum*	Mulberry	Mula, Koycheiz	November
13	*G. adspersum*	Mulberry	Karabalar, Mula	November

3100 × g for 2 min, and extraction yield was calculated.

Soxhlet extraction. After methanol extraction, 50 g of material were extracted in a Soxhlet apparatus with 1 l water for 4 h. Finally, concentrated under vacuum to V = 100 ml using a rotary evaporator. 400 ml of ethanol was added to concentrated hot water extracts. Polysaccharides were precipitated overnight at +4 °C. The precipitated polysaccharides were collected after centrifugation (Nüve NF800) at 3100 × g for 2 min, and extraction yield was calculated.

Determination of antioxidant activity. β-Carotene-linoleic acid assay

The procedure was done according to Ferreira et al. (2006) [7]. A stock solution of β-carotene and linoleic acid was prepared by dissolving 0.5 mg of β-carotene in 1 ml of chloroform and adding 25 µl of linoleic acid with 200 mg of Tween-40. The chloroform was evaporated at 40 °C under vacuum using a rotary evaporator. Aerated water (100 ml) was added to the residue.

4 ml of this mixture were transferred into different test tubes containing different concentrations of the sample in ethanol. The zero time-absorbance was measured at 470 nm. The samples were incubated for 2 h at 50 °C together with a blank solution, and four others containing the antioxidants Butylated hydroxyanisole (BHA), α-tocopherol. The absorbance was measured at 470 nm. The bleaching rate (R) of β-carotene was calculated according to the following equation:

$$R = (\ln a/b)/t,$$

where ln — natural log, a — absorbance at time zero and b — absorbance at time t (2 h). Antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control, using following equation:

$$AA = [(R_{control} - R_{sample})/R_{control}] \times 100.$$

DPPH free radical scavenging activity

The free radical scavenging activity of extracts was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay described by Blois [11] with slight modifications. In its radical form, DPPH absorbs at 517 nm, but on reduction by an antioxidant or a radical species its absorption decreases. 0.1 mmol·L^{-1} Solution of DPPH in methanol was prepared and 4 ml of this solution was added to 1 ml of sample solution in methanol at different concentrations. Thirty minutes in the dark later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical of an antioxidant was calculated using the following equation:

$$\text{DPPH } \textit{scavenging effect (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100,$$

where $A_{control}$ is the absorbance of the DPPH solution and A_{sample} is the absorbance of the sample.

ABTS cation radical decolorization assay

The spectrophotometric analysis of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) scavenging activity was determined according to the method of Re et al. [12] with slight modifications. The ABTS was produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. Oxidation of ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 h had elapsed. The radical cation was stable in this form for more than 2 days in storage in the dark at room temperature. Before usage, the ABTS solution was diluted to get an absorbance of 0.708 ± 0.025 at 734 nm with ethanol. Then, 160 µl of ABTS solution was added to 40 µl of sample solution in ethanol at different concentrations. After 10 min the absorbance was measured at 734 nm by using a 96-well microplate reader. The percentage inhibitions were calculated for each concentration relative to a blank absorbance (ethanol). The scavenging capability of ABTS was calculated using the following equation:

$$\text{ABTS } \textit{scavenging effect} = [(A_{control} - A_{sample})/A_{control}] \times 100,$$

where $A_{control}$ is the initial concentration of the ABTS and A_{sample} is the absorbance of the remaining concentration of ABTS in the presence of sample. The extract concentration providing 50% radical scavenging activity (EC_{50}) was calculated from the graph of ABTS scavenging effect percentage against extract concentration. BHT, α-tocopherol were used as antioxidant standards for comparison of the activity.

Cupric reducing antioxidant capacity (CUPRAC). The cupric reducing antioxidant capacity of the extracts was determined according to the CUPRAC method [13] with slight modifications. To each well, in a 96 well plate, 50 µl of 10 mM Cu (1 l), 50 µl of 7.5 mmol neocuproine, and 60 µl of NH_4Ac buffer (1 M, pH 7.0) solutions were added. 40 µl of extract at different concentrations was added to the

initial mixture so as to make the final volume 200 µl. After 1 h, the absorbance at 450 nm was recorded against a reagent blank by using a 96-well microplate reader. Results were given as absorbance and compared with BHA, a-tocopherol used as antioxidant standards.

HPLC analysis. A high performance liquid chromatographic system with multiwavelength spectrophotometer was used for measuring. Analytical RP-column Separon shim-pack VP-ODS (5 µm, 4.6 mm × 150 mm) was used for chromatographic separations. Chromatographic conditions used for methanol extracts were as follows. The mobile phase was a mixture of acetonitrile (A) and 0.1% CH_3COOH in water (B). 0 min — 2.0% A; 10 min — 2.0% A, 20 min — 5.0% A, 30 min — 20% A, 60 min — 100% A, 62 min — 100% A, 65 min — 2% A, 68 min — 2.0% A. The mobile phase was degassed in a sonicator, and pumped in gradient mode at a flow rate of 1.5 ml/min at 35 °C. The UV detection was accomplished at 245 nm. Samples of 20 µl were injected into column. The qualitative identification of the compounds present in the samples was based on comparison of retention time and UV spectrum with standards.

Chromatographic conditions used for acetone extracts were as follows.

The mobile phase was a mixture of methanol (A) and water (B). 0 min — 50.0% A; 10 min — 50.0% A, 65 min — 100.0% A, 67 min — 100% A, 70 min — 50% A, 75 min — 50% A. The mobile phase was degassed in a sonicator. The mobile phase was pumped in gradient mode at a flow rate of 1.5 ml/min at 35 °C. The UV detection was accomplished at 245 nm and samples of 20 µl were injected into column. The qualitative identification of the compounds present in the samples was based on comparison of retention time and UV spectrum with standards.

Statistical analysis. All the data on antioxidant activity tests were the average of triplicate analyses. The data were recorded as mean ± standard deviation. Significant differences between means were determined by Student's *t*-test, P values < 0.05 were regarded as significant.

Results and Discussion

The results for antioxidant activities of extracts collected under different sample preparation techniques are represented in the Tables 2–4. All extracts were tested in the range of their concentrations from 6.25 to 800 $mg \cdot l^{-1}$. Absorbance for those ones varies in the interval of 10÷90 absorbance units with the standard deviation 0.34÷4.

The antioxidant activity of mushroom extracts was compared with those of BHA and α-tocopherol that are used as standards in food and pharmaceutical industry. In β-carotene-linoleic acid assay, petroleum ether and choroform extracts of *G lucidum* demonstrated the best antioxidant activity with an IC_{50}: 33.66 ± 3.69 µg/ml, followed by acetone 36.97 ± 2.64 µg/ml, aqueous supernatant (IC_{50}: 76.03 ± 7.96 µg/ml), methanol (IC_{50}: 130.68 ± 28.05 µg/ml), and water extract (IC_{50}: 2966.67 ± 793.85). In DPPH assay, acetone extract of *G. lucidum* demonstrated the best antioxidant activity with an IC_{50}: 135.24 ± 8.94, followed by methanol 409.94 ± 10.09µg/ml, aqueous supernatant (IC_{50}: 586.51 ± 20.05 µg/ml), petroleum ether and choroform extracts (IC_{50}: 1195.25 ± 88.64). In general, the antioxidant activity of acetone extracts of all mushroom species was found as the highest. The DPPH free radical scavenging activity of the *G. lucidum* was better for solid-liquid extraction in acetone extracts (IC_{50}: 83.79 ± 1.37). The best activity was found to be in acetone extract of *G. lucidum* using Soxhlet extraction with β-carotene-linoleic acid assay (IC_{50}: 18.54 ± 2.38 µg/ml). The best ABTS scavenging activity was found in acetone extract of *G. lucidum* Soxhlet extraction with an IC_{50} of 25.07 ± 2.83 µg/ml. The best CUPRAC activity was found in acetone extract of *G. lucidum* using solid-liquid extraction (IC_{50}: 25.28 ± 0.14 µg/ml).

In β-carotene-linoleic acid assay, methanol extract of *G. adspersum* demonstrated the best antioxidant activity with an IC_{50}: 1.70 ± 1.13 µg/ml (Table 4), followed by acetone 9.79 ± 5.73 µg/ml, aqueous supernatant (IC_{50}: 69.19 ± 0.30 µg/ml), petroleum ether and choroform (IC_{50}: 157.17 ± 14.80 µg/ml), and water extracts (IC_{50}: 426.90 ± 24.27). In DPPH assay, acetone extract of *G. adspersum* demonstrated the best antioxidant activity with an IC_{50}: 10.36 ± 0.69 (Table 4), followed by methanol 36.54 ± 1.15 µg/ml, aqueous supernatant (IC_{50}: 282.85 ± 41.17 µg/ml), petroleum ether and choroform extracts (IC_{50}: 9950.60 ± 100.69). The DPPH free radical scavenging activity of the *G. adspersum* was better for ultrasonic extraction in acetone extract (IC_{50}: 10.36 ± 0.69). The best ABTS scavenging activity was found in acetone extract of *G. adspersum* in Soxhlet extraction with an IC_{50} of 3.18 ± 0.17 µg/ml. The best CUPRAC activity was found in acetone extract of *G. adspersum* using ultrasonic extraction (IC_{50}: 7.58 ± 1.33 µg/ml).

Table 2. **Antioxidant activity of the extracts of *G. lucidum, G. adspersum, G. applanatum* by the β-carotene-linoleic acid, DPPH, ABTS, and CUPRAC, obtained by solid-liquid extraction**

Mushrooms/ standards	Extracts	β-carotene-linoleic acid assay IC_{50a} (μg/ml)	DPPH assay IC_{50} (μg/ml)	ABTS assay IC_{50} (μg/ml)	CUPRAC IC_{50} (μg/ml)
G. lucidum	Petroleum ether and choroform	120.70±8.52* **	1448.76±42.06* **	890.02±199.93* **	477.50±14.85* **
	Acetone	22.84±1.78* **	83.79±1.37* **	27.14±2.24* **	25.28±0.14* **
	Methanol	24.94±0.07	249.09±7.23* **	35.78±20.56	199.25±48.44* **
	Water	260.18±10.00 * **	N.A.	150.70±17.62* **	26.15±0.68
	Aqueous supernatant	124.79±5.82* **	498.54±119.83	94.61±2.99* **	86.40±2.26* **
G. adspersum	Petroleum ether and choroform	367.35±0.41* **	6402.63±74.95* **	N.A.	436.33±12.90* **
	Methanol	41.20±2.42**	95.09±4.22**	23.23±1.05	31.89±0.77
	Water	24.97±3.34*	702.30±32.06* **	133.93±26.41* **	40.10±1.14**
G. applanatum	Petroleum ether and choroform	265.87±8.47* **	3653.56±242.66* **	N.A.	422.22±5.17* **
	Acetone	20.19±9.50**	3.50±1.68**	4.27±0.97**	1.16±0.17**
	Methanol	8.25±0.88	42.17±1.57* **	3.35±0.94	11.56±0.60*
	Water	41.79±1.28* **	231.62±0.25* **	79.36±10.54* **	47.50±2.12* **
Control α-Tocopherol b (standard)	Ethanol	0.81±0.01	28.99±0.87	15.37±0.50	64.50±3.94
Control BHA b (standard)	Ethanol	0.54±0.04	16.82±0.11		

Hereinafter: a — IC_{50} values represent the means ± standard deviation of three parallel measurements ($P < 0.05$); b — reference compounds; N.A. — not bioactive; * — level of confident probability between average value, statistical significance of the differences comparing to the first control (α-tocopherol), $P < 0.05$; ** — level of confident probability between average value, statistical significance of the differences comparing to the second control (BHA), $P < 0.05$.

In β-carotene-linoleic acid assay (Table 2), methanol extract of *G. applanatum* demonstrated the best antioxidant activity with an IC_{50}: 8.25 ± 0.88 μg/ml (Table 4), followed by acetone 20.19 ± 9.50 μg/ml, water (IC_{50}: 41.79 ± 1.28 μg/ml), petroleum ether and chloroform extracts (IC_{50}: 265.87±8.47 μg/ml). In DPPH assay, acetone extract of *G. applanatum* demonstrated the best antioxidant activity with an IC_{50}: 3.50 ± 1.68 (Table 2), followed by methanol 42.17 ± 1.57 μg/ml, water (IC_{50}: 231.62 ± 0.25 μg/ml), petroleum ether and choroform extracts (IC_{50}: 3653.56 ± 242.66). The best ABTS scavenging activity was found in methanol extract of *G. applanatum* in solid-liquid extraction with an IC_{50} of 3.35 ± 0.94 μg/ml. The best CUPRAC activity was found in acetone extract of *G. applanatum* using solid-liquid extraction (IC_{50}: 1.16 ± 0.17 μg/ml).

The acetone fraction of *G. adspersum* showed similar antioxidant activity in the

Table 3. **Antioxidant activity of the extracts of *G. lucidum, G. adspersum, G. applanatum* by the β-carotene-linoleic acid, DPPH, ABTS, and CUPRAC, obtained by Soxhlet extraction**

Mushrooms	Extracts	β-carotene-linoleic acid assay IC_{50} (μg/ml)	DPPH· assay IC_{50} (μg/ml)	ABTS·+ assay IC_{50} (μg/ml)	CUPRAC IC_{50} (μg/ml)
G. lucidum	Petroleum ether and choroform	88.57±0.67* **	1566.24±79.64* **	1045.57±342.46* **	356.00±5.57v
	Acetone	18.54±2.38*	94.72±1.18**	25.07±2.83**	27.68±1.11* **
	Methanol	29.08±1.94*	267.45±16.74**	191.22±5.38*	246.17±61.33* **
G. adspersum	Petroleum ether and choroform	59.27±4.06* **	4808.94±296.19* **	N.A.	399.50±0.71* **
	Acetone	17.50±2.41*	28.94±5.05*	3.18±0.17**	11.23±0.33*
	Methanol	31.35±4.71*	61.76±2.65**	15.53±1.86**	20.78±0.36*
G. applanatum	Petroleum ether and choroform	162.59±27.95* **	5108.02±376.58* **	N.A.	404.50±21.92* **
	Acetone	30.72±8.39*	11.94±0.60*	11.94±0.60*	9.40±0.25**
	Methanol	70.64±13.73* **	11.33±0.53**	15.09±0.14**	6.30±0.69*

Table 4. **Antioxidant activity of the extracts of *G. lucidum, G. adspersum, G. applanatum* by the β-carotene-linoleic acid, DPPH, ABTS, and CUPRAC, obtained by ultrasonic extraction**

Mushrooms	Extracts	β-carotene-linoleic acid assay IC_{50} (μg/ml)	DPPH· assay IC_{50} (μg/ml)	ABTS·+ assay IC_{50} (μg/ml)	CUPRAC IC_{50} (μg/ml)
G. lucidum	Petroleum ether and choroform	33.66±3.69**	1195.25±88.64*	N.A.	316.00±17.35**
	Acetone	36.97±2.64*	135.24±8.94**	39.66±1.89*	35.72±0.63*
	Methanol	130.68±28.05**	409.94±10.09* **	40.49±23.20**	370.00±12.53*
	Water	2966.67±793.85* **	N.A.	672.02±54.18* **	566.00±50.09* **
	Aqueous supernatant	76.03±7.96* **	586.51±20.05* **	119.58±1.34* **	101.88±11.68*
G. adspersum	Petroleum ether and choroform	157.17±14.80* **	9950.60±100.69* **	N.A.	379.67±3.79**
	Acetone	9.79±5.73*	10.36±0.69* **	17.72±1.60* **	7.58±1.33* **
	Methanol	1.70±1.13**	36.54±1.15* **	7.67±1.36* **	13.20±0.33*
	Water	426.90±24.27*	N.A.	66.38±4.93**	201.50±17.68* **
	Aqueous supernatant	69.19±0.30*	282.85±41.17	52.09±2.61*	26.15±0.68* **
G. applanatum	Petroleum ether and choroform	724.75±32.58	N.A.	N.A.	458.67±7.51**
	Acetone	N.A.	7.68±0.51* **	13.08±1.17**	6.08±1.31* **
	Methanol	67.84±0.25* **	5.42±0.83* **	11.15±2.48*	2.69±0.97* **
	Water	338.58±0.92* **	1566.68±615.75**	116.66±20.34**	52.00±0.45*
	Aqueous supernatant	267.85±22.91**	247.89±14.94*	42.67±3.32*	25.07±0.66**

β-carotene-linoleic acid assay in our (IC_{50} = 9.79 ± 5.73, Table 4) and other scientists research (IC_{50} = 7.89 ± 0.91 μg/ml, [10]). Authors of work [10] stated that among the extracts, the ethyl acetate fraction of *G. adspersum* demonstrated the highest activity in the β-carotene-linoleic acid assay (IC_{50} = 5.63 ± 0.66 μg/ml). In our research the highest antioxidant activity for *G. adspersum* was observed in methanol fraction (IC_{50} = 1.7 ± 1.13 μg/ml).

As an example, chromatograms of dry components of acetone extracts of *G. lucidum* ilustrated in the Fig. 1. Chromatogram in red color shows fingerprint for it. Chromatogram in black color shows the result of treatment of *G. lucidum* sample dissolved in methanol (40 000 ppm) with ABTS solution in ethanol. Solutions were mixed in ratio of 1 to 1. Concentration of *G. lucidum* in solution for both samples were the same (20 000 ppm).

Chromatogram of dry components of methanol extracts of *G. lucidum* ilustrated in the Fig. 2. Chromatogram shows fingerprint for it.

Thus, three-sample preparation techniques were used for obtaining mushrooms extracts. Antioxidant activity was estimated for all extracts. Antioxidant activities depend on investigated extract and method used for their measuring. Using solid-liquid extraction of *G. applanatum* and CUPRAC assay, optimal IC_{50} value is up to 1.16 ± 0.17 μg/ml. Soxhlet extraction of *G. adspersum* and ABTS assay gives the best IC_{50} value for this mushroom equal to 3.18 ± 0.17 μg/ml. Using ultrasonic

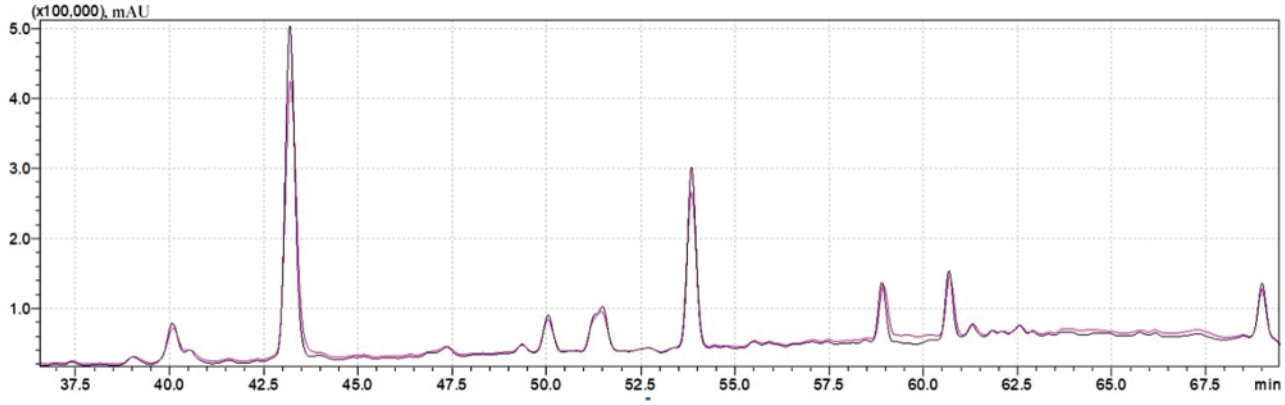

Fig. 1. **Chromatograms of dry components of acetone extracts of *G. lucidum***

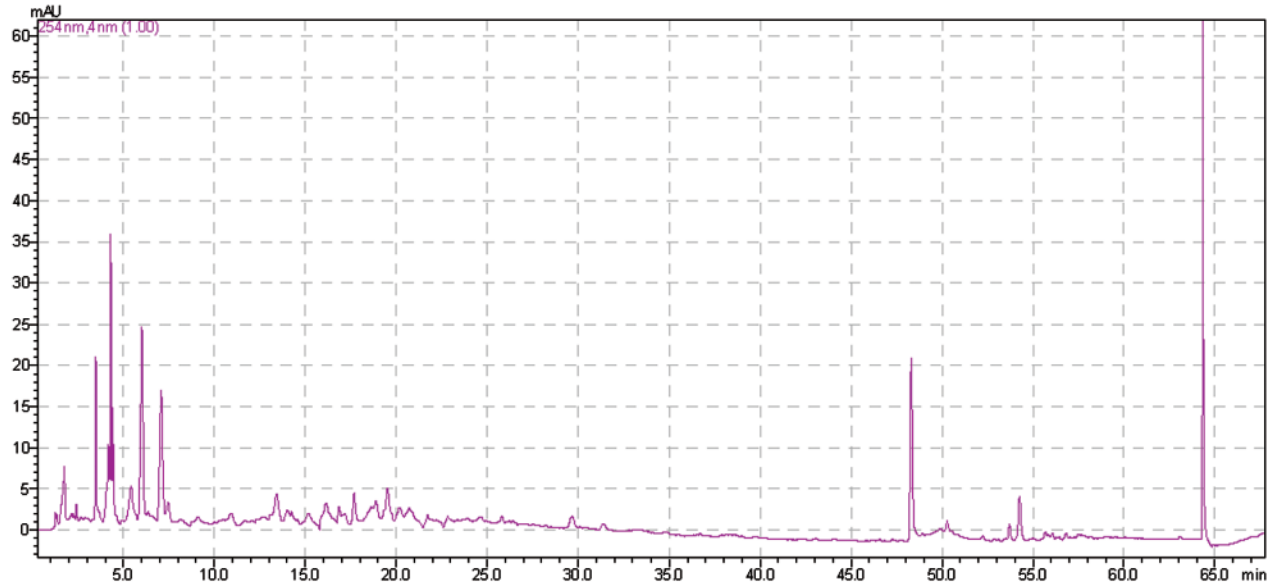

Fig. 2. **Chromatogram of dry components of methanol extracts of *G. lucidum***

extraction of *G. adspersum* and β-carotene-linoleic acid assay, methanol extract with the highest activity was found (IC_{50} 1.70 ± 1.13μg/ml).

HPLC conditions were developed for getting the chromatograms of extracts. Such chromatograms might be used to detect the presence of presence active components in various mushrooms species without usage of expensive standards.

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ХАРАКТЕРИСТИКА ЕКСТРАКТІВ ГРИБІВ РІЗНИХ ВИДІВ РОДУ *Ganoderma*: АНТИОКСИДАНТНА АКТИВНІСТЬ ТА ХРОМАТОГРАМИ

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Метою роботи було виділення біологічно активних сполук із плодових тіл грибів *Ganoderma lucidum*, *Ganoderma adspersum* і *Ganoderma applanatum* та оцінювання їхньої антиоксидантної активності. Для виділення біологічно активних сполук використовували різні методи. Антиоксидантні властивості визначали спектрофотометрично, вимірюючи активність захоплення вільних радикалів. Для аналізу хроматограм виділених екстрактів застосовували високоефективну рідинну хроматографію. В результаті аналізу з використанням β-каротин-лінолевої кислоти було визначено високу антиоксидантну активність метанольних екстрактів. Напівмаксимальне інгібування IC_{50} для *G. applanatum* і *G. adspersum* становило 8,25 ± 0,88 мкг/мл та 1,70 ± 1,13 мкг/мл відповідно. Водночас, екстракти петролейного ефіру і хлороформу *G. lucidum* мали вищу антиоксидантну активність: IC_{50} — близько 33,66 ± 3,69 мкг/мл. Отримано хроматограми компонентів ацетонових і метанольних екстрактів *G. lucidum*. Основною перевагою таких хроматограм є можливість виявлення активних компонентів різних видів грибів без використання високовартісних стандартів.

Ключові слова: гриби видів *Ganoderma*, антиоксидантна активність, високоефективна рідинна хроматографія.

ХАРАКТЕРИСТИКА ЭКСТРАКТОВ ГРИБОВ РАЗНЫХ ВИДОВ РОДА *Ganoderma*: АНТИОКСИДАНТНАЯ АКТИВНОСТЬ И ХРОМАТОГРАММЫ

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Целью работы было выделение биологически активных соединений из плодовых тел грибов *Ganoderma lucidum*, *Ganoderma adspersum* и *Ganoderma applanatum* и оценка их антиоксидантной активности. Для выделения биологически активных соединений использовали различные методы. Антиоксидантные свойства определяли спектрофотометрически, измеряя активность захвата свободных радикалов. Для анализа хроматограмм выделенных экстрактов применяли высокоэффективную жидкостную хроматографию. В результате анализа с использованием β-каротин-линолевой кислоты была определена высокая антиоксидантная активность метанольных экстрактов. Полумаксимальное ингибирование IC_{50} для *G. applanatum* и *G. adspersum* составило 8,25 ± 0,88 и 1,70 ± 1,13 мкг/мл соответственно. В то же время экстракты петролейного эфира и хлороформа *G. lucidum* имели большую антиоксидантную активность: IC_{50} — около 33,66 ± 3,69 мкг/мл. Были получены хроматограммы компонентов ацетоновых и метанольных экстрактов *G. lucidum*. Основным преимуществом таких хроматограмм является возможность выявления активных компонентов различных видов грибов без использования дорогостоящих стандартов.

Ключевые слова: грибы видов *Ganoderma*, антиоксидантная активность, высокоэффективная жидкостная хроматография.

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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 from 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 zeolite filter loading.

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 $(NH_4)SO_4$, $NaNO_3$, 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*.

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**

Notice: 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%.

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 cm^3 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 (Fig.) by different genuses of microorganisms have been constructed.

According to bar charts on Fig., *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 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

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

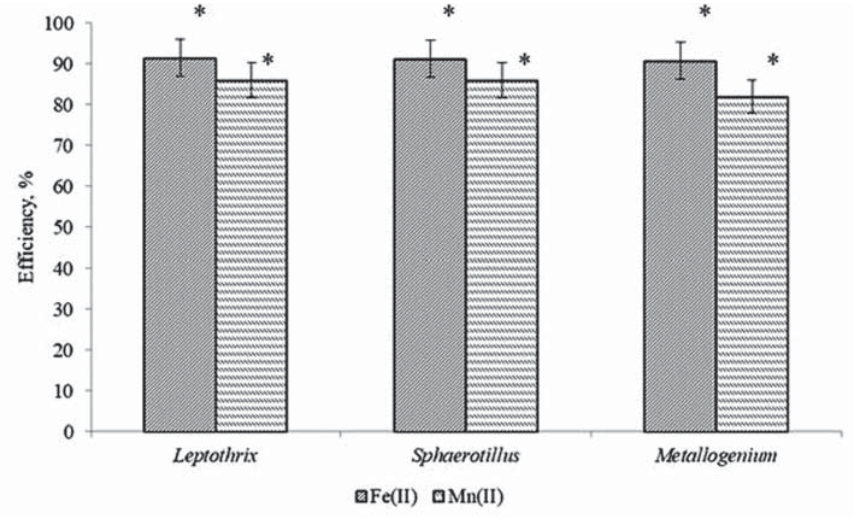
Genus	TMN, CCU/dm^3	The efficiency of removing Fe(II),%	The efficiency of removing Mn(II),%
Leptothrix	300*	91.5**	86.0**
Sphaerotillus	300*	91.2**	86.0**
Metallogenium	250*	90.8**	82.0**

Notice: * — $P < 0.05$, 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%.

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.



Average efficiency of iron and manganese removing

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

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ВИДІЛЕННЯ ЧИСТИХ КУЛЬТУР ЗАЛІЗО- ТА МАРГАНЕЦЬ-ОКИСНЮВАЛЬНИХ БАКТЕРІЙ ІЗ ШВИДКИХ ФІЛЬТРІВ

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Метою дослідження було виділення з питної води чистих культур мікроорганізмів, здатних окислювати залізо та марганець, з подальшим оцінюванням їхньої ефективності з метою видалення цих речовин із цеолітового завантаження швидких фільтрів. Для оцінювання ефективності вибрані штами вирощували на твердому живильному середовищі, готували суспензію та обробляли нею цеолітове завантаження. Виділено 10 чистих культур мікроорганізмів, здатних окислювати 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 окислювати залізо та марганець, швидкі фільтри.

ВЫДЕЛЕНИЕ ЧИСТЫХ КУЛЬТУР ЖЕЛЕЗО- И МАРГАНЕЦ-ОКИСЛЯЮЩИХБАКТЕРИЙ ИЗ БЫСТРЫХ 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 из цеолитовой загрузки быстрых фильтров. 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, быстрые фильтры.

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"REGOPLANT" AND "STIMPO" INFLUENCE ON THE CONTENT OF FREE AMINO ACIDS, PROLINE AND ON THE LIPID PEROXIDATION REACTION INTENSITY IN HELIANTHUS ANNUUS L. GROWN ON TECHNOSOL

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The aim of study was to investigate the effect of new growth regulators "Stimpo" and "Regoplant", produced by State Enterprise Interdepartmental Science and Technology Center — ISTC "Agrobiotech", on the proline and free amino acids content, and lipid peroxidation reactions intensity in comperison to the action of gibberellic acid and "Treptolem" (a second-generation growth regulator) in 14 day sunflower sprouts. The plants were cultured on soil substrates made from the coal mine rock waste of the Central Concentrating Factory in the Chervonohrad mining region.

It was shown that the free amino acids and proline content increased, and the intensively of lipid peroxidation reactions (evaluated by the content of malonic dialdehyde) decreased. This indicated to the increase of plants resistance to unfavorable conditions of the coal mine rock under the action of the growth regulators studied.

One could assump that "Stimpo" and "Regoplant" are promising agents in phytochemical treatment of coal mine rock dumps.

Key words: *Helianthus annuus* L., "Stimpo", "Regoplant", "Treptolem", substrates of coal dumps, free amino acids, proline, lipid *peroxidation reactions*.

Plant growth regulators belong to the third generation of biotechnological breakthroughs. "Regoplant" (TU U 24.2-31168762-006) and "Stimpo" (TU U 24.2-31168762-005) have a wide spectrum of action and a bioprotective effect. Previously, "Stimpo" and "Regoplant" were not used as growth stimulators for the phytochemical treatment of rock dump technoland. Therefore, in this research their influence on the content of free amino acids and proline, and the intensity of lipid peroxidation reactions was investigated to evaluate resulting plant resistance to unfavorable edaphic conditions of rock dumps.

Effectively cleansing soils of pollution and technogenic changes caused by heavy metals is an important issue everywhere, including Chervonohrad mining region (ChMR). The recultivation of such soils is problematic, the process is multi-stage and has several disadvantages [1, 2]. Biologically, recultivation aims to improve soil productivity with plants [3]. Phytorecultivation is one of approaches used to improve soils of the Central enriching plant (CEP) using plants which accumulate heavy metals [1, 4]. These plants tolerate the effects of heavy metal in concentrations, toxic for most plant species [5]. Scientists have found how to identify such tolerant plants [6, 7]. Particularly, plants of the genera *Calamagrostis*, *Phragmites*, and *Brassica napus* L. were used to recultivate substrates of coal mine wastes at the CEP [8–10]. There are also studies of compounds which can potentially enhance plant tolerance during phytorecultivation [11–14].

Two growth regulator (GR) preparations, "Regoplant" (TU U 24.2-31168762-006) and "Stimpo" (TU U 24.2-31168762-005), were produced in ISTC "Agrobiotech" (Kyiv, Ukraine). The preparations have a wide range of action and bioprotection effect. The main components of "Stimpo" and "Regoplant" are metabolites of microscopic fungi and the bacterium *Streptomyces avermetilis*. These preparations were produced *in vitro* in fungal culture, isolated from root systems of ginseng plants. The preparations contain a complex of amino acids, fat acids, polysaccharides, phytohormones, microelements and metabolites of *Streptomyces avermitilis*, which include avermectins (complex anthelmintic macrolide antibiotics). Thus they enhance the physiological indices of plants grown on normal soils [12, 15–18]. Improvement of soils under heavy metal pollution is a long-term process and requires effective biotechnologies to reverse those disturbed habitats to their previous natural state. Hence, plant growth regulators are needed there to create optimal growth conditions and increase plant tolerance to unfavorable edaphic and microclimatic conditions.

Sunflower plants are relatively tolerant to heavy metals and can accumulate several of them [11, 19, 20]. However, their metabolism is not studied in conditions of growing the plants on substrates of rock dumps and treatment with the mentioned GR. Our work aimed to evaluate the effect of "Stimpo" (S) and "Regoplant" (R) compared to gibberellic acid (GA) and another GR of previous (second) generation, "Treptolem" (T), on free amino acid content (particularly, proline) and intensity of lipid peroxidation (LPO) reactions in sunflower plants grown on substrates of ChMR dumps.

Materials and Methods

Previously, we have determined the optimal concentrations of GR for sunflower plants, namely 0.5 ml/l of "Stimpo" and 0.1 ml/l of "Regoplant" . Optimal concentrations of GA (10 mg/l) and "Treptolem" (1 ml/l) were taken from other publications [11, 19, 21, 22]. Seeds were soaed in 1 hr in solutions of the mentioned concentrations and then washed in distilled water. Seed germination was performed in Petri dishes in darkened thermostat at 22 °C for 3 days, and then seeds were planted into black (not burnt-out) and red (burnt-out) substrates of rock dumps for 14 days. Sprouts soaked in distilled water and planted into garden soil were used as control. Substrates were collected at CEP dumps, in Silets village, Sokalsky district of Lviv region at the depth of 20 cm.

Biochemical indices were analyzed at the 14^{th} day. Free amino acid content was determined spectrophotometrically according to [23] on KFK-3 at 580 nm. Recalculated for mg/100 g of studied compound. Proline content was estimated by changes in optical density of reaction mixture at 520 nm according to [24], calculating for mg/100 g of raw mass. Lipid peroxidation reaction intensity was determined spectrophotometrically by malondialdehyde content at 530 and 600 nm following [25]. Data was statistically processed in programs MS Excel and Statistics.

Results and Discussion

Helianthus annuus L. plants are suitable for dump recultivation, due to their tolerance to heavy metals (HM) [8, 11], that is metallic elements with density more than 6 g/cm^3, atomic weight no less that 50 carbon units. Soils easily accumulate heavy metals and conversely are slowly and laboriously purged. The elements can induce diseases in plants, animals and humans.

The resistance of plants can increase due to changes in the synthesis of amino acids, which, under various stresses, perform regulatory and protector functions. Free amino acids are involved in the formation of various forms of nitrogen, and in maintaining the cellular osmotic potential. They also can respond to the environmental stress factors [26, 27]. They act as buffers, binding anions and cations, reducing their concentration in the cell [8]. This is definitely a positive phenomenon for plants that grow in environments with high contents of toxic substances [28]. Hence, we started with studying the effect of GR on the content of free amino acids in sunflower plants grown on the coal dump substrates (Fig. 1).

The content of free amino acids increased in sunflower seeds treated with GR and grown on the garden soil. This is a possible indication of increased protein synthesis. Plants treated with "Stimpo" and Regaplanet, had higher content of free amino acids than those treated with "Treptolem" and GA, grown in the garden soil or on the substrates of ChMR.

Free amino acid content was higher in plants grown on black substrate compared to those grown on red substrate. A possible reason for that is more acidic pH of the black substrate, where plants accumulate heavy metals faster and neutralize them with amino acids.

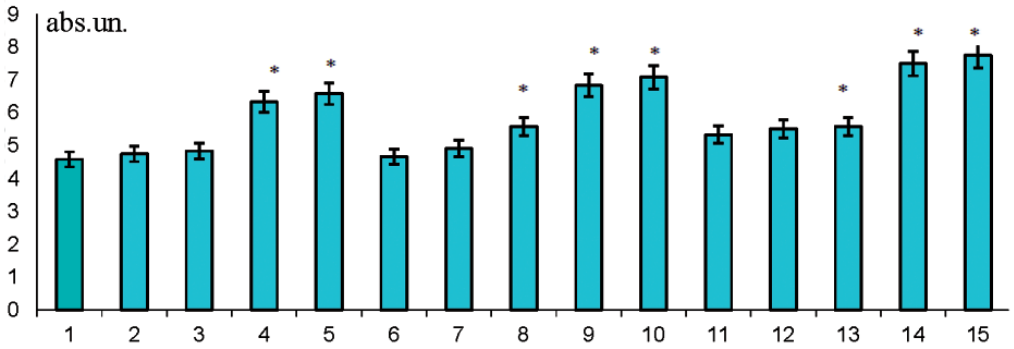

Fig. 1. **Influence of growth regulators on the content of free amino acids in *Helianthus annuus* L. grown on soil substrates (mg/100 g of raw mass)**

Hear and after: *1. Control — Garden soil; 2. Garden soil + GA; 3. Garden soil + Treptolem (T); 4. Garden soil + Stimpo (S); 5. Garden soil + Regoplant (R); 6. Red substrate; 7. Red substrate + GA; 8. Red substrate + T; 9. Red substrate + S; 10. Red substrate + R; 11. Black substrate; 12. Black substrate + GA; 13. Black substrate + T; 14. Black substrate + S; 15. Black substrate + R.

* — $P < 0.05$ compared with control.

The effect of "Regoplant" was stronger compared to "Stimpo". The difference between the increases in the content of free amino acids on the garden soil was 26%, 6% at the red substrate and 7% at the black substrate. Indices of plants grown on the garden soil and not treated with GR were used as control. Obviously, that increase in the content of free amino acids may be explained by adaptation to adverse edaphic conditions of substrates [9, 10, 29].

Stressed plant organisms, particularly influenced by HM, continuously adapt to preserve and restore the dynamic constancy of their internal environment [30]. One of the mechanisms by which plants adapt to adverse conditions is the accumulation of osmoactive substances, among which proline plays an important role. This compound is involved in protective reactions, in particular in stabilizing the cytoplasm [27]. Therefore, further work was to determine the content of proline (Fig. 2).

In plants treated with GR the proline content was higher compared to control. This may indicate increased photosynthesis and nitrogen accumulation on garden soil in experimental plants [31] and plants reaction to stress (heavy metals and the moisture deficit) on ChMR substrates [14, 24, 32]. In plants grown on garden soil, the effect of "Regoplant" was higher compared to "Stimpo": proline content increased by 491% in experiment with "Regoplant" compared to 352% under treatment with "Stimpo". The difference between indices was 139%. In plants grown on artificial substrates and treated with GR, the content of proline increased exceedingly: by 1011% and 1023% on the red substrate, and by 1112% and 1119% on the black substrate, for "Stimpo" and "Regoplant" respectively. That corresponded to the 12% difference in the effectiveness of "Regoplant" over "Stimpo" on red substrate, and the 7% difference on black substrate. The proline content in plants grown on garden soil without GR treatment was used as control in calculations.

The earliest stress reactions occur at the membrane level [33], therefore the next step in our work was to determine the activity of the lipid peroxidation, one of the main indicators of membrane integrity (Fig. 3).

Lipid peroxidation decreased in treated with GR sunflower plants grown on the garden soil or on the substrates of ChMR. Malonic dialdehyde content was lower after treatment with newer GR compared to GA and "Treptolem". Moreover, on the dump substrates, "Regoplant" caused the highest reduction of the MDA content, while the smallest reduction was caused by gibberelic acid.

MDA content was more reduced under treatment with "Regoplant" compared to "Stimpo". Interestingly, reduction percentage was almost the same in treated plants grown on garden soil, whereas there was an 8% difference of MDA content decrease in plants cultured on the red substrate, and an 11% difference on the black substrate respectively. Indicators of plants that grew on the garden soil without GR treatment were used as control.

In our opinion, the effects of Ukrainian growth regulators of different generations on plants are unequal because of the difference in their composition. Their basic component are growth substances of natural origin. The additives are different, for example in "Treptolem" those

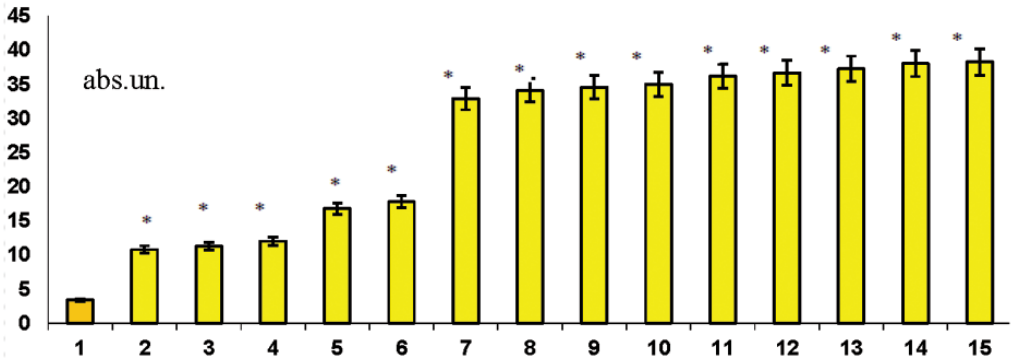

Fig. 2. **Proline content in *Helianthus annuus* L. grown on substrates of coal mines and treated with growth regulators (mg/100 g of raw mass)**

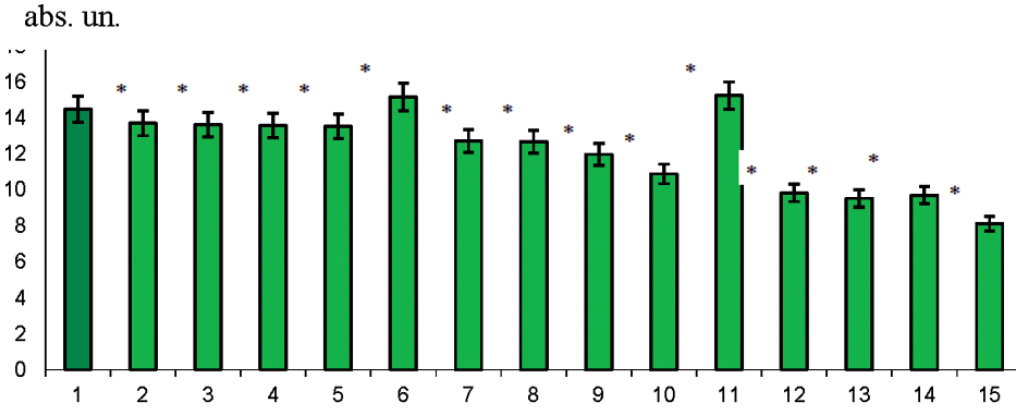

Fig. 3. **Influence of growth regulators on the content of malonic dialdehyde in *Helianthus annuus* L. grown on substrates of ChMR dump (mM/g of raw mass)**

are 2,6-dimethylpyridine-1 oxide and amber acid, without trace elements. There are no boron and molybdenum in "Stimpo" but they are present in "Regoplant" , which may explain the latter's greater effect on the plants, compared to "Stimpo" and "Treptolem" .

The obtained results indicate a decreased oxidative stress, as evidenced by an increase in the content of free amino acids and proline, and decreased content of malonic dialdehyde, which confirms lessened damage of membrane structures in the plants treated with GR and grown on substrates of the coal dumps waste mine.

Thus, the free amino acids and proline content increased, and lipid peroxidation decreased in sunflower plants treated with optimal concentrations of "Stimpo" and "Regoplant" and grown on substrates of coal mine rock dumps. Increased free amino acid and proline content, which are compounds with bioprotection functions, indicate lower oxidative stress in plants grown in unfavorable edaphic conditions of rock dump substrates, and preservation of cellular membrane integrity, which follows from decreased malonic dialdehyde content.

Thus, the optimal concentrations of "Stimpo" and "Regoplant" identified in the study can be recommended for use as stimulators of growth and metabolism in sunflower plants used for phytochemical treatment of coal mines in the Chervonograd mining region.

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ВПЛИВ «REGOPLANT» І «STIMPO» НА ВМІСТ ВІЛЬНИХ АМІНОКИСЛОТ, ПРОЛІНУ ТА ІНТЕНСИВНІСТЬ РЕАКЦІЙ ПЕРОКСИДНОГО ОКИСНЕННЯ ЛІПІДІВ У *Helianthus annuus* L. ЗА РОСТУ НА ТЕХНОЗЕМАХ

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Метою роботи було дослідити вплив нових регуляторів росту «Stimp»» та «Regoplant» (виробник Державне підприємство Міжвідомчий науково-технологічний центр — ДП МНТЦ «Агробіотех») порівняно з гібереліновою кислотою і регулятором росту другого покоління «Treptolem» на вміст вільних амінокислот, проліну та реакції пероксидного окиснення ліпідів у 14-добових паростків *Helianthus annuus* L. за росту на ґрунтових субстратах породного відвалу вугільних шахт Центральної збагачувальної фабрики у Червоноградському гірничопромисловому районі.

Встановлено збільшення вмісту вільних амінокислот, проліну та зниження інтенсивності реакцій пероксидного окиснення ліпідів (за вмістом малонового діальдегіду), що свідчить про збільшення стійкості рослин до несприятливих умов породного відвалу за дії регуляторів росту.

Одержані результати вказують на перспективність подальшого застосування «Stimpo» та «Regoplant» за фіторекультивації породних відвалів.

Ключові слова: *Helianthus annuus* L., «Stimpo», «Regoplant», «Treptolem», субстрати породних відвалів вугільних шахт, вільні амінокислоти, пролін, реакції пероксидного окиснення ліпідів.

ВЛИЯНИЕ «REGOPLANT» И «STIMPO» НА СОДЕРЖАНИЕ СВОБОДНЫХ АМИНОКИСЛОТ, ПРОЛИНА И ИНТЕНСИВНОСТЬ РЕАКЦИЙ ПЕРОКСИДНОГО ОКИСЛЕНИЯ ЛИПИДОВ У *Helianthus annuus* L. ПРИ РОСТЕ НА ТЕХНОЗЕМАХ

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Целью работы было исследовать действие новых регуляторов роста «Stimpo» и «Regoplant» (производитель Государственное предприятие Межведомственный научно-технологический центр — ГП МНТЦ «Агробиотех») по сравнению с гиббереллиновой кислотой и регулятором роста второго поколения «Treptolem» на содержание свободных аминокислот, пролина и активность пероксидного окисления липидов у 14-суточных проростков *Helianthus annuus* L. при росте на грунтовых субстратах породного отвала угольных шахт Центральной обогатительной фабрики в Червоноградском горнопромышленном районе.

Установлено увеличение содержания свободных аминокислот, пролина и снижение активности пероксидного окисления липидов (по содержанию малонового диальдегида), что свидетельствует об увеличении устойчивости растений к неблагоприятным условиям породного отвала при действии регуляторов роста.

Полученные результаты указывают на перспективность дальнейшего применения «Stimpo» и «Regoplant» при фиторекультивации породных отвалов.

Ключевые слова: *Helianthus annuus* L., «Stimpo», «Regoplant», «Treptolem», субстраты породных отвалов угольных шахт, свободные аминокислоты, пролин, реакции пероксидного окисления липидов.