BIOTECHNOLOGIA ACTA

Scientific journal

Vol. 15, No 1, 2022

Bimonthly

BIOTECHNOLOGIA ACTA

Науковий журнал

Том 15, № 1, 2022

REVIEWS

ACETONE-BUTYL FERMENTATION PECULIARITIES OF THE BUTANOL STRAINS-PRODUCER

O. O. Tigunova[1]
V. V. Bratishko[2]
A. V. Balabak[3]
S. G. Priyomov[1]
S. M. Shulga[1]

[1]SE "Institute of Food Biotechnology and Genomics
 of the National Academy of Sciences of Ukraine", Kyiv
[2]National University of Life and Environmental Science
 of Ukraine, Kyiv
[3]Uman National University of Horticulture, Uman, Ukraine

E-mail: Shulga5@i.ua

The aim of this review was to generalize and analyze the features of acetone-butyl fermentation as a type of butyric acid fermentation in the process of obtaining butanol as an alternative biofuel.

Methods. The methods of analysis and generalization of analytical information and literature sources were used in the review. The results were obtained using the following methods such as microbiological (morphological properties of strains), chromatographic (determination of solvent concentration), spectrophotometric (determination of bacterial concentration), and molecular genetic (phylogenetic analysis of strains).

Results. The process of acetone-butyl fermentation was analyzed, the main producer strains were considered, the features of the relationship between alcohol formation and sporulation were described, the possibility of butanol obtaining from synthesis gas was shown, and the features of the industrial production of butanol were considered.

Conclusions. The features of the mechanism of acetone-butyl fermentation (the relationships between alcohol formation and sporulation, the duration of the acid-forming and alcohol-forming stages during batch fermentation depending on the change in the concentration of H_2, CO, partial pressure, organic acids and mineral additives) and obtaining an enrichment culture during the production of butanol as an alternative fuel were shown. The possibility of using synthesis gas as a substrate for reducing atmospheric emissions during the fermentation process was shown. The direction of increasing the productivity of butanol-producing strains to create a competitive industrial biofuel technology was proposed.

Key words: producer strains, biofuel, biobutanol, acetone-butyl fermentation.

The process of acetone-butyl fermentation is associated with the transformation (oxidation) of the organic molecules of the substrate; consequently, the part of the energy is released and accumulated in the molecules of adenosine triphosphate (ATP) due to substrate phosphorylation [1−3]. As a rule, during fermentation, the carbon skeleton of the substrate molecule is split [4], and the fermentation products are formed, such as various organic acids (lactic, butyric, acetic, formic), alcohols (ethanol, butanol, propanol, acetone), as well as gases (carbon dioxide and hydrogen) [5]. In the process of fermentation, two stages can be distinguished — oxidative and reductive stage [6]. The oxidation process is based on the electron detachment from certain metabolites with the help of enzymes (dehydrogenases) and its attachment to other molecules (anaerobic oxidation) [7, 8]. The energy released during this process is stored in the form of ATP. The second stage is the reduction, in which the formed intermediate compound is reduced due to the transfer of electrons and protons to it from a temporary carrier. Reduced organic compounds are released by microorganisms into the external environment.

The present paper considers the stages of acetone-butyl fermentation as a type of anaerobic butyric acid fermentation, features of alcohol formation and sporulation, preparing an enrichment culture, butanol obtaining from synthesis gas, and selected aspects of industrial butanol production.

Acetone-butyl fermentation and butanol production

Acetone-butyl fermentation is a biochemical process of carbohydrates decomposition carried out by selected bacteria, which passes anaerobically (without oxygen access) and results in the formation of acetone, butyl alcohol, as well as acetic, butyric acids and fermentation gases, hydrogen and carbon dioxide.

There are several types of bacteria that are able carry out the process of butyric acid fermentation, e.g., in one of its subtypes, an acetone-butyl fermentation [9−11]. In butyric acid fermentation, glucose is oxidized to pyruvate via the glycolytic pathway, wherein pyruvate is further converted to acetyl-CoA. Acetone-butyl fermentation is carried out by microorganisms that belong to the genera *Clostridium, Butyrvibrio, Butyribacterium, Sarcina, Eubacterium, Fusobacterium* and *Megasphera* [3, 12].

The *Clostridium* genus belongs to the *Bacillaceae* family, together with other members of this family (*Bacillus, Sporolactobacillus, Desulfotomaculum* and *Sporosarcina*). Clostridia are gram-positive, spore-forming bacteria, their dimensions vary from about 2−3 to 7−8 μm in length and 0.5−1 μm in width. Spore-forming anaerobes include giant vegetative cells, reaching 15−30 μm in length and 1.5−2.5 μm in width. They are highly mobile due to peritrichous flagella. Vegetative cells are rod-shaped. However, their shape may vary depending on environmental conditions. Presence of the oval or spherical endospores changes the shape of the rod-shaped mother cell, since their diameter is usually greater than the width of this cell [13].

Physiologically, clostridia are distinguished by a pronounced fermentative type of metabolism, as well as sensitivity to oxygen: their growth is possible only under anaerobic conditions. However, there are also transitional forms from strictly anaerobic species (*C. pasteurianum, C. kluyveri*) to almost aerotolerant ones (*C. histolyticum, C. acetobutylicum*). Clostridia, as a rule, do not contain hemoproteins (cytochrome and catalase). Some species are able to form cytochromes if their precursors are contained in the nutrient medium. Among the reserve substances, starch-like polysaccharides are widespread [14].

The temperature optimum for the growth of most known *Clostridium* sp. lies between 30 and 40 °C. Along with those mesophilic microorganisms, there are many thermophilic species with an optimum of 60−75 °C (*C. thermoaceticum* and *C. thermohydrosulfuricum*). They are able to grow, as a rule, in a neutral or alkaline medium, and their growth almost completely stops in acidic conditions [15, 16].

Clostridia vary in their ability to ferment different substrates [17]. Some types of those microorganisms can ferment a wide range of different substrates, while others are highly specialized and are able to ferment only one or several types of raw materials (Fig. 1). *Clostridia* are able to convert polysaccharides (starch, glycogen, cellulose, hemicelluloses, pectins), organic acids, proteins, amino acids, heterocyclic compounds [18]. Selected microorganisms use complex nutrient media and/or growth substances, while others use molecular nitrogen as the only nutrient (*C. pasteurianum*) [19].

According to the ability to ferment various substrates, microorganisms can be divided into saccharolytic and proteolytic. Saccharolytic clostridia break down mainly mono- or polysaccharides, while proteolytic clostridia break down proteins and amino acids [20].

Butyric acid fermentation is mainly carried out by anaerobic microorganisms *C. butyricum, C. tyrobutyricum, C. lactoacetophilum* [22]. Their main fermentation products are butyric and acetic acids. Acetic acid fermentation of carbohydrates is observed in *C. aceticum* and *C. thermoaceticum* [23]. Propionic acid fermentation is inherent to *C. propionicum*, resulting in the formation of propionic and acetic acid and carbon dioxide as main products [24].

The most active pectinolytic species are *C. felsineum, C. laniganii, C. pectinolyticum, C. pectinovorum, C. virens,* and other pigmented and non-pigmented clostridia and plectridium [25]. Each species has its own specific details of metabolism, but their common property is the ability to decompose pectin substances with the formation of organic acids, alcohols and gases.

Some microorganisms have very stable pectinolytic properties and secrete pectinolytic enzymes into the media not containing
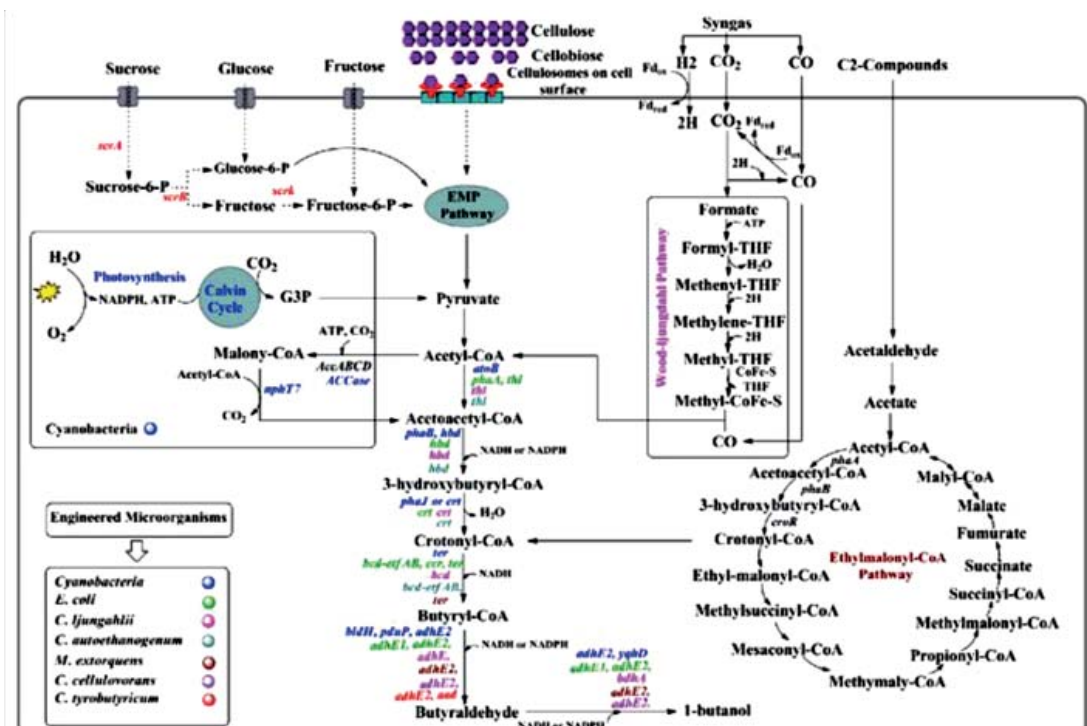
Fig. 1. Schematic of 1-butanol production in heterologous hosts from various feedstocks. Different colors represent heterologous genes expressed in different hosts [21]

pectins. In other anaerobes (for example, *C. multifermentans*), relevant enzymes are synthesized only when pectins are added to the media (induced enzyme synthesis) [26]. Pectinolytic anaerobes carry out the fermentation of sugars according to the butyric or acetone-butyl type. There is a group of highly specialized anaerobic spore-forming bacteria that obtain energy by fermenting cellulose with the fermentation end-products of acetic, propionic, butyric and lactic acids, ethyl alcohol, hydrogen and carbon dioxide, and intermediate products of glucose and cellobiose. For such bacteria, when glucose or sucrose is added to the nutrient medium, the fermentation process is practically absent (sugars are not assimilated), and when glucose and fiber are added simultaneously, mainly fiber is fermented. This indicates a high specialization of cellulose-decomposing microorganisms.

Cellulolytic bacteria differ not only in physiological but also in morphological features. Most cellulolytic spore-forming anaerobes have the appearance of thin long rods that form spores according to the plectridium type. Vegetative cells are usually present in an adsorbed state on cellulose fibers. Perhaps this is due to the fact that enzymes that hydrolyze cellulose (cellulases) are not released into the medium, but are attached to the cell surface. Spore-forming cells usually exist in solution; during spore formation, the nature of the cell connection with the environment changes, and spore formation occurs due to endogenous metabolism (due to intracellular nutrient reserves). Several specialized species of anaerobic bacteria have been identified that use organic acids and alcohols as a source of carbon and energy [27].

Microorganisms *C. kluyveri*, as a rule, obtain energy due to the conjugated oxidation-reduction system of ethyl alcohol−acetic acid, thus higher fatty acids are formed (mainly caproic and butyric acids). Not all *C. kluyveri* bacteria are capable of fermenting carbohydrates, amino acids, and purines. The accumulation of energy through ATP in such anaerobes occurs through the mechanism of oxidative phosphorylation [28].

There are three types of bacteria (*C. acidiurici*, *C. cylindrosporum* and *C. uracilicum*) that ferment heterocyclic compounds. They are able to destroy heterocycles with the formation of acetic acid, carbon dioxide and ammonia. The first two types of bacteria are not able to use carbohydrates and proteins (amino acids). These bacteria cleave xanthine, guanine, guanosine, 6,8-dioxipurine relatively quickly, and cleave hypoxanthine and inosine relatively slowly (even after adaptation) [29].

In spore-forming anaerobes, the specificity in relation to substrates is highly pronounced. The media containing a set of amino acids, carbohydrates, mineral salts, a complex of vitamins, and microbial growth activators may not be sufficient for selected proteolytic anaerobes (for example, *C. sporogens*) [30]. Such apparent heterotrophs grow only on the media containing proteins or products of their partial hydrolysis. However, there are anaerobes (sulfate-reducing bacteria) that ferment simple media, which include several mineral salts (including sulfates) and organic acid (atmospheric nitrogen can also be assimilated).

The ability to fix molecular nitrogen is widespread among spore-bearing bacteria. Such a process can be carried out by butyric, acetone-butyl and sulfate-reducing bacteria. The most active nitrogen fixers are saccharolytic anaerobes (clostridia). The relation to oxygen in different physiological groups of spore-forming anaerobes is not the same. Saccharolytic anaerobes are more resistant to oxygen. Some representatives of this group are aerotolerant forms of *C. carnis* and *C. histolyticum*, capable of weak growth on agar plates even under aerobic conditions. Sulfate-reducing bacteria are sensitive to oxygen and difficult to culture. Their growth is possible only under anaerobic conditions without oxygen in the cultivation medium [30].

Alcohol formation and sporulation

The mechanism of the regulation of alcohol formation has not been fully elucidated [31, 32]. This is especially relates to the switching phase of fermentation and the relationship between alcohol formation and sporulation (Fig. 2).

The spores represent specifically arranged resting germ cells that may withstand the action of high temperature, radiation, vacuum, various kinds of toxic substances and other unfavorable factors that lead to the death of vegetative cells. The formation of spores occurs at a certain stage of development at the moment when nutrient resources (sources of carbon and nitrogen) are exhausted in the environment, or toxic metabolic products accumulate [34]. The main purpose of spore formation is to transfer the culture to a resting (anabiotic) state, therefore, in mature spores, the metabolism occurs at extremely low level. This enables bacteria to survive in unfavorable environmental conditions, and when conditions change, they switch again to vegetative growth. For anaerobes (especially soil ones) it is also extremely important that the spores are not sensitive to oxygen. This allows them to survive under aerobic conditions that would have a detrimental effect on vegetative cells [35, 36].

Young, rapidly dividing anaerobic cells contain nucleoids in the form of dumbbells or V-shaped figures. Before sporulation, cell division stops, the cells sharply increase in size. At this time, an accumulation of a large amount of granulosa, a reserve nutrient, occurs, and it is being deposited in the form of granules, thus the cytoplasm becomes granular, and the cells swell, taking the form of a lemon (clostridium) or a drum stick (plectridium). In a minor part of proteolytic anaerobes cells do not change their original
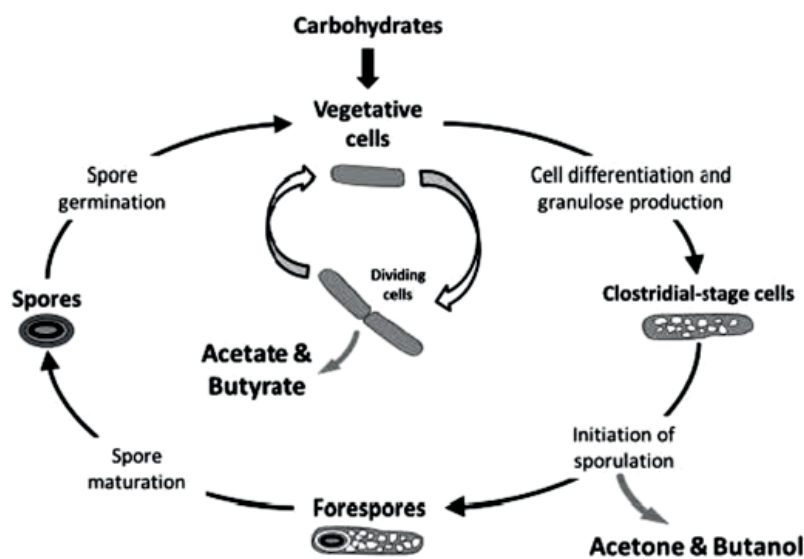
Fig. 2. The general cell cycle of *Clostridium acetobutylicum* depicting different cell forms and major products during acidogenesis and solventogenesis [33]

appearance, retaining the usual rod-shaped (bacillary) shape [37]. The first sign of the onset of spore formation is a change in morphology. Further, several nucleoids approach at one of the poles of the cell, merge and form a longitudinally located convoluted chromatin (nuclear) strand. The cytoplasm zone, where the nuclear cord is located, turns into a prospore [38]. Small cell bacteria usually have two separate nucleoids before sporulation, which fuse to form an axial chromatin strand. Subsequently, only part of this thread goes into a spore. The third type of nuclear behavior is found in many saccharolytic anaerobes. Their nuclear substance has the form of a chromatin mesh located throughout the cytoplasm. Part of this mesh is pulled together at one of the cell poles with the formation of a strand, which forms the center of the emerging prospore. Using a conventional microscope, three stages of spore formation can be observed [39]. At the first stage the sporogenous appears zone at one of the cell poles, in which the nuclear substance in the form of light rods is clearly visible. At the second stage, the sporogenic zone turns into a dark (optically dense) oval prospore with clearly defined contours [40]. In the prospores, the nuclear substance is no longer detected without the use of special methods (staining). At the third stage, the prospores gradually lighten, acquiring the ability to strongly refract light, and lose their ability to stain with dyes. Ripe spores look like light, sharply refracting light bodies with a strong shell. Prospore formation begins with invagination (ingrowth) of the cytoplasmic membrane closer to one of the cell poles [41]. In this case, the membrane moves to the center of the cell, and its poles merge to form a spore partition wall (septum). This process involves mesosomes, which help to stick together the converging sections of the invaginated membranes. The septum consists of two elementary membranes. At this moment, the second stage of spore formation is finished (if we take the formation of a chromatin strand as the first stage). The second stage can be considered as a modified cell division, which, as is known, also occurs due to the invagination of the cytoplasmic membrane and a septum formation. The next stage is an "absorption" process by the mother cell of the septate (cut-off) area of the cytoplasm with the nucleus [42]. This process is carried out by the growth and advancing of the peripheral sections of the membrane in the mother cell towards the cell pole. Then the converging sections of the membrane merge and a prospore

is formed, which has two elementary (three-layer) membranes, internal and external. In some species, the prospore later remains at the cell pole (terminal location); in others, it moves inside the cytoplasm, occupying a central or subterminal position [43]. Thus, at the end of this stage, a kind of bicellular organism is formed: inside the cytoplasm of the mother cell, a new cell arises, a prospore, surrounded, unlike the mother, by two membranes. From this moment, a new irreversible phase of the development and metabolism begins, ending with the maturation of the spore and the death of the mother cell [44]. Unlike the fourth stage, the second and, in part, the third stages of sporulation are reversible. Thus, when, after the formation of a septum, the antibiotic chloramphenicol is added to the sporulating culture, protein synthesis will be suppressed. The movement of the peripheral sections of the membrane that absorbs the cut-off section of the protoplast will be stopped [45]. As a result, the process of spore formation that has begun will turn into a normal process of vegetative cell division, and cell wall material will accumulate between the two septa membranes. Such accumulation does not occur in the normal course of sporulation. At the fourth stage of sporulation, a cortical layer (cortex) is formed between the inner and outer membranes of the prospore [46]. First, the cortex appears as a thin dark layer, similar in structure and density to the cell wall of a vegetative cell. Then this layer sharply increases in thickness due to the formation of more electron transparent (light) layer. At the fifth stage, the spore shell is formed. At the beginning, the areas of a dark (electron-dense) substance in the form of scales appear around the prospore at some distance from the outer membrane of the prospore in the cytoplasm of the mother cell. At the sixth stage, the individual sheets of the shell elongate and, in the end, merge, forming a solid continuous dense layer. Between this layer and the outer membrane of the prospore, a cut-off layer of the cytoplasm of the vegetative cell remains [47]. On top of the first layer of the shell, one or two more layers can be deposited. In this case, they are divided into inner, middle and outer layers of the shell. These layers differ from each other in structure. In some species, the inner layer of the shell is lamellar, while the outer layer looks like a dense thick layer [48]. In other species, on the contrary, the lamellar layer may be external, and the denser layer may be internal. If the structure of the core is very similar in different species, then

the structure of the spore shells in them varies greatly both in fine structure and in the number and thickness of layers [49]. After the final maturation of the spore, the lysis of the parent vegetative cell occurs: the cell wall is destroyed, and the spore enters the external medium (seventh stage). The shape of mature spores can be different in different types of anaerobes: spherical, oval, ovoid, cylindrical [50].

In many anaerobes, another structure is found on top of the spore membrane, i.e., the exosporium. The exosporium has the appearance of a multilayer sheath, which the spore is located in. Such structure is observed in *C. pasteurianum, C. bifermentans, C. tyrobutyricum* [51]. In the exosporium of many anaerobic species, the layers contain subunits which are placed in specific order. The spherical subunits in the lamellar layer of the exosporium have a hexagonal packing. Adjacent subunits may sometimes fuse, forming ring-shaped structures with pores in the center. Such exosporium layers comprise perforated membrane films. Exosporium occurs at an early stage of spore formation in the form of a small bubble on the outer membrane of the prospore. This vesicle grows, turning into a sheath covering the spore from all sides [52].

The core of the spore, surrounded by a layer of cortex, is a protoplast with its own membrane, nucleus, and cytoplasm. The core of a mature spore is a resting vegetative cell. It is characterized by a very low metabolic rate and although it contains all the necessary enzymes, their activity is somehow suppressed [53].

The cortex is composed of mucopeptides that are very similar to cell wall mucopeptides. The cortex also contains diaminopimelic acid. In spores, dipicolinic acid ($C_7H_5O_4N$) is found in fairly large quantities. It is an active chelating agent, forming the claw-like complexes with metals. This substance is absent in vegetative cells. Dipicolinic acid is released from spores in the form of calcium and magnesium salts, which play a major role in the thermal stability of spores. Dipicolinic acid is also involved in the process of transferring the spore protoplast to a dormant state [54]. The mechanism of these processes has not been elucidated yet. Possibly, dipicolinic acid is localized in the cortex, since there is a certain correlation between cortex formation and the accumulation of dipicolinic acid and calcium in the spore [55]. The cortex of mature spores plays a protective role. It protects the core from lytic enzymes that destroy cells. This assumption was confirmed for mutants that have lost the ability to form a cortex. At the

final stage of spore formation, there is a sharp increase in the activity of lytic enzymes, which completely destroy the parent vegetative cell. Spores without cortex are also lysed [56].

The shell (or cover) is a unique structure of bacterial spore that is not found in other microorganisms. It mainly consists of protein substances enriched with cystine. The volume of the shell reaches 50% of the total spore volume. The substance of the spore shell is not sensitive to the action of various lytic enzymes. The shape of the spores, specific for each type of bacteria, is maintained due to the structural rigidity of the membranes. The shell also plays the role of a protective structure that protects spores from premature germination. Spores of mutant strains lacking the shell usually germinate immediately after emerging from sporangia in an environment unfavorable for growth (even in distilled water), which leads to the death of germinated cells. However, the role of the spore membranes, as well as the cortex, remains largely enigmatic [57].

The exosporium is a membranous structure; it often has a multilayer composition. The exosporium probably plays the role of a barrier that regulates the penetration of various substances into the spore. In many anaerobic bacteria, the exosporium is not a confined system, as its polar part, immersed in the cytoplasm of the mother cell, contains very large pores up to 0.5 μm in diameter. After mechanical removal of the exosporium, the spores remain normal, their germination process is not disturbed. A feature of spores in anaerobes is the formation of special outgrowths of various structure. Each type of anaerobic bacteria tends to have its own type of outgrowth structure. This feature is strictly specific, hereditarily fixed and very stable. Even in defective spores that have lost the ability to form a shell, the outgrowths are preserved and do not change their specific structure [58].

On *C. taeniosporum* spores, the outgrowths have a ribbon-like shape. A bundle of such outgrowths is attached to the spore with the help of a special organ — the pad. The outgrowths appear at an early stage of prospore formation, before the initiation of the cortex and shell; then they grow, lengthen, and penetrate the cytoplasm until they reach the opposite pole of the cell. The cytoplasm around the outgrowths gradually lyses. The mother cell is destroyed. On the free mature spores emerging from the sporangium, the outgrowths bloom in the form of an umbrella [59].

The spores of *C. sporogenes* have a single large and complex outgrowth. It has an

appearance of a long thick bundle or trunk, forming a ring at the end, from which antennae extend, a tubular rod-shaped outgrowths. The trunk has a coarse-grained structure and transverse striation, fine-grained antennae have a capsular layer. The formation of outgrowths in this species can be traced on intact cells [60]. At first, the processes are poorly visible, since they are surrounded by dense areas of the cytoplasm, then the cytoplasm becomes lighter and the outgrowths become clearly visible. A ring-shaped structure and antennae are clearly visible at one of the poles of the cell [61]. The function of outgrowths on spores has not yet been finally elucidated. Some researchers suggest that the outgrowths on spores are specific sensitive (chemosensory) organelles that give the spore a "command" for germination (under favorable conditions). Others believe that outgrowths play an important role in the process of spore maturation, participating in the formation of spore covers and the cortex. Some studies postulate that outgrowths on spores are the result of some disturbances in normal metabolism. The question of the enzymatic activity of outgrowths is very important [62].

When spores are transferred to a fresh nutrient medium they begin to germinate. Firstly, they swell, darken, then, through the hole formed in the spore shell, the young cell exits into the outer medium. In this case, the cortex layer is destroyed, and the spore shell, together with outgrowths (if any), is shed. In anaerobes, it is rarely possible to study the germination by observing the same single spore. At the last stage of the exit, the vegetative cell is blasted off. The hole in the spore shell is formed not strictly at the pole of the spore, but somewhat on the side, and the young vegetative cell, when exiting, is located at an angle to the long axis of the spore. In other anaerobes, the germination process may look different [63].

Germination characteristics of *C. pasteurianum* are used to differentiate this species from other spore-forming anaerobes [64]. Finally, three species of Clostridium, *C. pectinovorum*, *C. butyricum*, and *C. tetani*, differ in that their spores germinate inside the sporangium [65]. The cell wall (or part of it) in these species is not lysed, but remains on mature spores, covering them in the form of a sheath. But this sheath is not identical in origin and structure with the exosporium described above. *C. acidiurici* and *C. cylindrosporum* are physiologically very close, but clearly differ by morphological

features. In the case of *C. acidiurici*, the spores are oval, located terminally, and the cells swell during sporulation [66]. In the case of *C. cylindrosporum*, the spores are cylindrical, located centrally or subterminally, and the sporangia do not swell [67].

Obtaining an enrichment culture

To obtain enrichment cultures of *Clostridium*, some of their features could be used. Their main feature is the thermal resistance of spores, which facilitates the isolation of microorganisms by the method of preliminary pasteurization of the inoculum [68]. To maintain the ability for intensive fermentation, pasteurized inoculums are used when working with isolated strains. Another feature is their anaerobicity or aerotolerance [69]. By creating strictly anaerobic conditions, the growth of all aerobic bacteria is excluded in advance. Strains can be isolated from soil, sewage, animal wastes, potatoes, roots of nitrogen-fixing legumes, milk, and cheese. When using a substance containing large particles as an inoculum, for example, starch grains or cellulose particles from the rumen of ruminants, those particles are firstly washed and then used as material for inoculation. Industrial strains isolated from natural sources are contained in different microorganism collections , such as ATCC (American Collection of Culture Types), DSM (German Collection of Microorganisms), NCIMB (National Collection of Industrial and Marine Bacteria, United Kingdom), NRRL (Northern Regional Research Laboratory — Agricultural Research Service Culture Collection, US Department of Agriculture) [70].

Most acetone-butyl bacteria share a similar phenotype, metabolic pathways, and end products. The taxonomy of these bacteria is quite complicated and time-consuming. Acetone- and butanol-producing strains are now divided into four species (Fig. 3), according to genetic features, namely *C. acetobutylicum*, *C. beijerinckii*, *C. saccharoperbutylacetonicum* and *C. saccharobutylicum* [71−73].

C. beijerinckii synthesizes solvents at approximately the same rate as *C. acetobutylicum*, but it synthesizes isopropanol instead of acetone [74, 75]. *C. aurantibutyricum* synthesizes isopropanol in addition to butanol and acetone [76]. The main source of carbon for the growth of *C. tyrobutyricum* bacteria is lactose, and the fermentation products are butyric acid, hydrogen, and carbon dioxide [77].

C. tetanomorphum is a relatively new producer that synthesizes almost equimolar amounts of butanol and ethanol and does not synthesize other alcohols [78].

Clostridium producer strains have different productivity and the end-product accumulation rates (Table 1). The accumulation of the corresponding products of microbial synthesis depends both on the strain itself and the cultivation medium, as well as on growth factors, pH, and temperature [79].

Production of butanol from synthesis gas

In addition to the classical scheme of ABE fermentation, it is necessary to note an alternative way of butanol production from synthesis gas using the *C. carboxidivorans* bacteria [81]. *C. carboxidivorans* binds CO, fixes CO_2 and convert them into acetyl-CoA according to the Wood-Ljungdahl scheme (Fig. 4).

In this scheme, two CO_2 molecules are used, but for completely different purposes: one molecule is used as a carbon source, and the second molecule is used as an electron acceptor. At the first stage, carbon dioxide is fixed with the help of tetrahydrofolate using the energy of ATP, and at the second stage, acetyl-coenzyme A (acetyl-CoA) is synthesized from CH_3-H_4-folate. The transfer of the methyl group to coenzyme A is carried out by a special methylase, a cobalt-containing iron-sulfur protein.

Two processes take place in parallel:

The first process. CO-dehydrogenase (CODH) reduces the CO_2 molecule to CO, and the electrons for reduction are usually taken from H_2:

$$CO_2 + 2\,e^- + 2H^+ = CO + H_2O.$$

The reduction of CO_2 to CO occurs at the so-called Fe_4NiS_5-active center of the "C-cluster"protein.

The second process. Acetyl-CoA synthetase condenses a carbonyl group with a methyl group at the so-called "A-cluster", giving a metal-bound acetyl group, which is released from the enzyme via thiolysis by acetyl-CoA.

$$CO + H_2O = CO_2 + 2e^- + 2H^+ \qquad (1)$$

$$CH_3\text{-}Co^{3+}FeSP + CO + CoASH = \\ = CH_3C(O)SCoA + Co^{1+}FeSP + H^+ \qquad (2)$$

$$H^+ + Co^{1+}FeSP + CH_3\text{-}THF = \\ = CH_3\text{-}Co^{3+}FeSP + THF \qquad (3)$$

$$CH_3C(O)SCoA + H_4SPT + H_2O = \\ = CH_3\text{-}H_4SPT + CO_2 + 2e^- + 2H^+ + CoASH, \qquad (4)$$
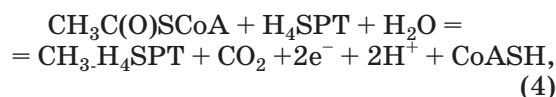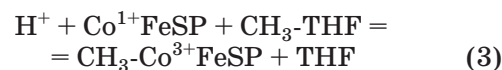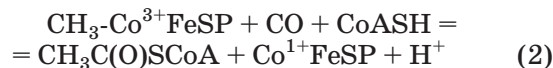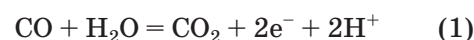
where H_4SPT is tetrahydrosarcinapterin, the archaeal analogue of tetrahydrofolate (THF). CoFeSP in reaction (2) is a corrinoid-[FeS]-protein, a heterodimer containing the nucleotide cofactor cobalamin in one subunit and the Fe_4S_4 cluster in the other. Reduced Co^{1+}cobalamin accepts the methyl group from CH_3-THF in the reaction (3).

This process contains fewer steps, does not require organic seeds such as citric acid to initiate reactions, and carbon fixation in it occurs in only one reaction. In addition, the Wood-Ljungdahl pathway is the only way to fix carbon without the use of ATP or other triphosphates. It should be noted that all genes encoding enzymes in the classical scheme of ABE fermentation are present in the genome of *C. Carboxidivorans*, and the genome also has the *sol* operon and alcohol formation genes, which, for some reasons, are not included in fermentation. [83–85]. It is possible that the same genetic process had occurred here, as in the degenerated (DGN) strains which lost the ability to produce solvents after repeated cultivation. For *C. acetobutylicum* ATCC 824, the complete loss of the pSOL1 plasmid, which

Table 1. **Productivity of butanol-producing strains [80]**

Strain	Substrate	Type of fermentation	Solvent accumulation, g/l
Clostridium sp. BOH 3	Xylose	ABE*	5.32; 14.94; 1.25
C. tetanomorphum DSM 665	Glucose	Butanol-ethanol (BE)	9.8; 1.01
C. pasteurianum DSM 525	Glycerol	Butanol-propanediol	7.13; 6.79
Clostridium sp. BT 10-6	Glucose	Isopropanol	5.26
Clostridium sp. NJP 7	Glucose	Isopropanol-methanol	12.21; 1.92
C. pasteurianum GL11	Glycerol	Butanol-ethanol	14.7; 0.01

Note: * — The name of the type of fermentation directly characterizes the products of fermentation, for example, ABE — acetone-butanol-ethanol.
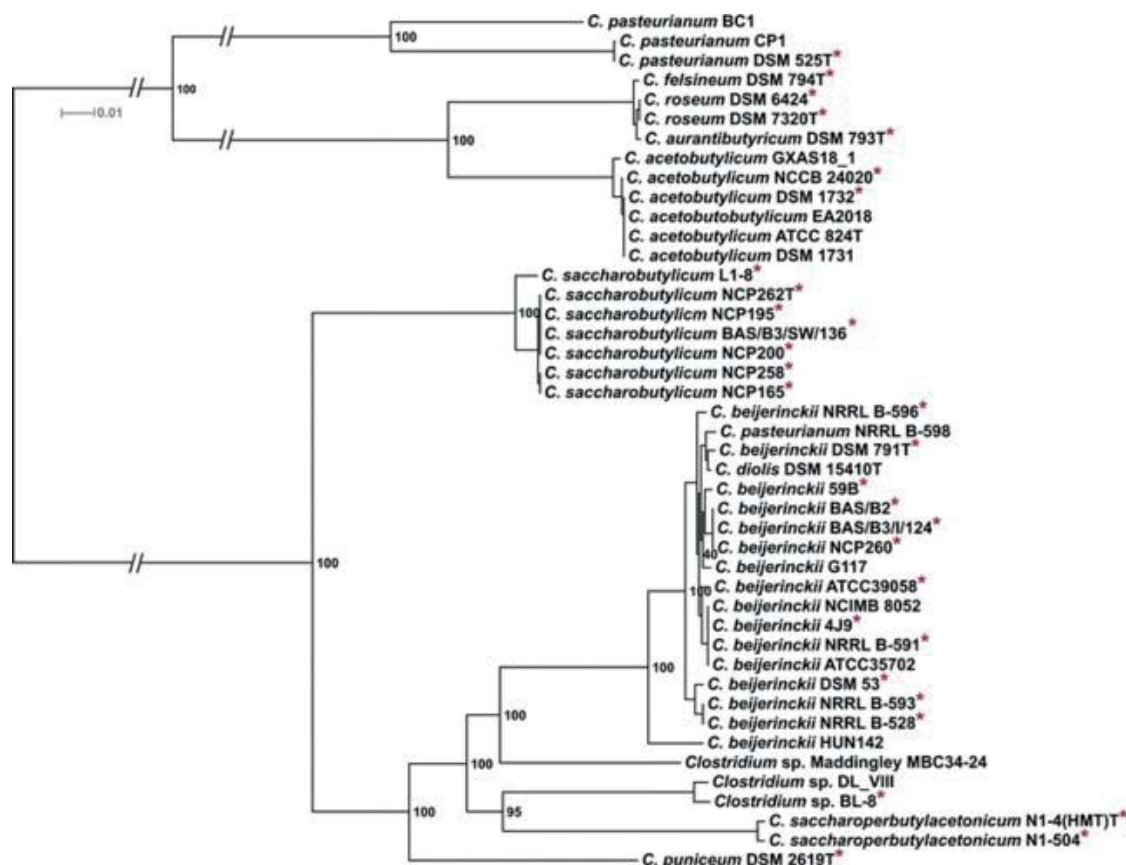
Fig. 3. **Phylogenetic tree of 44 sequenced solventogenic clostridia [10]**
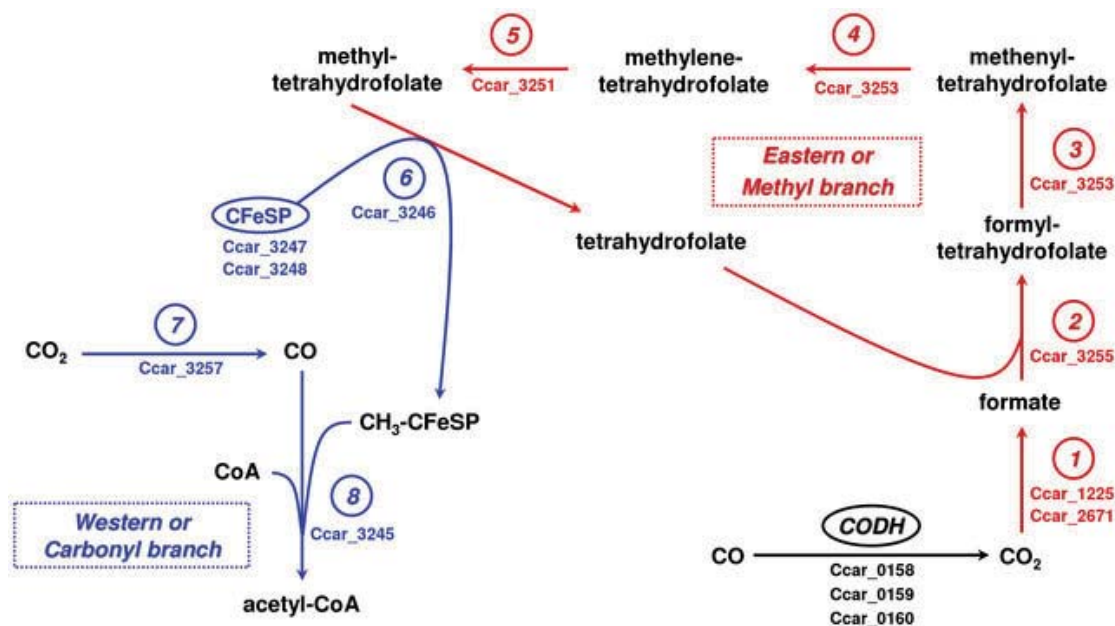
Fig. 4. **Scheme of the Wood-Ljungdahl pathway of** *C. carboxidivorans* **P7T [82]:**
where 1 is formate dehydrogenase; 2 — formate-tetrohydrofolate ligase; 3 and 4, bifunctional methenyltetrahydrofolate cyclohydrolase/methylenetetrahydrofolate dehydrogenase (NADP$^+$); 5 — 5,10-methylenetetrahydrofolate reductase; 6–5-methyltetrahydrofolate:corrinoid/iron-sulfur protein Co-methyltransferase; 7 — carbon monoxide dehydrogenase; 8 acetyl-CoA synthetase; CoFeSP is a cobalt-iron-sulfur protein that catalyzes the transfer of a methyl group from tetrahydrofolate to coenzyme A and carbon reduced to (+2), CODH is an additional carbon monoxide dehydrogenase complex. The corresponding genes of the strain are shown below the enzyme

contains the *sol* operon, consisting of the alcohol-producing genes *aad, ctfA, ctfB,* and *adc,* was shown during the degeneration [86, 87].

One species of Clostridia, *C. cylindrosporum*, is able to generate formyl tetrahydrofolic acid formate and tetrahydrofolate in a reaction that is accompanied by ADP phosphorylation. In this species, this reaction is the main way to obtain ATP. All reactions of substrate phosphorylation are localized in the cell cytosol, which suggests the simplicity of the chemical mechanisms underlying substrate phosphorylation. The degree of oxidation and the amount of free energy, as well as the nature of the products formed, are related to the nature of the final electron acceptors. In the process of fermentation, the final electron acceptors are mainly organic compounds: metabolites obtained from the original substrates (pyruvic acid, acetaldehyde), or the substances present in the cultivation medium. The main function of hydrogenases in Clostridia is to remove excess catabolic reactions of reducing equivalents (electrons) that have been produced and are removed from the cell in the form of molecular hydrogen. Other ways to obtain hydrogen are also possible. For example, $NADH_2$, which is produced in the glycolytic pathway, can reduce ferredoxin with the help of $NADH_2$: ferredoxin oxidoreductase, and H_2 is released from the reduced ferridoxin by hydrogenase [88].

Features of industrial butanol production

It is not only the genetic features of the producer strain are a key factor in the microbiological synthesis of butanol; substrate (raw materials) and, in fact, the technology also make a major contribution into the cost of the final product.

By selecting a producer strain and an appropriate substrate, pretreatment of the substrate, optimization of technological parameters (pH, temperature, aeration, and nutrient supply), one can change the productivity of the producer organism and the accumulation of the final product [89]. The following microorganisms of the genus Clostridium are used in the industrial production of butanol: *C. acetobutylicum, C. beijerinckii, C. saccharobutylicum* and *C. saccharoperbutylacetonicum.*

Some species of Clostridia are characterized by an altered metabolic pathway of synthesis and, and consequently, the yield of solvents differs significantly from that obtained in classical ABE fermentation. In this regard, the selected producer strain must be checked using metabolomic analysis. The first stage of such an analysis is the performing of classic "direct" fermentation with subsequent study of all intermediates and final products. For fermentation, pure cultures of bacteria, or preliminarily prepared spores for inoculation are used [90].

Spore preparation is an essential and necessary step for cultivation in industrial production [91]. The accumulation of spores is carried out on a 6% mash of corn or rye flour. For the formation of spores, tubes with fresh sterile mash are inoculated with a culture of bacteria and the entire fermentation cycle is carried out at 37 °C. Spores could be prepared by pouring as well. For each new portion, spores obtained from cultures that have shown the maximum results of product accumulation during fermentation under industrial conditions are used [92]. The first stage of fermentation is carried out in a vessel, from which the mash is poured into sterile test tubes where the process ends with the formation of spores. The tubes with spores are sealed and placed in a thermostat. After 18−60 hours, the fermentation gases are released, the test tubes are sealed again and stored at room temperature. After two months of storage, the spores are tested by test-tube fermentation. The spores satisfying the technological conditions are recognized as suitable and the passports are issued for them [93].

After the accumulation of spores, ABE fermentation is carried out directly. It can be proceeded both in batch, semi-continuous and continuous mode. Batch fermentation is a relatively simple process [94].

Large amounts of pure culture are needed to carry out a batch process under industrial conditions. The preparation of the required amount of pure bacterial culture for production begins with the inoculation of spores in a pure culture apparatus (PCA) [95]. After 28 hours, the fermentation contents of the PCA are sterilely transferred to a large inoculator (LIN). Sterile mash for LIN is taken when it is hot. It is cooled in a LIN or refrigerator. From the inoculator, the bacterial culture is transferred under sterile conditions to the fermenter-activator of the production battery. In batch fermentation, in the first hours after inoculation of the mash with active culture, fermentation is observed, noticeable by gas release from the surface. Gas release peaked after 24−26 hours and subsided towards the end of fermentation.

During the period of maximum gas release, a characteristic stratification of the substrate occurred: a "loose" mucous layer moved upward to the surface, a cloudy opalescent liquid remained in the lower layer, and the entire medium acquired a yellowish color. This phenomenon in production is referred to as the "rise" of the mash and is one of the signs of normal fermentation. By the end of fermentation, the solid part of the substrate precipitated to the bottom.

Along with the gas release, the shape of the titratable acidity curve is also a characteristic feature of ABE fermentation. The growth of bacteria was characterized by the increase in titratable acidity, reaching its maximum (4.0–4.6 mL of 0.1 NaOH per 10 mL of mash) by 12–16 hours of fermentation, and then sharply decreased by 24–25 hours, after which there was a slight increase in acidity towards the end of fermentation. In the process of increasing acidity, the pH of the medium decreased from 6.0 to 4.1 and virtually remained at this level. The formation of alcohols began starting from approximately the 6th hour of fermentation, but become the most intensive after the "break" in the acidity curve. Up to 35% of total carbohydrates were converted into alcohols, and the final mash contained about 2% of solvents. The cost of the substrate in the cost of butanol obtained by the classical method is 60%, which makes the process of obtaining butanol economically unprofitable [96–100].

To increase the accumulation of butanol in the process of "direct" batch fermentation, the duration of the acid-forming and alcohol-forming stages can be changed by changing the concentration of H_2, CO, partial pressure, organic acids and mineral additives. The addition of CO during the batch fermentation using *C. acetobutylicum* tends to inhibit hydrogenase activity [101]. During batch cultivation of *C. saccharoperbutylacetonicum*, it was demonstrated that the removal of hydrogen from the bioreactor leads to the accumulation of only H_2, while alcohols did not accumulate [102].

Increasing the culture productivity is possible at the stage of metabolites formation. Artificial electron carriers such as methyl viologen and neutral red drastically change the production of metabolites. Microelements of the environment can also influence the electron transfer. The conversion of pyruvate to acetyl-CoA involves the use of iron-sulfur proteins (ferredoxin oxidoreductases), and iron is also an important mineral supplement. A change in the iron concentration significantly affected the process of butanol synthesis [103–107].

A semi-continuous fermentation process was used to avoid fermentation inhibition by high substrate concentrations. However, due to the inhibitory properties of butanol, fed-batch culture is ineffective. In industrial production, a semi-continuous fermentation process is known as a battery fermentation. The battery consists of 6–8 bioreactors serially connected into an integral device. The main reactor (the activator) is inoculated with a culture from the inoculator and after the "break" of the acidity curve (after about 12 hours) they are loaded with flour mash. The entire battery is filled through the activator. The battery is unloaded (the worth is transferred for rectification) from the last "tail" bioreactor. After sterilization, the "tail" bioreactor becomes the activator of the next battery, consisting of the same bioreactors, but loaded in the opposite direction. To optimize the semi-continuous fermentation process, the technological system for a continuous fermentation process was created.

Continuous fermentation made it possible to reduce (up to 70%) the flour usage and replace it with cheaper raw materials: sugar beet molasses (syrup) and hydrolysates of vegetable waste [108].

In this scheme, the use of flour mash for the first phase is preferred. This ensures the rapid growth of bacteria and the formation of enzymes for the synthesis of solvents. Molasses and hydrolysates are introduced during the transferring of the fermentation to the second phase. The scheme of continuous two-phase fermentation has been introduced into industrial production. According to this scheme, for the process of continuous ABE fermentation, an additional stage was proposed, a pure production culture apparatus (PPCA), which was sown from LIN. After 10–12 hours of fermentation, the entire culture from it was transferred to the first bioreactor of the production battery, and its loading with flour mash had immediately began. The flour mash was transferred to the first (head) bioreactor of the battery, as well as for the further breeding of a pure culture in the inoculator and PCA. The molasses mash was sent to the second fermenter, where the transition to the second phase of fermentation took place.

This technology allowed the fermentation to continue for a long time and with a high productivity of the producer strain. The rate of butanol synthesis intensification in a

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continuous process is similar to that for a batch process. An increase in butanol accumulation can also be obtained by adding precursors to the cultivation medium [109–113].

Conclusions

The features of the acetone-butyl fermentation mechanism (the relationships between the alcohol formation and sporulation, the duration of the acid-forming and alcohol-forming stages during batch fermentation depending on the change in the concentration of H_2, CO, partial pressure, organic acids and mineral additives) and obtaining an enrichment culture were shown. The possibility of synthesis gas use as a substrate for reducing emissions into the atmosphere during the fermentation process was demonstrated.

The direction of increasing the productivity of butanol-producing strains to create a competitive industrial biofuel technology has been proposed.

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ОСОБЛИВОСТІ АЦЕТОНОБУТИЛОВОГО БРОДІННЯ ШТАМІВ−ПРОДУЦЕНТІВ БУТАНОЛУ

О. О. Тігунова[1], В. В. Братішко[2], А. В. Балабак[3], С. Г. Прийомов[1], С. М. Шульга[1]

[1]ДУ «Інститут харчової біотехнології та геноміки» НАН України, Київ
[2]Національний університет біоресурсів і природокористування України, Київ
[3]Уманський національний університет садівництва, Умань, Україна

E-mail: Shulga5@i.ua

Метою даного огляду узагальнення та аналіз особливостей ацетонобутилового бродіння як процесу отримання альтернативного біопалива — бутанолу.

Методи. Застосовано методи аналізу й узагальнення аналітичної інформації та літературних джерел. Результати отримано з використанням мікробіологічних (морфологічні властивості штамів), хроматографічних (визначення концентрації розчинників), спектрофотометричних (визначення концентрації бактерій) та молекулярно-генетичних (філогенетичний аналіз штамів) методів.

Результати. Досліджено процес ацетонобутанолового бродіння, розглянуто основні штами-продуценти, описано особливості взаємозв'язку спиртоутворення та споруляції, показано можливість отримання бутанолу із синтез-газу, розглянуто особливості промислового виробництва.

Висновки. Показано особливості механізму ацетонобутилового бродіння (взаємозв'язок спиртоутворення та споруляції, тривалість кислотоутворюючого та спиртоутворючих етапів за переодичної ферментації в залежності від зміни концентрації H_2, CO, парціального тиску, органічних кислот та мінеральних добавок) та отримання накопичувальної культури за отримання бутанолу, як альтернативного палива. Показано можливість використання синтез-газа як субстрата для зменшення викидів в атмосферу в процесі ферментації. Запропоновано напрямок збільшення продуктивності штамів-продуцентів бутанолу для створення конкурентноспроможної промислової технології бутанолу.

Ключові слова: штами-продуценти, біопаливо біобутанол, ацетонобутилове бродіння.

MATHEMATICAL MODELS OF RESPIRATORY AND BLOOD CIRCULATORY SYSTEM

N. I. ARALOVA[1], O. M. KLYUCHKO[2], V. I. MASHKIN[1], I. V. MASHKINA[3], P. A. RADZIEJOWSKI[4], M.P. RADZIEJOWSKA[5]

[1] V. M. Glushkov Institute of Cybernetics of the National Academy of Sciences of Ukraine, Kyiv
[2]National Aviation University Kyiv, Ukraine
[3]Boris Grinhenko Kiyv University, Ukraine
[4]Kazimiera Milanowska College of Education and Therapy , Poznan, Poland
[5]Czestochowa University of Technology, Czestochowa, Poland

E-mail: aralova@ukr.net

Aim. To analyze modern approaches to mathematical modeling of respiratory and blood circulatory systems of human organism.

Methods. Comprehensive review of scientific literature sources taken from domestic and foreign resources, databases.

Results. Historical information and contemporary data concerning mathematical modeling of functional respiratory and blood circulatory system were summarized and analyzed in present review; current trends in approaches to the construction of these models were revealed.

Conclusions. Two main approaches to the mathematical modeling of respiratory and blood circulatory systems exist for today. One of them is the construction of models of mechanics of respiration and blood circulation. They were based on the models of mechanics of solid deformable body, thermomechanics, hydromechanics, and mechanics of continuum media. This approach supposes the use of complex mathematical apparatus, including Navier-Stokes equation, which makes it possible to obtain a number of theoretical results, but it is hardly usable for real problems solutions at present time. The second approach was based on the model of F. Grodins, who represented the process of the breath as controlled dynamic system, written using ordinary differential equations, in which the control was carried out according to the feedback principle. There was significant number of modifications of this model, which made it possible to simulate various disturbing influences, such as physical activity, hypoxia and hyperemia, and to predict parameters characterizing functional respiratory system under these disturbing influences.

Key words: mathematical model of respiratory system; mathematical model of blood circulatory system; hypoxic state; theoretical analysis.

Human lives in the Nature during all his life and interacts with the environment. During this human receives various disturbing influences, both external (changes in gas composition and pressure of inhaled air, temperature of environment) and internal (changes in metabolism, viral infections). A number of laboratory and instrumental methods are developed to analyze the state of organism, with which one can get information about his current state, but nothing more. The task of adequate modeling of processes in living organism is one of the most relevant for contemporary medicine. The processes that ensure the viability of human organism are so complex and interrelated that the closest possible interaction of mathematicians, biologists and physicians is necessary for their modelling.

The purpose of the work was to analyze modern approaches to mathematical modeling of respiratory and blood circulatory systems of human organism.

Physical formulations of problems that arise during the study of normal and pathological processes in human organism have become significantly more complicated in contemporary reality [1]. Along with the expanding capabilities of computer modeling, the requirements for mathematical models that describe the processes occurring in human organism have increased. The objects of modern mathematical modeling are almost all major human systems and organs — respiration and blood circulation, heat exchange and thermoregulation, central and peripheral nervous systems, digestion, kidneys and liver, immune system and carbohydrate metabolism system, musculoskeletal system, organs of hearing, vision, leather, and etc. Simultaneously, modeling can be carried out at the cellular and gene levels. Mathematical models that allow the simulation of mechanisms of the start and course of diseases, from wounds and oncological diseases curing to issues of immunology, drug delivery, and the creation and functioning of various organs were of considerable interest too. Mathematical models of a number of organs and parts of organism (skin, bones, muscles, etc.) were based on mechanical models known from the mechanics of a solid deformable body. For the problems of hemodynamics, functioning of respiratory system, digestion, and excretory system, hydrodynamic formulations based on the Navier-Stokes equations were used. Significant part of mathematical models of thrombosis, functioning of stomach, skin, and treatment of a number of diseases by thermal or chemical effects was based on the equations of reaction-diffusion and heat conduction. The dynamic systems of ordinary differential equations formed the basis of models of respiratory and blood circulatory system, transmission of nerve impulses, cellular interactions, functioning of gene networks, and etc. Naturally, hybrid models that take into account the fullest possible interaction and interrelationships of the processes under the study were the most in demand.

The most characteristic numerical models for a number of biomedical processes constructed using the classical theory of continuum mechanics had been analyzed in review [1]. Reviews of this class of problems were included into [2−18] too; they were interesting, although partially not very recent ones. Brief, but rather capacious review was presented in [19].

Evolutionary models were of great interest too; they allowed simulating various disturbances in organism and predicting its stationary state at a given level of disturbances. It was possible to manage various influences, consider their combination, study and predict the functional state of human organism under various extreme influences by simulating these influences on mathematical model. Currently, this is especially important when choosing a strategy and tactics for medical cure of organism affected by the SARS CoV-2 virus.

Today, mathematical and simulation models are used widely to study the regulations of physiological processes. Mathematical modeling is an effective tool that allows simulating extreme disturbances on human organism and predicting its reactions to disturbances in the internal and external environment, while modern diagnostic methods characterize only the current state of organism. The advantage of using simulation models is the ability to obtain information at the level inaccessible to modern invasive methods. An overview of mathematical models of various organs and systems of organism one can find in [1, 20]. This direction was based primarily on the works of P.K. Anokhin [21, 22], whose main ideas were the theory of functional systems and application of systematic approach to the study of physiological functions. F. Meyerson built a coherent theory of organism adaptation, in which, among other functional systems, the respiratory and blood circulatory systems were singled out as most noticeably responding to the changes of human life conditions [23, 24]. It was also demonstrated [25−27] that if the human organism was presented as a chain with "weak link" in terms of reliability theory, the respiratory and blood circulatory system can be seen as such "weak link".

Widespread computerization had formed the bases for the development of theoretical foundations of any phenomenon or process by simulating this phenomenon or process using computer. It is clear that for the development of computer model the development of mathematical model had to be done previously. This is especially true for physiology and medicine. If in physics and chemistry the experimenter deals with inanimate objects with which any experiments can be performed, then here, in addition to ethical standards, there are quite a few limitations associated with inability to experiment with various extreme perturbations and limitations of diagnostic methods. Mathematical modeling allows
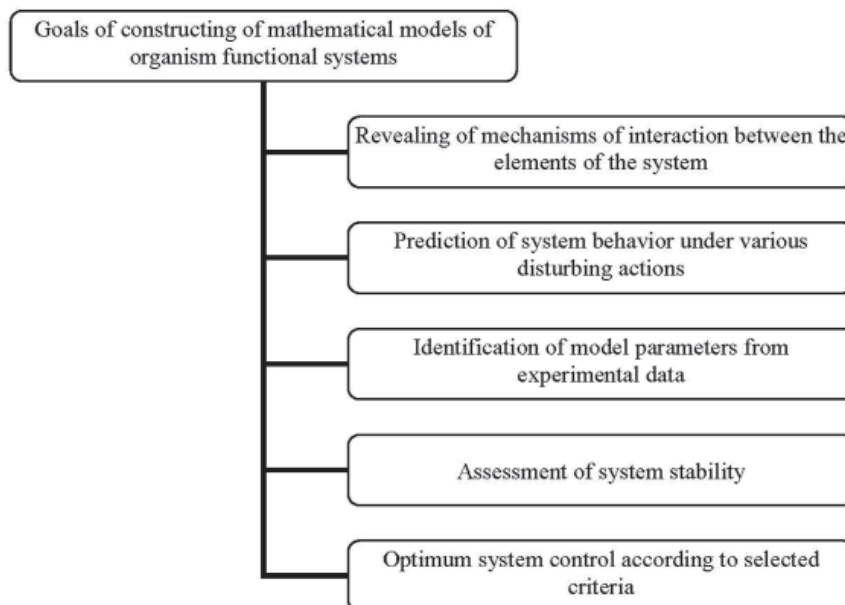
Fig. 1. **Goals of modeling of organism functional systems**

us to control various influences, study and predict the behavior of such complex system as human organism. The complexity of the task of mathematical models constructing of organism functional systems was primarily due to the extreme complexity of considered biological system, functioning of which depends nonlinearly on large number of factors, on almost every element of living organism, and these dependencies largely remain unformalized even at the level of physiological descriptions. Therefore, analytical methods of solution can be applied in rather narrow scope, and the main means for studying of real problems related to the study of respiratory and blood circulatory systems are computational methods for problems solution with computer.

When choosing and formulating a mathematical model, the determining factors are the object, purpose, method and methods of modeling [Anokhin]. For mathematical modeling methods of dynamic systems theory are used. Means — differential and difference equations, methods of qualitative theory of differential equations, computer simulation. The goals of mathematical modeling of organism functional systems were shown on Fig. 1.

The main principle of mathematical modeling of complex systems is the principle of optimality [28]. It is necessary to note that the principle of optimality has been used for a long time in biology.

Methods of theory of optimal control in physiology

The methods of the theory of optimal control of respiration had aroused the constant interest of researchers of physiological systems, primarily due to the ideas about the perfection of regulatory mechanisms in living systems. The specificity of application of methods of the theory of optimal control in the study of physiological systems is that the criteria for their optimality are unknown. The task of the study was to establish whether given physiological system was an optimal control system and what exactly the criterion for its optimality was. Accordingly, such studies include the following steps:

− selection of the control object and the construction of its mathematical model;

− selection based on the data of experimental studies of a hypothetical optimality criterion;

− construction of mathematical model of the optimal control system, including the control subsystem (optimizer) and the control object;

− study of the model of control in order to verify its adequacy.

At each of these stages, significant difficulties arise, both experimental and theoretical. Therefore, the number of works on this issue were insignificant and they had been done only recently [29, 30]. The indicators, linked with energy transformation in organism the most often played the role of criterion of optimality, since the economy of physiological

25

functions in general case is beyond the doubt. The model of regulation of parameters of external respiration and organism blood supply developed by Yu.M. Onopchuk, was based on the assumption concerning the optimality of the system for regulation of organism oxygen regimes [31]. The criterion for optimality of the functional state of oxygen transport system (OTS) can be the sum of energetic costs of organism [32]:

$$W = W_H + W_L + W_E + W_T,$$

where W_H, W_L, W_E, are the values of power consumed by the heart, lungs and hematopoietic system, respectively, W_T — is the power of extraction in the absence of physical activity. The values W_H, W_L, W_E, W_T are the functions of parameters φ_I, characterizing the functional state of the OTS. The optimal values of these parameters $\frac{dW}{d\varphi_i} = 0$ can be determined by solving equations.

Based on this approach, it is possible to investigate how close the OTS control algorithms are to optimality and within what limits of changes in the conditions of oxygen transport this closeness is maintained.

An adaptive neural network model had been developed; it detected the optimality and homeostatic characteristics of respiratory control system [33]. The theoretical analysis of the effect of main parameters changes of the inhaled air flow on the distribution of air and mean barometric pressure in the lungs was made [34]. A method has been developed to differentiate the process of lung ventilation into two images: the first characterizes the optimal distribution of inhaled air for each of the ventilated lungs; the second characterizes the optimal perfusion, which corresponds to the lowest possible average alveolar pressure. Using the concept of the work of the heart based on the end-systolic volume-pressure dependence, the conditions for optimal interaction of the left ventricle with the arterial system were determined [35]. A mathematical model of the left ventricle of the heart has been developed, reflecting both hemodynamics and the processes of myocardial enlargement depending on the load and metabolic conditions [36]. The model was used to determine the optimal myocardial function for given arterial load and vice versa. The optimal size of the left ventricle was determined from the conditions of maintaining normal oxygen consumption under the different conditions of chronic load on the heart rate. A mathematical model of the human circulatory system, which includes a description of the systemic and pulmonary circulation

and baroreflex regulator for heart rate and peripheral circulation [37], was used for theoretical analysis of the problem of optimal control of blood circulation with complete replacement of the heart or its partial unloading after the connection of auxiliary pump. An optimality criterion for blood circulation control was proposed. This criterion was based on minimizing the deviations of variables of the model state, for them the arterial and venous pressures in the systemic and pulmonary circulation were chosen. In Murray's model of optimal branching of vessels [38], the radii of the vessels were related to the viscosity, the rate of vascular exchange, and the rate of blood circulation in such a way as to minimize the overall (hydraulic and metabolic) work of the system.

In [39], the problem of optimal structural and functional organization of the external respiration and circulatory system was mathematically formulated as optimization problem with chosen objective function and restrictions. As a result of its solution, the optimal values of structural and functional parameters were found, which can be compared with the corresponding experimental values.

We would like to mention the work [40] as well, which analyzes publications related to extremal principles in mathematical biology.

Mathematical models of the respiratory system

According to [41], two approaches can be used for the mathematical analysis of physiological functions: the data models and system models. In the first case, the task is to build mathematical function that describes a set of input data more accurately, for example, a statistical data model. But physiological features of the structural and functional organization of the modeled object are not taken into account at all in it. Models of the second type are based on physiological principles and hypotheses regarding the structural and functional organization of the modeled object. The purpose of modeling is to test the physiological hypothesis, which is the basis of the model, and to study basic physiological mechanisms of the phenomenon or process under investigation. It should be noted that usually the results of analysis of physiological experiment with the construction of data models were used as initial data for the next stage of investigation — systemic analysis of these data models and development of mathematical model of studied

system functioning, aimed on studying of fundamental physiological mechanisms underlying its functioning.

Among the models of the respiratory system, a real breakthrough was the model of F. Grodins [42], in which the respiratory system is represented as dynamic system, so, this allows using appropriate mathematical apparatus. There are a large number of mathematical models of respiratory system, which are based on Grodins model, in which rather complex mathematical apparatus is used. Without touching of models of "black box" type, which also have the rights to exist because they allow us to identify cause-and-effect relationships and reliable dependencies at the population level, but do not allow us to analyze the processes occurring within the system, we will pay more attention to structural models. Such models were developed on the basis of the laws and hypotheses on the structuring and functioning of biosystems. Mathematical models of respiratory system differ depending on the purpose of investigations. There are widespread such models of respiratory mechanics, in which the lungs are represented by elastic shells connected to the atmosphere by a tube with some hydraulic resistance [43], which make it possible to obtain the simplest relationships between physical parameters characterizing the functioning of the lungs, but do not take into account spatial diagnostics in human lungs.

One of the most developed approaches was based on quasi-one-dimensional hydraulic models on graphs [1]. Detailed description of such models one can find in [44, 45], where a complex model of the respiratory system and closed model of blood flow was built on the basis of distributed dynamic system on graphs. The elastic properties of the tube walls, which determine the relationship between the pressure in the tube and its cross section, are determined by additional equation of state. Dynamic model of air entering the alveoli from the external environment with each breath (alveolar volume) was presented also in [45].

Brief review of human lung models that vary in complexity, starting from the simplest, which were presented as rigid container contacted with the atmosphere, to a model in which the volume and pressure that change under the influence of muscle work, taking into account gas exchange with blood and perfusion blood had been suggested in [46]. Two-chamber lung model consisting on alveolar space through which the blood was perfused and anatomical dead space was described in [47]. This model is used to estimate the minute volume of blood

circulation. One-dimensional model of air transfer from trachea to alveoli was analyzed in [48], taking into account the gas exchange of respiratory gases with blood and blood perfusion. An assumption about the correct dichotomy of airways and laminar flow of the air, and the explanation of the reasons of existence of exactly 23 generations of airways was done, although the Weibel model by itself was proposed much earlier [49]. However, we have to note that the assumption about laminar flow in airways was substantiated by domestic scientists on mathematical model [50] much earlier. Similar results were described too in [51]. With intensive development of computational methods of gas dynamics and means of their implementation three-dimensional models of air flow had been started to develop, the air by itself was considered as multicomponent mixture of gases. A review of these models can be found in [52].

The models that describe the process of gas exchange in the lungs and operate with averaged parameters were much more interesting. In [53], other version of the model was proposed in which the total mass of the lungs and chest were assumed to be distributed over the surface of any reservoir of variable volume. The mechanical properties of such reservoir were determined by integral characteristics that described the resistance of airways, the inertia of the air in them, the elasticity of the lungs and chest. The model was based on the ideal gas equation under isothermal conditions, the equation of the motion of reservoir shell and the integral equation of air movement in respiratory tract. This model was developed further in [52], where the equation of the model [53] was written not for the entire volume of the lung, but only for its individual elements, into which air enters through one of respiratory tubes.

It was written in [1] that in recent years, the flow of air in the upper respiratory tract and in whole respiratory tract had been studied intensively using realistic 2D and 3D [54−56]. The study of air movement in nasal cavity, in particular, has many analogies with classical problems of aerohydrodynamics and is of great interest to contemporary medicine. This is due to the facts that now new methods of drugs administration through the nose by inhalation into the lungs or directly on the mucous membrane of the nasal cavity are being actively developed. In [56] such studies were carried out on the basis of Navier-Stokes equations with reproduction of real geometry of respiratory tract, obtained on the basis of analysis of the results of computer tomography.

Mathematical model of human respiratory system was proposed in [52], which contained a set of three connected sub-models that describe the breathing process as a set of synchronized gas dynamics processes in large airways, air movement in a deformable saturated porous medium, and gas exchange (diffusion) through biological membrane. This model was positioned by the authors as sub-model of multilevel model of the entire human organism.

Linear graphs were uses for the development of dynamic model in dissertation [57]. Line graphs of respiratory system have been developed to include all energy domains with sensors linking them together to represent complex dynamic system. Mathematical model of lung mechanics had been developed, including the properties of alveolar tissue and surfactant, which created acceptable values of pulmonary pressure and volume in comparison with the data of healthy patients, patients with acute respiratory distress syndrome (ARDS). The model described time-varying alveolar compliance, which provided better understanding of lung diseases. Using analysis of sensitivity, it was shown that the concentration of surfactant and the parameter of collagen stiffness influenced strongly on the variables of lung mechanics. In addition, the model was proved to be stable and reliable under various perturbations. The model was a set of ordinary differential equations that could be implemented to allow testing of scenario "what if" by changing certain parameters. Using patient data and method of parameters estimation, a personalized version of the model can be obtained. One step more was done to personalized medicine in this dissertation with other physiology-based model and optimization algorithms that improve patient health estimation, diagnosis, and therapy. The dynamic system of the lungs was described, including:

· a module of lung mechanics that described quantitatively the changes in lung pressure and volume;

· alveolar elasticity module which determined alveolar compliance as a function of surfactant concentration and lung fiber elasticity;

· module of the respiratory and thoracic mechanics, which calculated chest movement and pleural pressure changes in process of respiratory muscles contraction and relaxation during breathing (diaphragm and intercostal muscles);

· system of microcirculatory exchange, described the transport of fluid (water) and mass (protein) between alveoli and pulmonary capillaries;

· lung gas exchange system, which quantitatively described the transport of carbon dioxide and oxygen from the lungs into the blood of pulmonary capillaries;

· pulmonary circulation module;

Each module was developed basing on the latest knowledge of lung physiology and validated using patient data when they were available, or published and validated physiology-based models when these data were not available. This dynamic respiratory system could be used to describe the state of healthy people and people with various pathologies. The model made it possible to enter individual patient data and test various therapeutic scenarios in order to select optimal therapy for the patient. In addition, systems identification techniques can be applied to this model or part of it to achieve personalized medicine for better diagnosis and treatment of diseases. For the estimation of the state of lungs health of particular patient, a simplified model of lung mechanics had been developed. Using this simplified model, the parameters which reflected the state of lung health and functionality, i.e. the mechanical properties of the lungs (resistance and compliance) and the efforts of patient's breathing, it was possible to evaluate pulmonary syndromes or diseases such as ARDS and COPD (chronic obstructive pulmonary disease); these diseases caused changes in lung resistance and compliance. Tracking of these two parameters can lead to better diagnostic of disease and easier monitoring of respiratory disease progression. Non-linear, model-based, constrained optimization algorithm has also been developed to estimate lung resistance, lung compliance, and patient inspiratory effort caused by inspiratory muscle activity.

The author also suggested to use linearized version of this model and system identification methods to evaluate not only changes in compliance, but also the properties of the fiber or surfactant that caused these changes. Thus, the model can also mimic the condition of some COVID 19 patients with ARDS. According to [58], 20−30% of patients with COVID 19 admitted to the Department of intensive therapy had severe hypoxemia associated with low values of corresponding parameters.

In some literature sources [59] one can find also such approaches to study of respiratory system as [60−78]: modeling of lung mechanics was given in [69, 70], and modeling of gas exchange — in [71, 72, 75]. Articles [70, 73,

74] provided examples of the development of controllers for the regulation of respiratory system and virtual laboratories designed to simulate the respiratory and cardiovascular systems [65, 66]. The article [59] suggested a simple model that provided a linearized description of pulmonary ventilation and gave some equations that described the basics of chemical regulation of pulmonary ventilation. The processes associated with lung ventilation were discussed, and this formed a basis for overview of other respiratory functions and physiology of some respiratory diseases [65]. The main role was to provide oxygen, which is essential for tissue metabolism, and to remove metabolic by-products such as carbon dioxide. In the nature, complete gas exchange is achieved by passive diffusion through the air-blood barrier between capillaries and alveoli. This requires, in the case of changing load conditions, the maintenance of an appropriate pressure gradient across the barrier, which is achieved by changing the rate of entry of fresh air into the alveoli. As a result of this exchange, the blood flowing from the lungs through the pulmonary veins contains a high O_2 concentration and a low CO_2 concentration. The reverse process of metabolism is carried out in the tissues, where O_2 is consumed and CO_2 is produced as well as metabolic by-products. Four main parts of the respiratory process were being considered:

· Pulmonary ventilation, which includes the flow of air between the atmosphere and alveoli of the lungs in both directions.

· O_2 and CO_2 diffusion between alveoli and perturbed elements of the blood

· O_2 transport and CO_2 ejection in blood and body fluids to the cells and from them.

· Regulation of ventilation and other aspects of breathing.

Only processes related to lung ventilation were discussed in [59]. One of the models describing the human cardiovascular system was represented by the system of 13 differential equations [61−63]. The model consists on two series-connected circuits (systemic blood and lung) and two pumps (left and right ventricle).

Mathematical models of circulatory system

As for mathematical models of blood circulatory system, there are many investigations in framework of this topic exist too. First of all, this is due to the fact that the study of physiological and pathophysiological processes in cardiovascular system is a relevant topic of many contemporary studies [79].

The main functional role of cardiovascular system is blood transportation. The heart provides blood flow in the system of blood vessels. The blood vessels through which the blood flows from the heart to periphery form arterial system. The vessels that collect blood and carry it to the heart form the venous system. A detailed description of the circulatory system and the formulation of the corresponding tasks was given in [4, 5]. In these monographs mechanical behavior of blood, mechanical behavior of the heart, static and dynamic properties of the heart, microcirculation, transcapillary transport of substances, mechanical properties of blood vessels and blood movement in them, as well as mechanics of pulmonary circulation were described.

The blood circulatory system is one of the most popular objects in medicine for all who study blood hydrodynamics [1]. Possible physical problem statements were associated with the description of general blood circulation in human organism [80−86], blood circulation in individual vessels [83, 87] and organs — in heart, kidneys, brain [80−82, 88, 89] for healthy organism or damaged one. To study the general patterns of blood flow in organism and individual organs, the most popular models on graphs are currently used. Classical hydrodynamic formulations based on the Navier-Stokes equations were primarily associated with 2D and 3D modeling of blood flow in large and small vessels, taking into account the elasticity and multilayeredness of vessel walls, multicomponent blood and complex rheology.

In review [1], the models of cardiovascular system were divided into such classes (Fig. 2).

Over the past thirty years, several key approaches have been formed. They made us possible to describe local and systemic processes associated with blood flow, which have different degrees of spatial representation, which depends on the applied problem being solved. Usually, for this was used mathematical apparatus, that included algebraic and differential equations [90]. Averaged models of this type are not demanding on computational resources and contain small number of parameters that are easily determined for particular organism; but, unfortunately, it reflects general physiological patterns only [79]. More complex models require the use of more complex mathematical apparatus. Thus, a detailed description of the blood flow in large vessels was carried out using the Navier-Stokes equation in two or three measurable approximations [91].
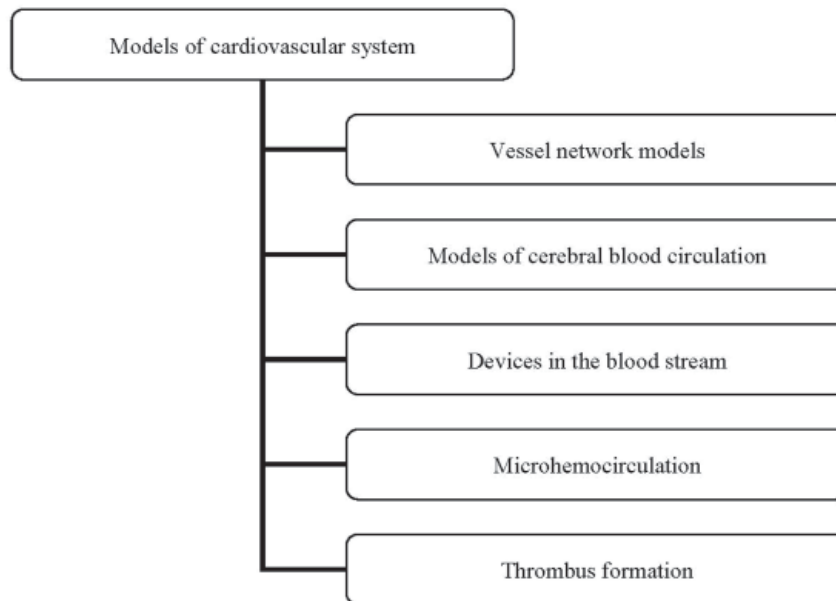
Fig. 2. **Models of the cardiovascular system analyzed in [1]**

The methods for solving of nonlinear partial differential equations in three-dimensional domains of complex shape were used in this approach [92, 93]. In this case, the problem of constructing of three-dimensional geometry corresponding to the shape of vessel or vascular bed aroused. The use of two-dimensional or three-dimensional models also required the setting of boundary conditions at the boundaries through which blood flows, the tasks of rheological properties of blood, taking into account the mobility of the vascular wall, the elastic properties of the wall, the pressure of surrounding tissues, etc. All this makes the use of such models quite inefficient; in addition, it requires the use of significant amount of computing resources. Although the area for the application of following models is typical: three-dimensional analysis of blood circulation parameters in aorta [94], in the main cerebral vessels [95], in aneurysm [96].

Current information on the functional interaction of respiratory and blood circulatory systems under various conditions of organism's vital activity was presented in [97]. Particular attention was paid to adaptive changes in respiratory and hemodynamic parameters in extreme conditions. Basing on the data from literature sources and our own research, physiological parallels were done between intersystem relationships of biomechanics of respiration and hemodynamics under conditions of normal gravity and weightlessness.

It was stated that all systems of organism are involved in maintaining of homeostasis at adequate level for metabolism, but external respiration and blood circulation play decisive role. These systems demonstrate certain independence, characteristic patterns of functional organization and they are in close connection with other parts of the gas transmission system. The often manifested synchronism in changes in respiratory movements and blood pressure under various influences indicates the anatomical and functional relationship between respiratory and vasomotor centers.

In [98] was represented mathematical model of hemodynamics under mechanical influence on the vessels, proved the existence of a smooth solution on the edge, presented numerical implementation of the model, discretized the compatibility conditions, verified the convergence of the numerical solution in uniform norm. There were also tested and numerically simulated following problems: test, autoregulation in separate vessel, occlusion test, blood flow in the coronary arteries, blood flow was also modeled in the lower extremities when running and walking, blood flow was simulated in stenosis of the femoral artery, blood flow in the coronary artery was simulated with increased external counterpulsation, the calculation of the fractional blood flow reserve with using a model of coronary circulation, calculations of coronary hemodynamics in multivessel lesions of the coronary arteries before and after stenting. In the model there were united such components as: models of the muscle pump, autoregulation, enhanced

external counterpulsation, as well as coronary circulation, taking into account the functioning of myocardium. The model is based on the previously developed one-dimensional model of hemodynamics and supplemented with models of mechanisms of autoregulation, elasticity of the vascular wall, coronary circulation, with increased external counterpulsation and muscle pump. The disadvantages of proposed model include the discreteness of the recalculation of parameters of vascular elasticity, while in real organism autoregulation occurs continuously. Input data were obtained from clinical examination, in particular angiography, computed tomography and magnetic resonance imaging, as well as the data of medical diagnoses. The lack of information about the structure of the networks was proposed to be solved by replacing the entire large circle with one integral vessel, which will have the same effect on the arteries of the heart as the entire network of vessels had.

The dissertation work [99] attracts the great interest because it represents developed global model of cardiovascular and respiratory systems. The cardiovascular part of this model was based on the four-component Grodins model and included modifications by Kappel and Peer. Respiratory part was based on two-chamber model developed in [100]. The basic models had been revised, expanded and generalized. As subsystems, the model included: systemic and pulmonary circulation, left and right ventricles, tissue and pulmonary compartments. Mechanisms such as Frank-Starling law, the Bowditch effect, and variable cerebral blood flow were included. In particular, the model was adapted to the situation of dynamic exercises. The initial anaerobic energy supply, the mechanism of metabolic autoregulation in peripheral regions, dilatation of pulmonary vessels were taken into account. In the model, the control parameters were the heart rate and alveolar ventilation. Simulation of rest and physical activity was possible. The disadvantage of this model was that it takes into account the control of the functional respiratory system only by linear feedback, in which the quadratic cost functional is minimized, while there are other control loops existed — by perturbation, by anticipation, etc. [101],

Integral mathematical model of the human cardiopulmonary system was presented in [102, 103], in which were described the problems of interaction between cardiovascular and respiratory systems. It should be noted that this approach was applied by a few authors only, and these works were a pleasant exception. Integrated cardiorespiratory model has been developed for mathematical description of interaction between the cardiovascular and respiratory systems, as well as their main short-term control mechanisms. The model was compared with the data from open-data sources of human and animal investigations. Article [102] was devoted to the development of models for normophysiology. It includes cardiovascular circulation, respiratory mechanics, tissue and alveolar gas exchange, and short-term neural control mechanisms acting on both cardiovascular and respiratory functions. The model has hundreds of parameters and variables representing physical and physiological properties of human cardiopulmonary system. It can simulate many dynamic states and scenarios. The model was able to simulate physiological variables commonly registered for adults under normal and pathological conditions and to explain the main mechanisms and dynamics.

Further development of this approach was [103], which emphasizes the importance of testing the model for abnormal or pathological conditions in order to prove its consistency and validates the model under conditions of hypercapnia and hypoxia. The authors of this article had focused themselves on testing of cardiopulmonary model under the conditions of hypercapnia and hypoxia by comparing the results of the model with population-averaged cardiorespiratory data presented in literature. The utility of this comprehensive model was demonstrated by testing of internal consistency of modeled responses for significant number of cardiovascular variables (heart rate, blood pressure, and cardiac output), respiratory variables (respiratory volume, respiratory rate, minute volume, alveolar ventilation), and partial pressures and stresses CO_2 over a wide range of perturbations and conditions; namely, hypercapnia at 3-7% CO_2 levels and hypoxia at 7−9% O_2 levels with controlled CO_2 (isocapnic hypoxia) and without control (hypocapnic hypoxia). Finally, the analysis of sensitivity was done to identify the role of main mechanisms of cardiorespiratory control triggered by hypercapnia and hypoxia.

Another extension of the Grodins model was the development described in [104], which is a computational model of human respiratory control system, which is the extension of the model in [105]. The model combined an accurate description of installation with new design of the control part, which considers minute ventilation as the sum of central and peripheral

components. To ensure that the developed model was stable and enough reliable to serve as a test platform for ventilation control hypotheses, the authors modeled a number of complex physiological conditions, in particular, the response to eucapnic hypoxia, development of periodic breathing during hypocapnic hypoxia, and the open-loop response to hypercapnic step. These stationary and transient responses of the model were compared with the results of similar physiological experiments. It was assumed that for a certain value of arterial pO_2, the steady-state difference between cerebral and arterial pCO_2 remained approximately constant depending on arterial pCO_2. The model shows that hypoxia-induced changes in cerebral blood flow contribute significantly to the reduction in ventilation observed during eucapnic hypoxia. The model demonstrates periodic breathing caused by hypoxia, which can be eliminated by small increase of carbon dioxide in breathing mixture. The dynamics of hypercapnic ventilation response of the open model approximated well the experimental data.

To predict changes in ventilation, blood gases, and other critical variables under conditions of hypocapnia, and these conditions in combination with hypoxia, a model [106] was developed, which was based also on the model [105]. Refinements of the model concerned the description of:
· influence of blood gases on cardiac output and cerebral blood flow;
· acid-base balance in blood and tissues;
· binding of O_2 and CO_2 with hemoglobin;
· respiratory-chemostatic controller.

The controller consisted on the central and peripheral parts. The ventilation response was induced by the central chemoreceptor, and it is a linear brain function of pCO_2 above the threshold. The peripheral response had both a linear term, similar to that for the central chemoreceptors, but depending on carotid body pCO_2 and with other threshold, and a complex, non-linear term, including the multiplication of individual terms, including the carotid body pO_2 and pCO_2. Being together, these terms form "bent-leg" ventilation curves plotted in dependence on pCO_2, which form a fan-shaped family for various values of pCO_2. With this chemical regulator, the model accurately describes a wide range of experimental data under conditions of exclusively pCO_2 changes and under conditions of short-term hypoxia combined with pCO_2 changes. This model can be used for precise descriptions of changes in ventilation and breathing gases during the ascent and brief-term stay at altitude.

Complex mathematical model [107] was proposed to simulate the exchange, transport, and accumulation of oxygen and carbon dioxide in adults; and model ability to provide realistic responses under various physiological conditions was evaluated. The model is three-compartmental (i.e. lungs, body tissues and brain tissues) and includes control part that regulates alveolar ventilation and cardiac output; the model integrated dynamically the stimuli from peripheral and central chemoreceptors. New realistic dissociation curve for CO_2 was included; it based on two-buffer model of acid-base chemical regulation. In addition, the model takes into account relevant physiological factors such as buffering, non-linear interaction between chemoreceptor reactions for O_2 and CO_2, lung shunt, dead space, variable time delays, and the Bohr and Haldane effects. When simulating hypoxia and hypercapnia modes using this model, obtained results were consistent with those obtained experimentally in the state of n as with dynamic, rest, load and recovery in terms of such parameters as kA ventilation and partial pressures of gases, concentrations of pCO_2, HCO_3, and hydrogen ions in the blood.

The model [108] allows simulating the response of respiratory and blood circulatory system to aerobic exercise for healthy individuals and individuals with heart failure. Physiological response to exercise is seen now as an important tool that can help in diagnosis and treatment of cardiovascular disease. That is why several mechanisms are needed to ensure higher cardiac output and higher oxygen delivery to the tissues. The paper presented fully closed cardiorespiratory simulator that reproduced the main physiological mechanisms occurred during aerobic exercise. The simulator provided also insight into the impairment of these mechanisms in heart failure and their impact on portability limitation of physical exercises. The simulator consists on a model of cardiovascular system, including the left and right part of the heart, the circles — pulmonary and systemic blood circulation. This latter was divided into exercising and non-exercising compartments and it is controlled by baroreflex and metabolic mechanisms. In addition, the simulator includes breathing model that reproduces gas exchange in the lungs and tissues, ventilation control and the effect of its mechanics on cardiovascular system. The simulator had been tested and compared with literature data at three different workloads while cycling (25W, 49W and 73W). The results demonstrated that the simulator was able to reproduce the reaction to the load in terms

of: heart rate (from 67 to 134 bpm), cardiac output (from 5.3 to 10.2 l/min), blood flow in the legs (from 0.7 to 3 .0 l/min), peripheral resistance (0.9 to 0.5 mmHg/(cm3/s)), central arteriovenous oxygen difference (4.5 to 10.8 ml/dL), and ventilation (6,1−25.5 l/min). The simulator was adapted further to reproduce the main disturbances observed in heart failure, such as reduced sensitivity of baroreflex and metabolic control, decreased perfusion in the training area (from 0.6 to 1.4 l/min) and hyperventilation (from 9.2 to 40.2 l/min). Thus, the simulator is a useful tool for describing the basic physiological mechanisms that operate during the training. The model can reproduce how these mechanisms interact and how their damages can limit physical performance in case of heart failure. Thus, the simulator can be used in future as testbed for various therapeutic strategies aimed at improving of physical performance in patients with cardiopathy.

In [109], an original mathematical model was proposed for studying the response of cardiovascular system to dynamic load, including pulsating heart, pulmonary and systemic blood circulation, separate description of vascular bed in active tissues, local metabolic vasodilation in these tissues, and the mechanical effect of muscle contractions on venous return. In addition, the model provides a description of respiratory response to exercise and various neural regulatory mechanisms that affect cardiovascular parameters. All parameters in the model were given in accordance with physiological data from scientific literature sources. The model was used to simulate a stationary value of main cardiorespiratory values at various levels of aerobic exercise and time pattern in transition phase from the rest to moderate exercises. The results showed that, with appropriate parameter settings, the model was able to mimic accurately the cardiorespiratory response over the entire range of aerobic exercises. The model may be useful for improving of understanding of exercise physiology and as educational tool for analyzing the complexity of cardiovascular and respiratory regulation.

Integrated mathematical model [110] focused on predicting the response of healthy person at rest and aerobic exercise to study the response of cardiorespiratory system to physical activity. The paper outlines the construction of the model and carried out comparative analysis with known models, like integrated cardiorespiratory model. The model includes cardiovascular circulation, respiratory mechanics and gas exchange system,

as well as cardiovascular and respiratory regulators. Each system was based on previously recorded physiological models and includes known mechanisms related to the dynamics of aerobic exercise. The simulation results were compared with experimental data in steady state and transient conditions. Predictions of proposed model replicate closely the experimental data, showing overall the smallest prediction error (10.35%), the fastest settling time for cardiovascular and respiratory variables, and overall the fastest and most similar transient responses. These results indicate that the proposed model was suitable for predicting the cardiorespiratory response of healthy adults under the conditions of rest and aerobic exercise.

An analysis of mathematical modeling of the response of blood pressure and heart rate to submaximal loads was carried out in [111]. Cardiovascular homeostasis was studied at rest and during physical loading.

Mathematical model of CO_2 influence on cardiovascular regulation was proposed in [112]. The effect of CO_2 pressure changes in arterial blood on cardiovascular system was analyzed using mathematical model. This model is an extension of previous one [109], which already included the main reflex and local mechanisms triggered by changes in O_2 and CO_2. New aspects covered by the model included O_2-CO_2 interactions at peripheral chemoreceptors, the effect of local changes of CO_2 on peripheral resistance, direct response of central nervous system (CNS) to CO_2, and control of central chemoreceptors on ventilation and respiratory movements. volume. Statistical comparison of the results of model simulation with various experimental data was carried out. This comparison suggests that the model is able to mimic the acute cardiovascular response to changes in blood gases under various conditions (normoxic hypercapnia, mechanically ventilated hypercapnia, hypocapnic hypoxia, and hypercapnic hypoxia). The model relates registered responses to complex overlay of many simultaneously operating mechanisms (baroreflex, peripheral chemoreflex, CNS response, lung stretch receptors, local effect of gas tension), which can be activated differently depending on the specific studied stimulus. However, while some experiments can be reproduced using one basic set of parameters, reproducing of other experiments requires a different combination of mechanism strength (in particular, different local mechanism of CO_2 strength on peripheral resistances and CNS response to CO_2). Based on these results,

some suggestions were presented to explain the striking differences reported in scientific literature. The model can provide reliable support for the interpretation of physiological data on acute cardiovascular regulation and can cause the synthesis of conflicting results into a single theoretical setting.

Integrated model of human ventilation control system was developed to determine the response to hypercapnia [112], which included such chains of mass transfer and mass exchange of respiratory gases as lungs, brain tissues, other tissues, and various types of feedback mechanisms. All these chains included peripheral chemoreceptors in carotid body, central chemoreceptors in medulla oblongata, and central respiratory depression. The latter acted by reducing responses of central nervous system to afferent activity of peripheral chemoreceptors during prolonged hypoxia of brain tissue. In addition, the model takes into account local adjustments in blood flow in response to O_2 and CO_2 changes in pressure in arterial blood. In this study, the model was validated by simulating the response to quadratic changes of alveolar pCO_2 at various constant levels of alveolar pO_2. The results demonstrated good agreement with the data given in scientific literature sources. Subsequently, the analysis of sensitivity of the main feedback mechanisms in the response of ventilation to CO_2 was performed. The results showed as well that the ventilatory response to CO_2 stimulation in hyperoxia can be almost entirely attributed to the central chemoreflex, while in normoxia peripheral chemoreceptors also made some contribution. Contrary to this, the response to hypercapnic stimuli during hypoxia involved complex overlay of various factors with incommensurable dynamics. Thus, the results indicated that the ventilatory response to hypercapnia during hypoxia is more complex than that provided by simple empirical models, and the distinction between central and peripheral components based on time constants can cause mistakes in understaning.

The same authors [113] developed an integrated model of human ventilation control system: to study the response to hypoxia, they simulated long-term isocapnic hypoxia on it at normal alveolar pCO_2 (40 mm Hg = 5.33 kPa) shows the occurrence of a two-phase response, characterized by an initial peak and subsequent hypoxic decrease in ventilation.

Improved dynamic model of respiratory response to physical activity was described in [114]. Modeling of respiratory system had been studied comprehensively in stationary conditions to simulate sleep disorders, predict its behavior in respiratory diseases or irritants, and model of its interaction with mechanical ventilation. The purposes of this study were following. Firstly, to analyze both the dynamic and static responses of two known breathing patterns to stimuli with physical loadings, using a sequence of increasing exercise stimuli (to analyze the response of the model when applying stepwise stimuli) and experimental data (for the estimation of possibility predictions by each model). Secondly, to propose changes in the structures of the models to improve their transient and stationary characteristics. Universalism of the resulting model compared to other two models was shown in accordance with the ability to mimic ventilatory stimuli such as exercises, with proper regulation of arterial blood gases, suitable time constant, and better adaptation to experimental data. The proposed model corrected the breathing pattern in each respiratory cycle using an optimization criterion based on minimizing the work of breathing by regulating the respiratory rate.

The monograph [78] provides a brief overview of human respiratory system and qualitatively describes the processes and components that will be combined for modeling. Ventilation was described by a linear differential equation with lumped parameters, taking into account of resistance R and elasticity E. Further expansion of the model assumed inertia, non-linear elastic behavior, variable resistance and some other characteristics. Future this model was integrated into more complex structures. The lung there was imagined as a set of interconnected pipes through which, according to Poiseuille's law, a viscous liquid flows, while the flow rate is proportional to the decrease of the pressure. The model also takes into account the inertia, which plays significant role in upper respiratory tract. The airways were divided into different zones and Navier-Stokes equation was solved in the upper zone. Further, phenomena of the movement of oxygen from the lungs into the blood were considered; further were taken into account the phenomena of diffusion through different barriers, and saturation of hemoglobin with oxygen.

Let's observe separately such aspect of circulatory system modeling as hemorrhagic shock. Concerning this the small review [115] attracted our attention; where seven mathematical models of hemorrhagic shock were analyzed. The authors noted that although the mathematical modeling of pressure and

flow dynamics in cardiovascular system had long history, finding an appropriate model for particular experimental situation was often a challenge by itself. The ideal model should be relatively easy to use and reliable, as well as ethically acceptable. In addition, it would help the pathogenic features of cardiovascular diseases that need to be investigated. No universal model had been identified, although many models had been developed. The purpose of the review was to describe several of the most relevant mathematical models of the cardiovascular system: to explain the physiological features of the dynamics of blood circulation and to compare their mathematical formulations. The focus was on whole-organism mathematical models that map the subject's response to hypovolemic shock. The models contained in this review differed from each other both in the accepted mathematical methodology and in described physiological or pathological aspects. In fact, each model mimics different aspects of physiology and pathophysiology of cardiovascular system to varying degrees: some of these models were aimed to better understanding of the mechanisms of vascular hemodynamics, while others focus more on disease states in order to develop therapeutic standards of care or test new approaches.

Continuation of these problems studying one can find in [116]. Hemorrhagic shock is the leading reason of the deaths of militaries on the battlefield as well as civilian injuries. This article presents an updated version of Zenker model (2007) for hemorrhagic shock, called the ZenCur model, which allows better description of the time course of phenomena relevant observations. The study provides a simple but realistic mathematical description of the dynamics of cardiovascular system, which may be useful in estimation and predicting of hemorrhagic shock. This model was able to reproduce changes in mean arterial pressure, heart rate and cardiac output after the beginning of bleeding (as observed in four experimental laboratory animals). It provided a reasonable compromise between too detailed description of relevant mechanisms, on the one hand, and the simplicity of the model, on the other. The first one was required significant modeling and entail cumbersome interpretations. From clinical point of view, the goals of new model were to predict survival and optimize the timing of therapy in both civilian and military scenarios.

Finally, an article [117] two different cardiovascular models in hemorrhagic shock scenario were compared. Hemorrhagic shock is a form of hypovolemic shock caused by rapid and large loss of intravascular blood volume and is the world's leading cause of death, whether on the battlefield. or in civil traumatology. For this reason, the ability to prevent hemorrhagic shock remains one of the largest challenges in medicine and technology. The use of mathematical models of cardiovascular system had expanded the possibilities: on one hand, for predicting the risk of developing hemorrhagic shock, and, on other hand, for determining effective treatment tactics. This article presents material of comparison between two mathematical models that model multiple hemorrhagic scenarios. Guyton and Zenker models were observed. In the vast panorama of existing mathematical models of cardiovascular system, we decided to compare these two models because they seem to be extreme cases due to their complexity and details of information they analyze. The Guyton model is complex and well-structured model that represents a milestone in the investigation of cardiovascular system; the Zenker model is newer, developed in 2007, and it is relatively simple and easy to implement. Comparison of these two models opens us new prospects for improving of mathematical models of cardiovascular system, which may be more effective in the study of hemorrhagic shock.

Thus, the development of mathematical models of physiological processes in healthy organism at rest and under various disturbing influences is rapidly developing area of mathematical modeling. At the same time, numerical simulation seems to be exceptional in terms of its efficiency and availability of tools for studying the problems of physiology and medicine. An analysis of literature has shown that the complexity and detailed elaboration of physical and mathematical formulations of the problems in modern conditions is directly related to the rapid growth of computing resources, and the revolution in the field of instrumental and diagnostic equipment plays significant role too, providing penetration to fundamentally new levels of understanding of the processes occurring in human organism. At the same time, modern invasive methods, no matter how perfect they are, give only some "slice" of the current state of a person, while in modern society, in particular, in occupational medicine and sports, there is a request to predict the functional state of a person under the certain or other extreme disturbing influences. In addition, there is a gap between works in which the fundamental scientific component comes to the fore and works that can be used in

real time to solve applied problems in medicine and physiology.

Summarizing all above written, it can be argued that the proposed models require the use of rather complex mathematical apparatus and significant computational resources. In addition, they were not always justified from mathematical point of view, they were based on a number of significant limitations, and there were also issues of checking the adequacy of such models. In particular, the adequacy of such models was checked by comparative analysis of the temporal implementations of the models and data from natural experiment in healthy subjects and patients. Therefore, the scope of their application seems to be rather limited, there are certain difficulties in the practical application of such models associated with obtaining of input data. In addition, such models, at least some of them, although they were parts of more complex formations, it was not clear how they took into account the interaction and mutual influence with other functional systems of organism. At the same time, there was specific request for mathematical models on which it would be possible to study the processes occurred in human organism at the level of predicting the stationary state of organism during disturbances of various etiologies, the input data for which would be obtained from experiment.

The emergence and development of models of individual oxygen transport systems of organism naturally led to the consideration of their interconnected holistic functioning, united by common task — to ensure an adequate supply of oxygen to organism. In this regard the concept of Profs. A. Z. Kolchinskaya and N. V. Lauer about oxygen regimes of organism and mechanisms of their regulation were formulated in time [17]. Based on the analysis of large amount of experimental data, they had concluded that the regulation of oxygen regimes of organism was carried out by single system that coordinates the most complex work of wide variety of mechanisms and subordinates them to the main task of maintaining of oxygen parameters at optimal level throughout the entire path of oxygen in organism — for the most economically, efficiently, effectively, and reliably matching of oxygen delivery to tissue in accordance to demands in oxygen.

This approach is currently in demand. In particular, automated workstations for functional diagnostics were developed [ta 2019Ak], in [Masha],

Basing on this concept, a simulation model of the dynamics of gases in organism was developed, which described the mass transfer of respiratory gases in human organism by all oxygen transport systems in the dynamics of respiratory cycle [22, 23]. Regulation in the system is carried out according to the principle of the optimal choice of control parameters (respiration volume, duration of respiratory cycle, volumetric velocities of systemic and regional blood flows), i.e., the problem of regulation was formulated as classical optimization problem according to given criterion, which is the minimum squared deviation of O_2 delivery value from oxygen request. The model was used to study the dynamics of respiratory gases mass transfer in organism under the conditions of hypoxic hypoxia, physical activity, and hyperbaria [24–26]. Transient processes were studied during changes in the composition of inhaled air, as well as for the transition from the rest to exercise — so, conversely, pressure changes, and the process of controlling the level of gas homeostasis in organism. A qualitative analysis of the model was carried out: the existence and uniqueness of solutions, non-negativity, boundedness, and asymptotic stability were demonstrated. This model was used to solve a number of applied problems of occupational medicine and sports.

Currently, there are two main approaches to mathematical modeling of respiratory and blood circulatory systems. One of them was the construction of models for the mechanics of respiration and blood circulation, which were based on models of mechanics of rigid solid deformable objects, thermomechanics, hydromechanics, and continuum mechanics. This approach supposes using of complex mathematical apparatus, including Navier-Stokes equation, which makes it possible to obtain a number of theoretical results, but it is hardly possible for real problems solutions today. The second approach was based on the model of F. Grodins, who imagined the process of the breath as controlled dynamic system, described using ordinary differential equations, in which the control was carried out according to the feedback principle. There are significant numbers of modifications of this model, which make it possible to simulate various disturbing influences, such as physical activity, hypoxia and hyperemia, and to predict the parameters characterizing the functional respiratory system under these disturbing influences.

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МАТЕМАТИЧНІ МОДЕЛІ СИСТЕМИ ДИХАННЯ І КРОВООБІГУ

Н. І. Аралова[1], *О. М. Ключко*[2], *В. І. Машкін*[1], *І. В. Машкіна*[3], *П. Радзійовські*[4], *М. Радзійовська*[5]

[1]Інститут кібернетики ім. В.М. Глушкова Національної академії наук України, Київ
[2]Національний авіаційний університет, Київ, Україна
[3]Київський університет імені Бориса Грінченка, Україна
[4]Коледж педагогіки та терапії Казіміра Мілановського, Познань, Польща
[5]Ченстоховський технологічний університет, Польща

E-mail: aralova@ukr.net

Метою цієї роботи було проаналізувати сучасні підходи до математичного моделювання системи дихання та кровообігу.

Методи. У якості методів були використані комплексний аналіз та огляд літератури з використанням вітчизняних та зарубіжних баз даних.

В результаті виконаної роботи в огляді було узагальнено та проаналізовано історичні відомості та сучасні дані, що стосуються математичного моделювання функціональних систем дихання та кровообігу, виявлено сучасні тенденції у підходах до побудови цих моделей.

Висновки. В результаті необхідно зроблено висновки про те, що в даний час існує два основних підходи до математичного моделювання систем дихання та кровообігу. Один з них — це побудова моделей механіки дихання та кровообігу, що ґрунтуються на моделях механіки твердого деформованого тіла, термомеханіки, гідромеханіки, механіки суцільних середовищ. Цей підхід передбачає застосування складного математичного апарату, зокрема й рівняння Нав'є-Стокса, що дозволяє отримати низку теоретичних результатів, але навряд чи він є можливим нині для вирішення реальних завдань. Другий підхід полягає в моделі Ф. Гродінза, який представив процес дихання як керовану динамічну систему, записану за допомогою звичайних диференційних рівнянь, управління у якій здійснюється за принципом зворотного зв'язку. Існує значна кількість модифікацій цієї моделі, які дають можливість імітувати різні збурювальні впливи, такі як фізичне навантаження, гіпоксія, гіперкпнія й спрогнозувати параметри, що характеризують функціональну систему дихання при цих збурювальних впливах.

Ключові слова: математична модель дихальної системи; математична модель системи кровообігу; гіпоксичний стан; теоретичний аналіз.

EXPERIMENTAL ARTICLES

METABOLIC PROFILE AND MECHANISMS REACTION OF RECEPTOR GABA-TARGETED PROPOXAZEPAM IN HUMAN HEPATOCYTES

M. Golovenko[1]
A. Reder[2]
V. Larionov[1]
S. Andronati[1]

[1]Physico-Chemical Institute
 of the National Academy of Sciences of Ukraine

[2]SLC "INTERCHEM", Odessa, Ukraine

E-mail: n.golovenko@gmail.com

The aim of this study was to identify the Propoxazepam metabolites, formed by suspension of human cryopreserved hepatocytes using the method accurate mass LC-MS/MS analysis. A suitable chromatographic method was developed for the profiling of Propoxazepam and its metabolites. Samples were analyzed using a Waters Vion high resolution LC-MS/MS instrument and data examined using Waters Unifi software to determine the identity of the most abundant metabolites. Following a 4 hour incubation with human hepatocytes, Propoxazepam accounted for 96.0% of the profile. The most abundant metabolite formed was oxidized Propoxazepam (3-hydroxyderivative), which accounted for approximately 2.5% of the total peak response in the 4 hour sample. Two minor components were also observed, each accounting for <10% of the total peak response. Glucuronic conjugates have not been found under these conditions. All metabolites formed represented less than 10% of the total chromatographic peak response. The data obtained indicate the absence of reactive electrophilic derivatives among the metabolites of Propoxazepam.

Key words: Propoxazepam; human hepatocytes; metabolism; LC-MS/MS analysis.

Introduction

A number of 3-substituted 1,4-benzodiazepines have been synthesized at the A.V. Bogatskiy Physico-Chemical Institute of the National Academy of Sciences of Ukraine, and their structure−activity relationships studied. Their pharmacological effect was unusual, because unlike most drugs in this class, in the models of nociceptive and neuropathic pain these substances showed significant analgesic activity [1]. One of them, Propoxazepam, 7-bromo-5-(*o*-chlorophenyl)-3-propoxy-1,2-dihydro-3H-1,4-benzodiazepin-2-one, is considered a promising drug and is undergoing preclinical studies [2]. Similar to gabapentin and pregabalin, which are well-known drugs used in general medical practice in the treatment of neuropathic pain [3], propoxazepam also has an anticonvulsant effect [4, 5], which explains the analgesic component in the pharmacological spectrum of compound. Our data suggest that the mechanism of propoxazepam analgesic and anticonvulsant properties includes GABAergic and glycinergic systems [6, 7].

Metabolism (biochemical transformation) is an essential biological process that converts xenobiotics including medicines to water-soluble substances, and eliminates toxic compounds from the body. As a result of metabolism process, the parent compound can be converted to more (activation) or less (deactivation) active. To investigate how metabolites act in body, screening systems require *in vitro* and *in vivo* metabolism methods. Human *in vivo* toxicity data for new chemicals are usually not full and require preliminary data, obtained in a variety of *in vitro* models. One key initiative is the National

Toxicity Program (NTP), a U.S. federal government organization, started in 1978 to coordinate toxicological testing programs for the strengthening of toxicological sciences and development and validation of methods for testing of metabolites with potential toxicity. Various *in vitro* approaches have been described in the literature to screen for toxicity [7, 8].

Hepatocytes suspension can be used for short-term assays in a collagen-coated 24-well plates [9] or culture tubes [10]. In addition, hepatocytes demonstrated strong correlation between *in vitro* and *in vivo* hepatotoxicity studies [11]. Activity of different cell enzymes (cytochrome P450 (CYP), UDP-glucuronosyltransferase (UGT), and sulfotransferase (ST) have been characterized for their ability to be cultured *in vitro*.

The study was aimed to determine Propoxazepam metabolism in human hepatocytes. The metabolic profile of Propoxazepam and its metabolites analyzed by LC-MS/MS instrument. Our results will provide a reference for the clinical safety and rational use of this drug.

Materials and Methods

Propoxazepam and 3-hydroxy derivative (7-bromo-5-(2chlorophenyl)-3-hydroxy-1,3dihydro-2H-1,4-benzodiazepin-2-one, major metabolite) was synthesized according to the method described in [12]. Using the IR, mass spectroscopy and X-ray diffraction analysis the structure of the substance was determined and approved. Chemical purity was confirmed by elemental analysis (99%). General purpose reagents and solvents were of AnalaR® grade (or a suitable alternative). LC/MS grade reagents were used for preparation of mobile phases with developed chromatographic conditions for parent compound and reference standard. Samples were analyzed using reverse phase LC-MS analysis to generate high and low energy mass spectra (MSE). Each sample was analysed using accurate mass LC-MS to determine relative levels of parent compound at each time-point to determine the profile of metabolites formed. Appropriate no cells control samples were also analyzed.

All cryopreserved hepatocytes were obtained from BioIVT (formerly Bioreclamation IVT) and delivered stored frozen in liquid nitrogen. On receipt, the hepatocytes were transferred to permanent storage in liquid nitrogen. Human hepatocytes were supplied as a mixed-gender pool. The required vials of cryopreserved hepatocytes were removed from storage in liquid nitrogen. They were then immersed in a water bath set at 37 °C ± 1 °C for approximately 75-90 sec. The contents of each vial were then decanted directly into a tube which contained 15 mL of pre-warmed (37 °C ± 1 °C) *in vitro* GRO™ hepatocyte thawing medium per vial of hepatocytes with further re-suspension by gentle tube inverting followed by centrifugation at 50 g for 5 min at 20 °C. The supernatant was then poured off and the cell pellet was then loosened and then re-suspended in a total of ca 1.5 mL of supplemented Williams' Medium E per vial of thawed hepatocytes. Cells were kept on ice while not being actively prepared. Supplemented Williams' Medium E comprised Williams' Medium E (500 mL) supplemented with 10 mM dexamethasone (5 µL) and cell maintenance 'cocktail B' (solution of penicillin-streptomycin, ITS (insulin, transferrin, selenium complex) + BSA and linoleic acid), GlutaMAX™, and HEPES, 20 mL) which had been pre-gassed with (95% O_2/CO_2) for 30 minutes at room temperature. The cell suspensions were then made up to give required cell density at 1×10^6 cells/0.990 mL with supplemented Williams' Medium E. Trypan blue exclusion methods were used to determine the cell viability and density. Cell viability in the hepatocyte preparations was ≥70%.

The incubation components were mixed together in untreated 12-well plates as follows: Supplemented Williams' Medium E containing 1.52×10^6 viable cells in 1485 µL; Well plates were maintained at 37 °C ± 1 °C using a heated well plate incubator; Propoxazepam (15 µL of 10 mM solution in DMSO: Super pure water (1:1, v/v)) for hepatocyte-containing samples. Following the final addition of Propoxazepam, the well plates were then placed on a tilting mini rocker-shaker in an incubator (maintained at 37 °C ± 1 °C, 5% CO_2/95% O_2) to commence the incubation. At the requisite incubation period, for Propoxazepam samples, an aliquot (500 µL) was removed from each incubate and transferred to an aliquot of chilled acetonitrile to stop the reaction (incubation : acetonitrile ratio = 1:1 (v/v)). Each sample was vortexed and then sonicated in an ultrasonic bath for approximately 10 minutes to fully disrupt the cells. The samples were then stored at −70 °C ± 10 °C prior to transfer to the test site for analysis.

For the main incubation, a stock solution of Propoxazepam was prepared nominally at 2 mM in DMSO, followed by 1:1 dilution

with ultrapure water to give 1 mM stock solution. The 1 mM stock solution gave a final concentration of 10 µM in the final incubation and 0.5% (v/v) organic content. A portion of the stock solution was added to separate portions of the incubation mixture. For the remainder of the Propoxazepam samples after 4 hours incubation, the residual incubates, a further 1 mL aliquot of chilled acetonitrile added, mixed with a pipette and 2×1 mL aliquots were transferred into microcentrifuge tubes and treated as described above. These additional samples were prepared for the development of a suitable analytical method check/method development for the analysis of parent compound and metabolites. True' time zero incubations were prepared in parallel. Hepatocyte suspensions of each species (495 µL) were placed in a clean microcentrifuge tube followed by the immediate addition of 500 µL of chilled acetonitrile and vortex-mixing. Propoxazepam (5 µL) was then added to the hepatocyte suspension/acetonitrile mixture and then sonicated in an ultrasonic bath for approximately 10 minutes to fully disrupt the cells. The following control incubations were conducted in 12-well plates, in duplicate, in parallel to the main incubation: Incubation of Propoxazepam for 4 hours in the absence of hepatocytes.

Positive control samples incubating 7-ethoxy[3^{14}C]coumarin (7-EC) at a concentration of 50 µM for 4 hours in duplicate with hepatocytes. All samples were stored at −70 °C ± 10 °C. The samples incubated with test item were then transferred (frozen on dry ice, at −80 °C) to test site for characterization of parent and metabolites in the incubation medium by LC-MS/MS analysis [13]. The percentage of sample radioactivity associated with 7-EC and known metabolites, 7-hydroxycoumarin glucuronide (7-HCG), 7 hydroxycoumarin sulphate (7-HCS) and 7 hydroxycoumarin (7-HC) was determined for all samples

The nature and identity of Propoxazepam and its metabolites, present in representative aliquots of the incubation medium from human samples at 'true' zero and 4 hours as well as parent were investigated using accurate mass spectrometry LC-MS/(MS) analysis. A suitable chromatographic method was developed for the profiling of Propoxazepam and its metabolites. Samples were analysed using a Waters Vion high resolutions LC-MS/MS and data examined using Waters Unifi software to determine the identity of the most abundant metabolites. Samples were analyzed using reverse phase LC-MS analysis to generate high and low energy mass spectra (MSE). Each sample was analyzed using accurate mass LC-MS to determine relative levels of parent compound at each time-point, and determine the profile of metabolites formed. Appropriate no cells control samples were also analyzed. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for detection and characterization of reactive metabolites due to the high sensitivity and selectivity of this combined technique and its ability to separate, detect, an identify metabolites in the presence of endogenous materials [14].
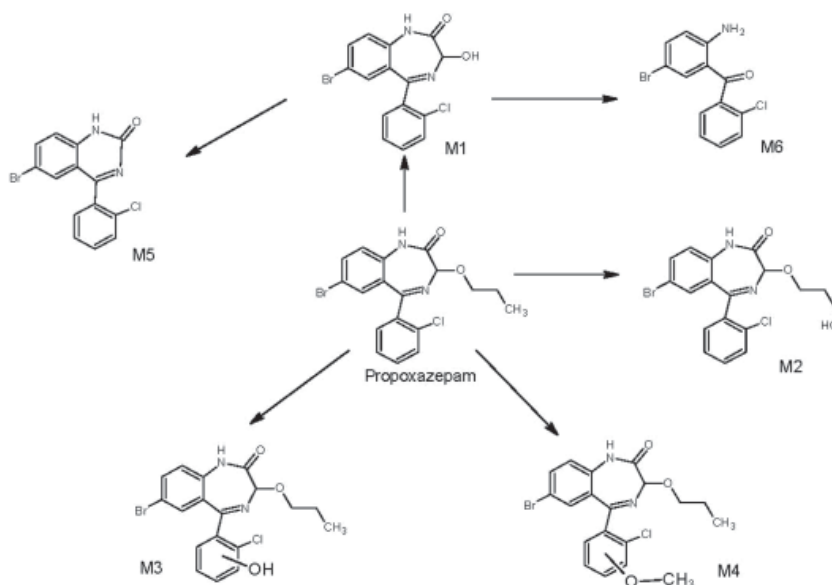
No statistical analyses were performed on this study.

Fig. 1. **Theoretical scheme of Propoxazeam metabolism routes**

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Results and Discussion

Based on the concept of regioselectivity and stereoselectivity of xenobiotic biochemical transformation [15], we proposed theoretically possible metabolism route of propoxazepam.

According to the results of previous studies [16], Propoxazepam metabolism leads to formation, at least, the following metabolites: 3-hydroxyderivative (M1) and aromaticaly oxidized (hydroxylated) parent compound (M3) as well as its methoxyderivative (M4) and oxidized in the alcoxyradical (M2). The presence of corresponding quinazolinone (M5) and benzophenone (M6) is also possible.

In this metabolite profiling data are comparative only, as these values were not generated using quantitative analytical methods. The proportions of parent compound and its metabolites assume equivalent mass spectrometer detector response. As the ionization characteristics of the parent compound and its metabolites are likely to be different; the actual proportions of these components cannot be confirmed, but the data can be considered to be comparable across metabolites. In addition to the accurate masses associated with the parent compound, the LC-MS data from the study samples were also examined for the possible metabolite transformations.

An extracted ion chromatogram (EIC) for Propoxazepam and 3-hydroxyderivative (M1) in a standard solution are presented in Fig. 2. EICs for selected 4 hour incubations are presented in Fig. 3. The peak response for each component was determined from the relevant EICs and was expressed as a percentage of the total evaluated peak response. These results are presented in Table 2.

In addition to the parent compound 3 metabolites were detected across the samples analyzed, these were assigned as M1, M2 and M2N based on retention time. Only Propoxazepam was observed following incubation of the test substance for 4 hours in the absence of hepatocytes, indicating that it was stable under the test conditions and absence spontaneous nonenzymatic (chemical) transformation.

Proposed structures, parent masses and characteristic fragment ions of metabolites observed in matrix samples are presented in Table 1.

A summary of representative accurate mass data is presented in Table 2.

Consequently Propoxazepam underwent moderate biotransformation in human cryopreserved hepatocytes after 4 hour incubation. Overall three metabolites were identified (M1, M2, M2N). All these metabolites formed represented less than 10% of the total chromatographic peak response.

The changes in levels of parent 7-ethoxycoumarin (positive control) and formation of associated Phase I and Phase II metabolites indicated that all hepatocytes were metabolically viable and were capable of integrated Phase I/II metabolism under the incubation conditions used on this study. Therefore, the results generated for the incubation of these hepatocytes with Propoxazepam considered valid.

Earlier we have analyzed the Propoxazepam metabolites formed in the organism of animals using the synthesized $[2^{14}C]$Propoxazepam [13,

Item name: 8455-192 Analysis2 Run 19
Channel name: 2: +364.9687 (5.0 PPM) + 407.0156 (5.0 PPM) : HD TOF MSe (100-800) 4V ESI+
Item description: Mixed Std 2 µg/mL
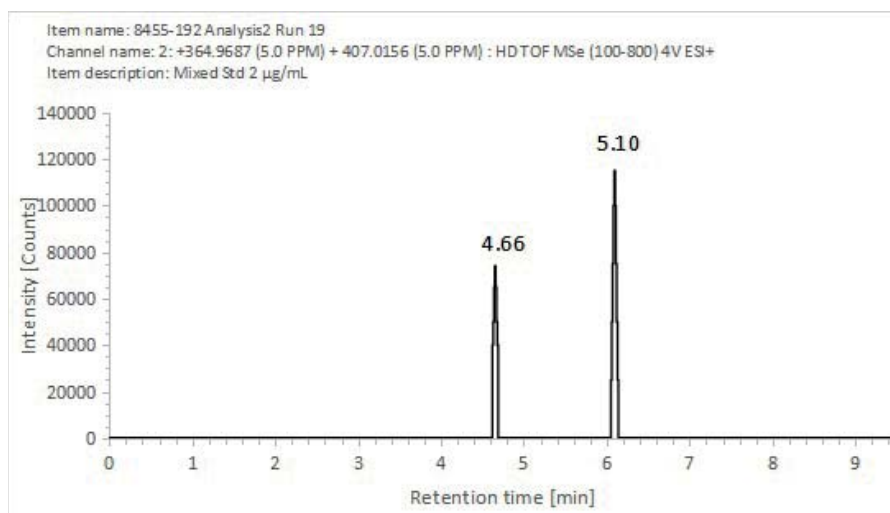
Fig. 2. **Extracted ion chromatogram from the analysis of a 2 µg/mL standard solution of Propoxazepam (retention time 6.10 min) and 3-hydroxyderivative (retention time 4.66 min)**
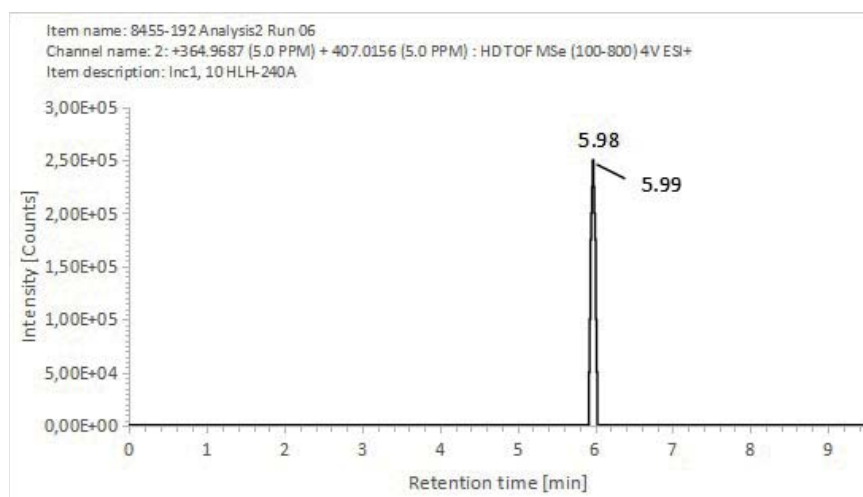
Fig. 3. Extracted ion chromatogram of human hepatocyte incubation of Propoxazepam (4 hours).
Retention time: 4.53 min Propoxazepam+O (M2); 4.55 min 3-hydroxyderivative (M3);
4.78 min Propoxazepam+O (M4); 5.98 min Propoxazepam

Table 1. **Summary of protonated molecular ions and characteristic fragment ions for Propoxazepam and identified metabolites**

Peak	Retention Time, min	[M+H]$^+$	Structure	Characteristic Fragment Ions
Parent Propoxazepam	6.12	407		347*, 319, 273, 239, 205
M1 (3-hydroxyderivative)	4.69	365		347*, 273, 194
M2 (Propoxazepam +O)	4.66	423		347*, 319, 239, 205
M2N (Propoxazepam +O)	4.91	423		347*, 319, 239, 205

Note: Retention times are representative and may differ from extracted chromatograms

Table 2. **Summary of representative accurate mass data for Propoxazepam and identified metabolites**

Peak	Measured m/z	Theoretical m/z	Proposed neutral formula	ΔmDa	Δppm
M2	423.0101	423.0106	$C_{18}H_{16}BrClN_2O_3$	−0.5	−1.2
M1	364.9676	364.9687	$C_{15}H_{10}BrClN_2O_2$	−1.1	−3.0
M2N	423.0107	423.0106	$C_{18}H_{16}BrClN_2O_3$	0.1	0.2
Propoxazepam	407.0155	407.0156	$C_{18}H_{16}BrClN_2O_2$	−0.1	−0.2

Note: values are representative unless otherwise stated ΔmDa = (Measured Mass — Theoretical Mass)*1000 Δppm = (ΔmDa/Theoretical Mass)*1000

17]. Using UPLC-MS/MS method [18] it was found that M1, M2, M3, M4 metabolites are formed in rat organism. Thus, only metabolites M1 and M2 are similar for human and rat.

The molecule of Propoxazepam has some reaction centers, which undergo transformation during metabolism. In theory any structural fragment or functional group can be a reaction center, but in particular enzymatic reaction it is formed by the limited quantity of atoms, what is due to the nature of reaction center and the nature of its nearest environment. The particularities of structural selectivity and stereochemical changes are valuable criteria for determination of reaction mechanism.

Each reaction of Propoxazepam metabolites formation has its own mechanism (Fig. 1). So the main metabolite M1 is formed during the oxidative dealcylation of the aliphatic moiety of molecule, which is facilitated by the transformation of stable ether group to ester one, which is more capable for hydrolysis, during which the formation of 3-hydroxyderivative takes place (Fig. 4).

There is a possibility that one of intermediates (either M7 or M8) in this scheme is metabolite M2N (Table 2).

This process differs from the process of 3C-hydroxylation of 1,4-benzodiazepindes with methylene group in the position "3" of heteroring, which is catalyzed with the corresponding monooxygenazes CYP3A4 and CYP2C19 [19]. In this case takes place the stereoselective formation of 3-hydroxymetabolites due to the asymmetrical carbon in position "3".

M2 metabolite formation proceeds through the aliphatic oxydation of methylene group. In the simplest way nonactive carbon of the alcyl group undergoes hydroxylation, catalyzed by CYP. The penultimate position ω-1 is the preferred site, but hydroxylation can also take place at the ultimate position or in another positions in case of spatial difficulties of with specific cytochromes as in case of Propoxazepam. Based o the isotope exchange data [20] during the aliphatic hydroxylation the reaction mechanism involves the inclusion of singlet oxygen in the bond carbon-oxygen.

The enzymatic oxidation of Propoxazepam, which takes place only *in vivo*, passes through the epoxides stage. It can be the way of direct intrusion of oxygen in the bond carbon-hydrogen with the formation of corresponding phenol, or by joining of oxygen to the formally-aromatic double bond with subsequent epoxide isomerization to phenol [15]. Aromatic structures hydroxylation according to the electrophilic substitution does through the joining of CYP450-activated oxygen to π-electron system and the orientation of the formed compound is determined with the nature of already existing substituent [19] — either to ortho- or para- positions.

Methoxyderivative of Propoxazepam(M4) belongs to rare enough metabolites of xenobiotics. The mechanism of its formation is unknown but possibly the first step is aromatic hydroxylation followed by methylation performed by methyltransferase [15].

Both in present study and in previous one [13] metabolites M5 and M6 haven`t been found (Fig. 1). We have earlier identified in the rat organism the processes of narrowing of seven-membered heterocyclic ring of 1.4-benzodiazepines to six-membered quinazolinone (M5) and the mechanisms of this transformation were described [19, 21]. The hydrolytic cleavage to the corresponding 2-aminobensophenones had also been detected in the organism of rats [22].
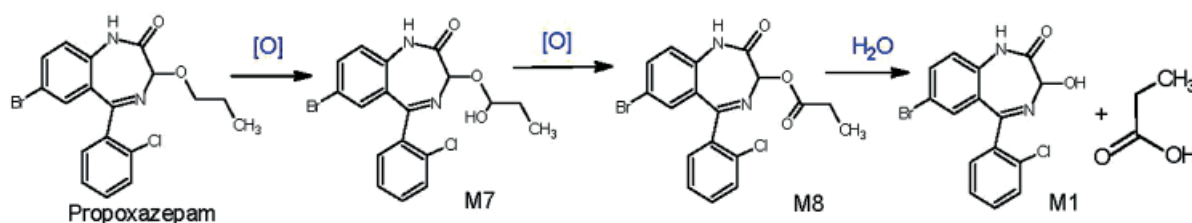
Fig. 4. **Scheme of Propoxazepam transformation to 3-hydroxyderivative (M1)**

Despite the presence of both UDP-glucuronosyltransferase (EC 2.4.1.17) and sulfotransferase (EC 2.8.2.29) in human hepatocytes, catalyze the conversation of 7-ethoxycoumarine (positive control) to glucuronide and sulfate, the corresponding conjugates of 3-hydroxymetabolite haven`t been found. The possible explanation of this is that this reaction goes through phenolic types of conjugates but in our studies such metabolites (M3) doesn`t form.

The common purpose of metabolism is adding of ionozable groups and formation of compounds with lower lipophilicity. This allows the faster elimination (detoxication) of xenobiotics from the body. Metabolites are commonly less toxic than the corresponding parent drug, though in some cases the more reactive electrophilic compounds, possessing higher toxicity are formed (bioactivation).

One of isoforms of cytochrome P450 (CYP3A4) is the predominant isoform in liver and metabolizes more than 50% of the clinical drugs commonly used. However, CYP3A4 is also responsible for the formation of reactive metabolites of nitrobenzodiazepines, such as flunitrazepam, nimetazepam, nitrazepam, and clonazepam [23]. Flunitrazepam was reported to induce hepatotoxicity and nitrazepam and clonazepam were reported to cause drug-induced liver injury [24]. In the reductive pathways from nitro to the reduced amine catalyzed by P450 and/or reductase, several reactive metabolites including nitroso- and N-hydroxylamine derivatives could be produced. Such reactive metabolites seem to bind covalently to nucleophile targets of proteins and nucleic acids, leading to the cytotoxic effects [25]. On the other hand, electrophilic N-hydroxylamine reacts with intracellular molecules, which induce various types of toxicity including hepatotoxicity.

Reactive metabolites are often short-lived and are not usually detectable in circulating blood/plasma. *In vivo* (acute and chronic toxicity, gene mutations) approaches are generally employed to examine the bioactivation potential of drug candidates, which may provide some indirect but valuable information that drug undergo bioactivation to form reactive electrophilic species. LD_{50} of Propoxazepam is greater than 5000 mg/kg and, therefore, it belongs to the category V of relatively non-toxic substances according to the GHS [26]. In our studies [27], the possible induction of gene mutations by the effects of Propoxazepam on the *S. typhimurium TA98* (frameshift mutation type) and *TA 100* (mutations of the base substitution) without and with metabolic activation (fraction S9) showed that Propoxazepam did not the ability to induce gene mutations in the test system used by us. The metabolic activation was also not effective, that is, Propoxazepam is neither a "direct" nor an "indirect" mutagen for Ames strains. Thus, the data obtained during the microplate version of the Ames test (Muta-ChromoPlate kit) on the strains *Salmonella typhimurium TA 98* and *TA 100* indicate that mutagenic activity of Propoxazepam in the concentrations studied is not revealed.

Conclusions

Under *in vitro* conditions Propoxazepam showed relatively high metabolic stability with ~90 % of unchanged compound after 4 hours incubation with human hepatocytes. The main detected metabolites detected were 3-hydroxyderivative and oxidized in alcoxy moiety. At the same time, no glucuronic or sulfate conjugates were found. The presented data indicate the absence of reactive chemicals among the metabolites of Propoxazepam.

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МЕТАБОЛІЧНИЙ ПРОФІЛЬ ТА МЕХАНІЗМИ РЕАКЦІЇ ГАМК-РЕЦЕПТОР ОРІЄНТОВАНОГО ПРОПОКСАЗЕПАМУ В ГЕПАТОЦИТАХ ЛЮДИНИ

М. Головенко[1], *А. Редер*[2], *В. Ларіонов*[1], *С. Андронаті*[1]

[1]Фізико-хімічний інститут ім. О.В.Богатського НАН України, Одеса
[2]ТДВ «ІНТЕРХІМ» Одеса

E-mail: n.golovenko@gmail.com

Мета. Виявлення метаболітів Пропоксазепаму, що утворюються суспензією кріоконсервованих гепатоцитів людини, за допомогою методу точного масового LC-MS/MS аналізу.

Методи. Для профілювання Пропоксазепаму та його метаболітів розроблено відповідний хроматографічний метод. Зразки аналізували за допомогою приладу WatersVion високої роздільної здатності LC-MS/MS, отримані дані обробляли за допомогою програмного забезпечення WatersUnifi для визначення та ідентифікації найбільш поширених метаболітів.

Результати. Після 4-годинної інкубації з гепатоцитами людини Пропоксазепам становив %96,0 складу від вихідної кількості препарату. Найпоширенішим метаболітом, який утворився, був окиснений Пропоксазепам (3-гідроксипохідне), що становило приблизно 2,5% загальної пікової реакції у 4-годинному зразку. Також спостерігали два мінорні компоненти, кожен з яких становив менше 10% загальної площі піків. Глюкуронові кон'югати за цих умов не було ідентифіковано. Усі утворені метаболіти становили менше 10% загальної хроматографічної пікової відповіді.

Висновки. Отримані дані свідчать про відсутність серед метаболітів Пропоксазепаму реакційно здатних електрофільних похідних.

Ключові слова: Пропоксазепам; гепатоцити людини; метаболізм; LC-MS/MS аналіз.

CARDIOPROTECTIVE EFFECT OF ENKEPHALINS UNDER IMMOBILIZATION STRESS

I. J. Shcheniavskyi

Institute for Problems of Cryobiology and Cryomedicine
of the National Academy of Sciences of Ukraine,
Kharkiv, Ukraine

E-mail: ivanhou11@gmail.com

Objective: The aim of this study was to investigate the cardioprotective effect of dalargin, a synthetic leu-enkephalin.

Methods: The induction of myocardial infarction in rats, which were kept on a diet with excess fat and calcium/sodium salts for two months, by the use of immobilization stress. The experimental results indicated that the applied model allowed to induce the development of myocardial infarction within one three days, which was confirmed by electrocardiography, enzyme-linked immunosorbent assay and histological examination.

Results: Pre-treatment of rats with dalargin had no prevented myocardial infarction, however, it increased the resistance to immobilization stress and reduced infarction-induced myocardial lesions. Simultaneous administration of naloxone, an opiate receptor antagonist, together with dalargin eliminated its cardioprotective effect in experimental animals.

Conclusion: The use of synthetic leu-enkephalin dalargin significantly reduced the risk of myocardial infarction caused by excessive neuromuscular stress. The dalargin effect on the myocardium was mediated by opiate receptors.

Key words: myocardial infarction; immobilization stress; dalargin.

The diseases of cardiovascular system comprise one of the biggest problems of modern medicine. The most common forms of cardiovascular disease include hypertension, atherosclerosis, coronary heart disease and myocardial infarction [1]. There are many common links in the etiopathogenesis of cardiovascular diseases. Thus, the pathogenesis of ischemic heart disease is based on the impaired microcirculation in the heart muscle due to atherosclerotic vascular damage. Myocardial infarction occurs when the level of heart muscle supply does not correspond to the increased functional load on the bloodstream. Myocardial infarction is an acute irreversible ischemia, mostly due to coronary artery thrombosis with insufficient compensatory function of the collaterals, resulting in the necrosis of a certain portion of the heart muscle. All myoglobin-bound and physically dissolved oxygen is consumed during 8−10 seconds in the ischemic area, when the later occurs due to absolute or relative insufficiency of coronary blood flow [2]. The main factors increasing the risk of myocardial infarction are the following: 1) imbalanced nutrition, in particular, with the excess of fats and sodium/calcium salts; 2) increased carbon dioxide concentration and decreased oxygen concentration in the air inhaled by a person; 3) hypodynamia and prolonged stay in a confined space, usually attributed to the occupational activities; 4) infectious diseases; 5) psychological and emotional stress; 6) excessive neuromuscular tension. The efforts of many researchers were aimed at creating therapeutic means and methods that can increase the resistance of the myocardium to these factors in at risk patients (i. e., those having ischemic heart disease, cardiosclerosis). This would enable minimizing myocardial infarction area and reducing the risk of complications, including lethality. A significant number

of biopharmacological studies are focused at the study of cardioprotective properties of biologically active substances of peptide nature, derived from animal and plant tissues and cells [3−6].

Noteworthy, the publications on the cardiotropic properties of low molecular weight peptide fractions derived from tissues of cold-adapted animals are scarce [7−9]. One can assume that the cardioprotective effect of those fractions, at least in part, is due to the presence of biologically active peptides in their composition. It is known that an important role in curbing stress-induced pathological processes is played by anti-stress substances, such as, i.a., endogenous opioids. Their protective effect on the cell is mediated by opiate receptors, which are found in the membranes of cardiomyocytes [10−12]. Regulatory neuropeptides play an important role in the adaptation of animals to environmental factors. Opioid peptides play, in particular, a significant role in the regulation of hibernation. Quite a number of peptides acting as endogenous regulators of seasonal physiological changes in hibernating animals are known: bombesin, opioids, neurotensin, cholecystokinin, and others. The ability to hibernate has evolved in mammals as a special condition of energy preservation in unfavourable environmental conditions. The winter (or, in some species, summer) hibernation is inevitably accompanied by depletion of energy reserves, intracellular acidosis and hypoxia, similar to those that occur in ischemia. However, despite the presence of all those potentially detrimental factors, the myocardium remains undamaged for months. While circulating opioid peptides are known to increase dramatically during deep sleep, they are potential substances which may provide resistance to hypoxia in cells and tissues in hibernating animals. This opens up the prospect of using the protective potential of opioids against ischemia or hypoxia [12, 13]. There is an evidence of participation of such neuropeptides as leu- and met-enkephalins in the maintenance of hibernation [14]. The group of leucine enkephalins also includes the synthetic hexapeptide Lei-Tyr-D-Ala-Gly-Fen-Lei-Arg, known as dalargin [12]. Kyotorphin, a Tyr-Arg dipeptide having an analgesic effect, is considered to be one of the endogenous regulators of the transition of hibernating animals from active wakefulness to hibernation, which is a kind of tolerant strategy of the adaptation to unfavorable environmental conditions [15, 16]. Although

kyotorphin is unable or almost unable to interact with opiate receptors directly, it interacts with them indirectly by stimulating the release of methionine enkephalins into the bloodstream where they interact with opiate receptors, [17]. The study of cardioprotective effects of low molecular weight extracts of umbilical cord blood and tissues of newborn animals could also be a promising area of modern biopharmacology; this is supported by the studies of the biological activity of those substances [18−21]. Elevated levels of endogenous opiates (β-, α- and γ-endorphins, leucine- and methionine-enkephalins) are known to be present in the fetus blood at birth, which is considered to be a reaction that protects newborn from the pain and oxygen deficiency, i.e. potentially damaging factors of so-called intranatal hibernation. Therefore, the cardioprotective effect of those extracts and low molecular weight fractions may also be due to the presence of neuropeptides.

According to the aforementioned, the excessive neuromuscular tension is one of the common causes of myocardial infarction in at-risk patients. Given this fact, the aim of this work was to study the cardioprotective effect of enkephalins under conditions of immobilization stress.

Matherials and Methods

In this study we used our own model of myocardial infarction [22]. Provided that the mechanism of human myocardial infarction pathogenesis in almost all cases involves sclerotic changes in the heart muscle and blood vessels [23], rats were pre-kept on a special electrolyte-fat diet until they developed hyperlipidemia and a critical increase in atherogenic index.

The animals were pre-divided by their behavior: low-active — supposed to be highly sensitive to exogenous influences, highly active — supposed to be low-sensitive, and moderately active — supposed to be moderately sensitive [24]; the latter group accounted for the vast majority of tested animals and were selected for the further experiment. To that end, 60 *Wistar* rats at the age of 6 months weighing 240−270 g had been selected. Animal fats in the amount of 10% of the total feed weight were added to the standard diet for rats. Instead of water, 0.9% NaCl solution was poured into the drinkers, thus provoking excessive consumption of sodium by animals, which is harmful to the cardiovascular system. The rats received 20 mg of calcium

gluconate (PJSC "Kyivmedpreparat", Ukraine) orally per animal per day. Vitamin D_2 was also administered at 2,000 IUs per animal (PJSC "Vitamins", Ukraine). Normally, ergocalciferol favors the absorption of calcium by bone tissue, but in conditions of hypervitaminosis it causes excessive calcium accumulation in the blood [25]. There is a known positive association between high serum calcium levels and the incidence of cardiovascular diseases, including myocardial infarction [26, 27]. In particular, the risk of coronary artery calcification increases, which contributes to the formation of atherosclerotic plaque. It was also borne in mind that systemic inflammation may be one of the reasons for the initiation of proatherogenic modification of blood lipoproteins, and vascular calcification [28]. To induce the systemic inflammatory process during the 8th week animals were twice injected intraperitoneally with the bacterial lipopolysaccharide "Pyrogenal" (N. F. Gamaliia RIEM, Russia) with the interval of three days, at a dose of 5 µg of dry weight of the substance per animal. The development of systemic inflammation was monitored by the rectal temperature at a depth of 1 cm. Within three days after the second injection of «Pyrogenal», a steady increase in body temperature, by approximately 1.5 degrees (39.2−39.7 °C), was registered in all rats. During the implementation of the model in rats, their blood samples were periodically collected from the tail vein to study the lipid profile. Starting from the 58th day of the experiment, the stressful impact was applied on the rats. To that end, animals were subjected to immobilization stress, by immobilizing the four limbs and lower jaw in the abdominal position for 3 hours daily, with the break of 24 hours.

The animals were divided into 6 groups. Group 1 consisted of animals kept on a standard balanced diet (normal group), and group 2 consisted of the rats, in which the state of blood hyperlipidemia was reproduced by keeping them on a special diet. Animals from groups 1 and 2 were not subjected to immobilization stress. The immobilization stress to the group 1 rats was not applied due to the fact that in those completely healthy animals the risk of myocardial infarction due to the stress was much lower than in rats from the other groups, and therefore the duration of stressful impact should be many times longer. This could lead to the death of the animals due to the stress-induced ulcers of the stomach and intestines. Rats of groups 1 and 2 were used to determine the serum content of cardiac troponin I in normal (group 1) and in hyperlipidemic animals, i.e. before the applying of the stressful impact (group 2). In addition, rats from group 2 were used for histological examinations of the impact of the pathogenic diet only (without immobilization stress) upon the myocardium state. Rats from group 3 were injected with 200 µl of 0.9% NaCl immediately before the immobilization. Group 4 consisted of rats, which received dalargin in the form of 0.25% solution of the pharmaceutical substance ("Peptidnyie Tekhnologii", RF) at a dose of 100 µg/kg body weight immediately before the immobilization. Group 5 consisted of rats, which, together with dalargin, were injected with naloxone, an inhibitor of opiate receptors, in the form of naloxone hydrochloride ("Sigma-Aldrich", USA) at a dose of 500 µg/kg body weight. Groups 1−3 included 10 rats each; groups 4 and 5 included 5 rats each. An electrocardiogram was registered in rats immediately after immobilization, and every hour during the immobilization and before the release of the animal. The cardiography used three standard (I, II, III) and three augmented (aVR, aVL, aVF) leads. Cardiograms were analyzed for abnormalities indicative of the occurrence of acute cardiovascular pathology. Such deviations were as follows: the appearance of a negative Q-wave, a significant decrease or absence of the R-wave (QrS or QS complexes), displacement of the ST complex relative to the isoline, high-amplitude «coronary» T-waves. The detection of these abnormalities in several leads was considered as the reason for a preliminary diagnosis of acute coronary syndrome, and the stressful impact on the animal was stopped. Eighteen hours later, blood sample was collected from the rat's tail vein, and serum was prepared from it, in which the content of cardiac troponin I was determined by enzyme-linked immunosorbent assay using the "Rat Cardiac Troponin I (cTn-I) ELISA kit" (Cusabio Technology LLC, USA). The increase of the serum cardiac troponin I in rats, that was at least 2-fold higher than the predetermined average value for the animals kept on a pathogenic diet but not exposed to immobilization stress (group 2) provided a basis for the diagnosis of a myocardial infarction. After that, the animal was excluded from the experiment by decapitation under ether anesthesia, its heart was removed and fixed in 10% neutral formalin (pH 7.4), followed by tissue processing and embedding in paraffin. Histological sections of 5−7 µm thickness

were cut and stained with hematoxylin and eosin [29]. Slide mounts were photographed and analysed using an "Axio Observer Z1" microscope (Carl Zeiss, Germany) in bright field mode. For comparison, histological samples were analyzed from the hearts of animals kept on pathogenic diet but not exposed to immobilization stress.

Experimental data were processed statistically using the statistical module of the "Excel" software (Microsoft, USA). Differences between the samples were estimated using Student's two-sample t-test. The data were presented as «sample mean ± sample standard deviation» ($\bar{X} \pm s$).

Results and Discussion

The results published earlier [21] showed that rats kept on a diet rich in fats and sodium/calcium salts for two months developed a hyperlipidemia. The level of total cholesterol in the blood from rats used for the heart infarction modeling exceeded that in animals kept on a standard diet by almost a quarter (3.32 ± 0.17 vs. 2.67 ± 0.19), and the content of low-density lipoprotein was almost three times higher (1.34 ± 0.09 vs. 0.49 ± 0.08). As a result of such changes in the lipid profile, the atherogenic index was twice as high as normal (1.84 ± 0.35 vs. 0.91 ± 0.13).

During the first minutes of immobilization stress the cardiograms of all animals without exception showed signs of severe tachycardia. Analysis of the cardiograms of animals from the control group (group 3) 1 hour after the onset of stressful impact revealed the changes in the width and amplitude of the T-wave in 9 out of 10 animals. In five rats, the T-wave was reduced or smoothed, and in four rats it was biphasic. Such changes in the cardiogram indicate the occurrence of myocardial ischemia.

Two hours after immobilization, the cardiogram of one of the rats showed a negative Q-wave in combination with a positive high-amplitude "coronary" T-wave (a sign of transmural infarction), on the basis of which a preliminary diagnosis of "acute coronary syndrome" was made, and this animal was released and transferred to a cage. Six animals had T-wave changes: four rats showed a negative symmetrical "coronary" T-wave, and two others had elevations above the ST-segment isoline. These changes could indicate unstable angina, myocardial damage, or even necrosis, so the immobilization of those animals was stopped. Cardiograms of other animals had signs of myocardial ischemia (T-wave was broadened and lowered, and was smoothed in one rat). An hour later, another rat was pre-diagnosed with acute coronary syndrome. The reasons were the same as in the first case: a negative Q-wave in combination with a positive high-amplitude "coronary" T-wave. No changes allowing to diagnose acute coronary syndrome were detected in the cardiograms of the two other rats, and after 3 hours of immobilization stress they were returned to the cages (after the application of the stress, all rats were kept in separate cages), and the next day they were repeatedly subjected to immobilization stress. However, the QS complex (with negative Q- and no R-wave) was detected in the cardiogram of one of those rats, which occurred in the first minutes after immobilization. That rat was released, and its blood was immediately tested for troponin I. In the remaining animal, the pathological changes in the cardiogram, which allowed to pre-diagnose an acute coronary syndrome (negative symmetric «coronary» T-wave) were detected three hours after the onset of the repeated immobilization stress.

Administration of dalargin to rats significantly increased the resistance of their hearts to excessive neuromuscular load, as evidenced by the increased duration (by $1.5-2$ times) of their ability to resist the immobilization stress before the acute coronary syndrome was registered (Fig. 1). Simultaneous administration of dalargin and naloxone, the opiate receptor antagonist, to the animals (group 5) eliminated the protective effect of the leu-enkephalin in that test. This was evidenced by both the reduction of the time during which animals from those groups were able to resist immobilization stress until they were preliminary diagnosed with an acute coronary syndrome (Fig. 1); and the increase in cardiac troponin I in the blood to the levels found in control (group 3) animals. Micromorphological analysis also supported these findings (see below).

In the group of rats treated with dalargin before the onset of stress impact (group 4), the acute coronary syndrome was diagnosed in two cases during the second immobilization stress, and in three cases during the third immobilization stress. Preliminary diagnosis was based on the following abnormalities in the cardiograms: negative Q-wave in combination with positive high-amplitude "coronary" T-wave (3 cases), or lowered R-wave and the elevation above the ST segment isoline (2 cases).
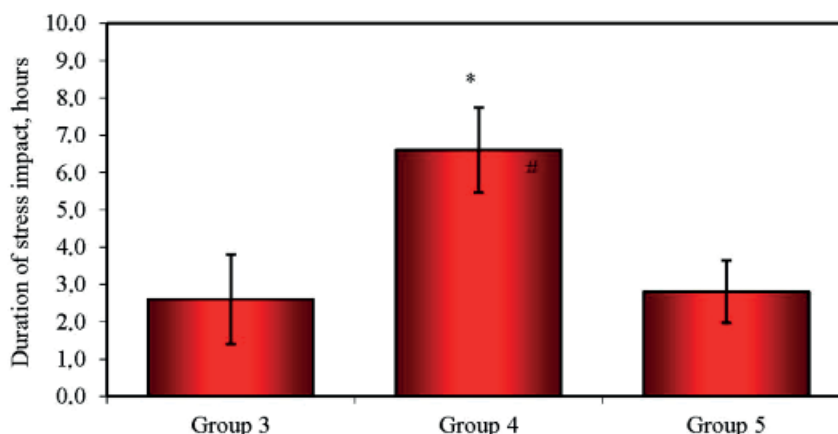
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Fig. 1. The effect of dalargin and naloxone on the total duration of rats resisting the impact of immobilization stress until the moment of registration of acute coronary syndrome signs in the cardiograms

Note: * — statistically significant differences compared to the group 3 and group 5, $P \leq 0,01$.

It is known that QS and QrS cardiogram complexes may indicate large focal transmural myocardial infarction. Sera cardiac troponin I levels (Fig. 2) in the rats from all groups which were preliminarily diagnosed with acute coronary syndrome were significantly increased in all animals without exception, as compared to both the intact animals and animals kept on a pathogenic diet but not exposed to stress. This allowed us to refine the diagnosis and suppose that those animals had suffered a myocardial infarction (the preliminary diagnosis was an "acute coronary syndrome"). It should be noted that animals that were not exposed to stressful impact after being kept on a pathogenic diet also had a significant increase in this indicator compared to intact animals, but those changes were much less pronounced (16.2 ± 1.9 vs. 6.62 ± 0.29, $P \leq 0.05$). Lower (38.19 ± 1.09 vs. 54.08 ± 4.01; $P \leq 0.05$) levels of cardiac troponin I in the blood of rats injected with dalargin (group 4), compared with animals from the control group (group 3) may indicate a smaller heart damage.

Histological examination of heart tissue showed that keeping animals on a diet with high fats and calcium/sodium salts caused changes in the myocardium and blood vessels which increase the risk of myocardial infarction. Thus, the microphotographs clearly demonstrated the signs of myocardial dystrophy in the form of small intermittent groups of slightly hypertrophic or atrophic cardiomyocytes (Fig. 3, *a*). Myocardial muscle fiber bundles were thinned due to the atrophy, or demonstrated a wavy deformation. Cross sections of some cardiomyocytes showed that the cytoplasm contained small round

whitish vacuoles which were similar to fat droplets. The areas with poor blood supply, and the foci of venous-capillary congestion occured intermittently. It could be seen that large and small intramural coronary arteries had uneven wall thickening due to mild coronary sclerosis and initial stages of perivascular cardiosclerosis. Clusters of lymphocytes around blood vessels, and focal infiltration of the myocardial stroma were also detected. Apparently, both atherosclerotic and myocardial forms of cardiosclerosis were present, as injection of bacterial liposaccharide ("Pyrogenal") induced systemic inflammation in the animals.

Because the aforementioned pathological abnormalities in the cardiograms in rats from all groups occurred in the leads I and aVL, and in their reciprocal leads III and aVF, those findings indicated the infarction located in the lateral wall of the left ventricle and the thrombosis of the anterior descending artery.

Histological examinations confirmed that all rats subjected to immobilization stress had a large focal transmural myocardial infarction. Myocardial tissues demonstrated similar pathological changes: focal hemorrhages, congested vessels, loss of striated muscle fibers (individual muscles took the form of so-called "minced meat"), and vascular thrombosis (Fig. 3, *b*, *c* and *d*). Necrotic muscle cells in the infarction area lacked nuclei. Blood flow in the necrotic area was completely absent, and congestion in the veins and sinuses, and blood stasis in the capillaries were registered in peri-infarct zone. Areas of intermuscular hemorrhage have been found in many tissue samples. A small focal parenchymal dystrophy of cardiomyocytes, and clusters of lymphocytes
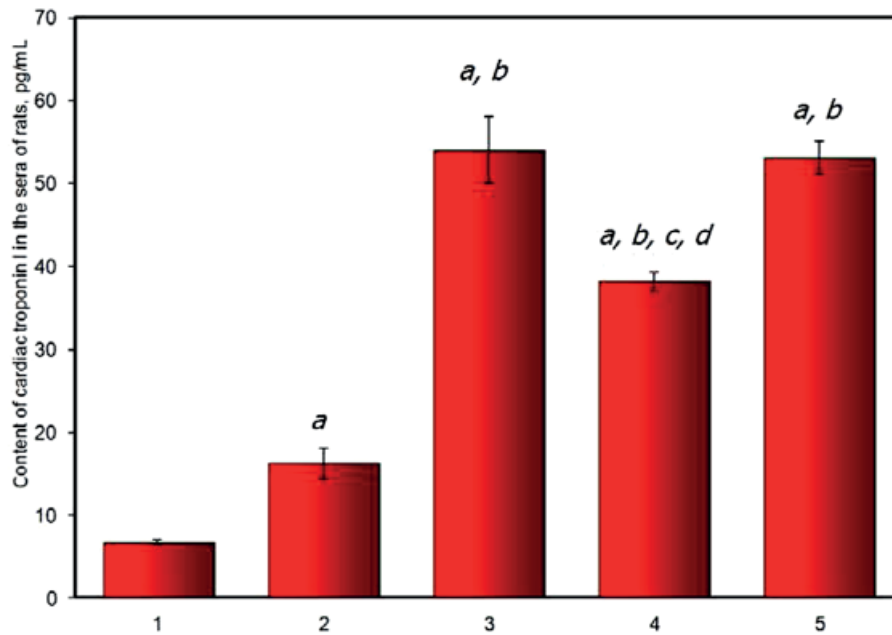
Fig. 2. **The content of cardiac troponin I in the sera of rats**

Notes:

1) Numbers on the abscissa denote groups of experimental animals;

2) a — statistically significant differences compared to the group 1, $P \leq 0.01$; b — statistically significant differences compared to the group 2, $P \leq 0.01$; c — statistically significant differences compared to the group 3, $P \leq 0.01$; d — statistically significant differences compared to the group 5, $P \leq 0,01$
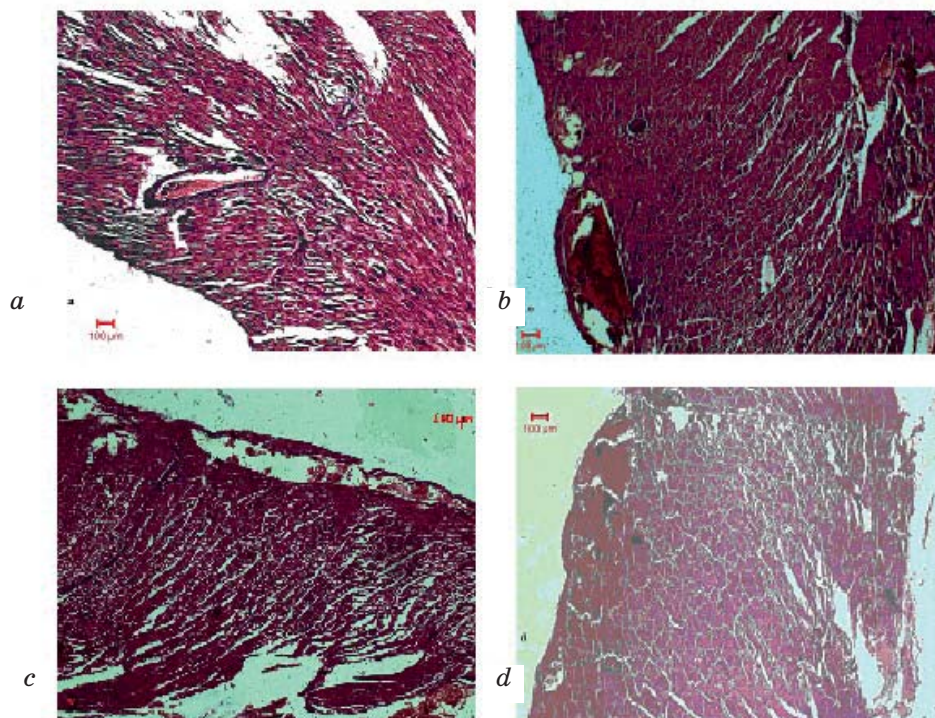
Fig. 3. **Left ventriclular tissue sections from rat hearts, stainined with hematoxylin-eosin:**

a — the animal was on a pathogenic diet, but not exposed to immobilization stress (group 2); b — the animal with developed myocardial infarction as a result of immobilization stress (group 3); c — the animal received dalargin before the application of immobilization stress (group 4); d — the animal received naloxone and dalargin simultaneously (group 5)

were seen. In the samples from rats which didn't receive dalargin (Fig. 3, *c*) before the onset of immobilization stress, or received dalargin and naloxone simultaneously (Fig. 3, *d*), the organized thrombi in epicardial coronary artery and small subendocardial arteries were clearly visible, as well as the destruction of cardiomyocytes in the infarction area, and formation of collaterals in peri-infarct zone. In the rats which received dalargin without concomitant administration of naloxone prior to immobilization stress (Fig. 3, *c*), the resorptions and recanalizations of the thrombi in epicardial coronary artery and in individual small subendocardial arteries were significantly more frequent. The collaterals in the peri-infarct zone were also formed oftener.

Many previous studies have shown that dalargin acts on the cells through opiate receptors [30, 31]. Therefore, the inhibition of this opiate receptor agonist by naloxone, an opiate receptor antagonist, was rather predicted.

Thus, based on the results obtained, we can conclude that enkephalins possess a cardioprotective effect. The use of dalargin, a synthetic leu-enkephalin, significantly reduced the risk of myocardial infarction due to excessive neuromuscular stress.

Animal studies were performed in accordance with the Law of Ukraine "On Protection of Animals from Cruel Treatment" (№3447-IV of 21.02.2006) in compliance with the requirements of the Institution's Bioethics Committee, which are consistent with the provisions of "The European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Strasbourg, 1986), as well as the recommendations "Bioethical expertise of preclinical and other scientific studies conducted on animals" (Kyiv, 2006).

The study was performed within the scientific and research work of the Cold Adaptation Department of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine "Study of the dependence of cord blood fractions composition and biological activity under cold exposure" (state registration № 0115U000093).

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КАРДІОПРОТЕКТОРНА ДІЯ ЕНКЕФАЛІНІВ ЗА УМОВ ІММОБІЛІЗАЦІЙНОГО СТРЕСУ

І. Й. Щенявський

Інститут проблем кріобіології і кріомедицини НАН України, Харків

E-mail: ivanhou11@gmail.com

Мета. Вивчення кардіопротекторної дії синтетичного лей-енкефаліну даларгіну.

Методи. Розвиток інфаркту міокарду у щурів, яких протягом двох місяців утримували на дієті з надлишковим вмістом жирів та солей кальцію і натру, ініціювали шляхом застосування іммобілізаційного стресу. Результати експериментів свідчать про те, що застосована модель дозволила протягом одного-трьох днів викликати розвиток інфаркту міокарда, який було підтверджено методами електрокардіографії, іммуноензимнго аналізу та гістологічним.

Результати. Попереднє введення піддослідним тваринам даларгіну, хоча й не запобігало інфаркту, підвищувало витривалість до впливу іммобілізаційного стресу та зменшувало ступінь ураження міокарда. Введення піддослідним тваринам разом з даларгіном антагоніста опіатних рецепторів налоксону усувало кардіопротекторну дію.

Висновки. Використання синтетичного лей-енкефаліну даларгіну істотно зменшує ризик інфаркту міокарду внаслідок надмірного нервово-м'язового навантаження. Дія даларгіну на міокард опосередкована опіатними рецепторами.

Ключові слова: інфаркт міокарда; імобілізаційний стрес; даларгін.

CYTOTOXIC, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF *Peganum harmala* L. EXTRACTS

Dehiri Mounira[1]
Diafat Abdelouahab[1]
Fatmi Widad[2]
Ben Mansour Riadh[3]
Bouaziz Farid[1]
Bahloul Ahmed[2]

[1]Laboratory of Characterization and Valorization
 of Natural Products, Faculty of Nature and Life Sciences,
 University El-Bachir El-Ibrahimi, Bord jBou Arreridj
[2]Laboratory of Health and Environment, Faculty of Nature
 and Life Sciences, University El-Bachir El-Ibrahimi,
 Bord jBou Arreridj
[3]Laboratory Analysis, Valuation of Food Securities,
 Biotechnology Research Group and Pathologies, National
 School of Engineering of Sfax (ENIS), Tunisia

E-mail: abdelouahab.diafat@univ-bba.dz

Peganum harmala L., known as ′Harmel′, is a plant widely used in the traditional Algerian medicine.
Aim. The purpose of this work is to study the antioxidant, antiproliferative and antimicrobial potential of *Peganum harmala* extracts.
Methods. Colorimetric methods were used to quantify phenolic compounds, while the antioxidant activity was estimated *in vitro* using DPPH/ABTS radical scavenging assay, ferric reducing power, β-carotene bleaching assay, total antioxidant capacity, and ferrous iron chelating assay. The agar well diffusion and the broth microdilution method were used to evaluate the antibacterial activity and the MTT assay was used to test the cytotoxicity of the extracts.
Results. The ethanolic extracts of *Peganum harmala* L. showed the highest polyphenols content and the potent antioxidant, gave a good activity against Gram+ and Gram− bacteria and good antifungal effect and were more cytotoxic to the HeLa cell line.
Conclusions. It is concluded that selected plants could be a potential source of bioactive compounds with antioxidant, antimicrobial and antiproliferative potential. Hence, it is indicated to further investigate this plant *in vitro* as well as *in vivo* for new drug discovery.

Key words: Peganum harmala; polyphenols; antioxidant activity; antimicrobial effect; cytotoxic
 effect.

Natural products are attracting growing interest by phytotherapy researchers due to their safety and medicinal properties [1]. Medicinal plants have been used for many years as remedies for both human and animal ailments [2]. Aromatic and medicinal plants as the key source of complementary and alternative medicine have been recently bring many hopes in alleviating of symptomatology and curing associated with so many diseases [3]. The World Health Organization (WHO) reported that around 80% of the world's population uses traditional medicine for their primary health care [4]. The beneficial health effects of these plants are mainly related to the presence of phenolic compounds [5].

Polyphenols of medicinal plants are powerful antioxidants capable of fighting against free radicals (Reactive Oxygen Species — ROS), which are involved in the appearance of several pathologies such as cancer, diabetes, Alzheimer's disease, and cardiovascular disorders [6]. Polyphenols present a wide range of other bioactivities, including anti-inflammatory, anticarcinoma, antiviral and cardioprotective effects [7]. Phenolic compounds may also play a critical role as antimicrobial agents, a property that has received increased attention due to their potential effects against antibiotic-resistant bacteria [8].

Algeria, given its privileged biogeographical position and its extent

between the Mediterranean and sub-Saharan Africa, is considered among the countries known for their floristic diversity to which is added a secular tradition of traditional use of plants. Among the medicinal plants, the genus *Peganum* commonly called harmel, is a plant of the family *Zygophyllaceae* [9], is widely distributed in arid and semi-arid regions [10].

Peganum harmala L. is known for its wide use in folk medicine for the treatment of various diseases including diabetes and hypertension, lumbago, asthma, colic, hepatitis B, jaundice, and as an emmenagogue stimulant [11]. *P. harmala* has anti-carcinogenic [12], vasodilator, anti-protozoal, and anti-inflammatory effects [13, 14]. Because of their analgesic and antibacterial properties [15], their fruits are used in traditional medicine. In addition, alkaloid from *P. harmala* has antihemosporidian, antinociceptive and antineoplastic effects [16]. The seeds of *P. harmala* contains a large number of alkaloids and β-Carboline alkaloid and used in fever, abortion, red dye, diarrhea and many other human chronic diseases [17]. *Peganum harmala* seeds also comprises a large extent of total phenolic and total flavonoids component and act as strong antioxidant plant [18].

The main objectives of this study were to determine the antioxidant, cytotoxic and antimicrobial activities of extracts of the plant *P. harmala* L.

Materials and Methods

Plant material

Peganum harmala L. (Zygophyllaceae) seeds were collected during flowering stage El Hammadia, wilaya of Bordj Bou Arreridj. The identification of the plant was carried out by Pr. Boudjelal Amel and a voucher specimen (No. AB-65, 2018) was deposited at the University Mohamed BOUDIAF —M'Sila. The seeds of *P. harmala* L. were dried in dark, at room temperature for two weeks and powdered using electrical grinder and resulting powder was then stored until use.

Extracts preparation

To obtain the *aqueous extract (AqE)* of *P. harmala* L. following the method of Mbiantcha *et al.* [19] maceration has been made with 50 g of the seed powder in 500 mL of distilled water for 3 days. *The ethanolic extract (EtE)* was prepared from a mixture of 50 g of *P. harmala* L. powder in 200 mL of ethanol 80%; the mixture was shaken for 8 h and incubated overnight at room temperature. *The decoction extract (DcE)* was prepared

according to the method of Robeson and Strobel [20] with some modification; 50 g of powdered plant material were mixed with boiling distilled water (200 mL) for 1 hour. All extracts were filtered through a Whatman No. 3 filter paper and evaporated using a rotary evaporator under vacuum at 40 °C until completely dried, to obtain AqE, EtE and DcE, which were stored at 4 °C until use.

Spectrophotometric estimation of phenolic compounds

The total phenolic content of the various extracts of *P. harmala* L. was determined using the Folin-Ciocalteu reagent. The results were expressed in µg of Gallic acid equivalent per mg of dry extract (µg of GAE/mg extract) [21].

Total flavonoid content was determined following the trichloride aluminum assay [22]. The results were expressed as µg quercetin equivalent per mg of dried extract (µg of QE/mg extract).

Total flavonols content in *P. harmala* L. extracts were determined according to the method of Kosalec et al. [23]. 0.5 mL of standard solution or extracts was separately mixed with 1.5 mL of ethanol and 0.1 mL of 10% methanolic aluminum chloride solution, then 0.1 mL of sodium acetate and 2.8 mL of water were added and incubated for 30 min at room temperature. The absorbance was measured at 415 nm. The results were expressed as µg of quercetin equivalent per mg of dried extract (µg of QE/mg of extract).

Antioxidant activity

The antioxidant activity was measured by using the phosphomolybdenum assay [24], which is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green Phosphate / Mo (V) complex at acidic pH. The greenish color is measurable at 695 nm in the presence of reducing agent. The total antioxidant capacities were expressed as a µg equivalent of ascorbic acid per mg of dry extract (µg AAE/mg).

The 1.1-diphenyl- 2-picrylhydrazyl radical (DPPH) were also used for antioxidant activity. 375 µL of extracts or standard at the various concentrations was mixed with 125 µl of a methanol solution of DPPH (0.004%). After 30 min of incubation, the absorbance was recorded at 517 nm [25]. L'hydroxyanisolebutylé (BHA), L'hydroxytoluènebutylé (BHT) and Quercetin were used as standards; the scavenging activity was calculated according to equation (1):

$$\text{Inhibition} (\%) = [(\text{Abs}_{control} - \text{Abs}_{sample})/ \text{Abs}_{control}] \times 100. \qquad (1)$$

To assess the scavenging activity of the extracts against the ABTS radical cation (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)),the reaction mixture included ABTS (7 mM) and potassium persulfate (2.45 mM). The extracts or standard (100 µL) were homogenized with 1.9 mL of ABTS solution and incubated for 7 min. The absorbance was measured at 734 nm [25]. The capacity of the extract to scavenge the ABTS radical was calculated according to the previous formula (1). Trolox was used as positive control, and the results were expressed in µmol g^{-1} of Trolox-equivalent antioxidant capacity (TEAC).

To assess the extract antioxidant properties in mechanisms involving iron, both *ferric reducing power (FRP)*and *ferrous iron chelation assays* were used. FRP was used to assess the extracts capability to reduce iron ions [26]. The absorbance was recorded at 700 nm and the EC_{50} value (effective concentration for that has an absorbance of 0.5) was calculated.

To evaluate extract-iron interaction, the ferrozine test was performed following the method of Decker and Welch [27], modified by Le et al. [21]. The red chromophore of the Fe^{+2}-ferrozine complex was measured spectrophotometrically at 562 nm against a blank. Ethylene diamine tetra acetic acid (EDTA) was used as reference chelator. The inhibition of ferrozine$-Fe^{2+}$ complex formation was calculated according to equation (1). An IC_{50} value is defined as the inhibition concentration of tested material, which produces 50% of the maximal scavenging effect.

To assess the extracts antioxidant properties in lipidic like-systems, *the* β−*carotene/linoleic acid assay* was used. The antioxidant capacity of each extract was evaluated by measuring the inhibition of the volatile organic compounds and the conjugated diene hydro-peroxides arising from linoleic acid oxidation [28]. The antioxidant activity of samples and standards BHT (2 mg/mL) are calculated after 24h at 490 nm according to the following equation (2):

$$AA\% = (Abs_{test} / Abs_{BHT}) \times 100 \qquad (2)$$

where Abs_{test}: Absorbance of the extract; Abs_{BHT}: Absorbance at t = 0 in the presence of positive control BHT.

The results were expressed as IC_{50} (the concentration required to inhibit 50% of the β-carotene bleaching). Synthetic antioxidant BHT was used as positive control and methanol and distilled water were used as a negative control.

Antiproliferative activity

HeLa cells(cervical cancer line, adherent) were used to investigate the cytotoxicity effect of *P. harmala* extracts, using MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) [29]. The cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) fœtal calf serum(FCS) and 2 mM L-glutamin with 400 µL antibiotic 50 U/mL of gentamycin and penicillin and 50 µg/mL of streptomycin (Sigma). The cultures were then maintained at 37 °C in an incubator at 5% CO_2 and saturated with water vapor 95%.

HeLa cells (4.44×10^5 cells/well) were seeded in 96-well cell culture plates for 48 hours in the presence or absence of extract (10 µL). After this period, 10 µL of MTT solution (1 mg/mL in phosphate-buffered saline; PBS) were added to each well and incubated for 4 h at 37 °C in a CO_2-incubator. 180 µL of the medium was removed from every well without disturbing the cell clusters. Formazan crystals were dissolved using 180 µL methanol/DMSO solution (50:50), and the preparations were thoroughly mixed on a plate shaker for 30 min in the room temperature with the cell containing formazan crystals.

Finally, after the dissolution of all crystals, the absorbance of each well was determined with a microplate reader (ELx 800) at 570 nm. The results were expressed as a percentage inhibition of cell proliferation and calculated according to the following formula (3):

$$\% \text{ Inhibition} = 100 - \% \text{ viability}, \qquad (3)$$

where % viability $= (Abs_{extract} \times 100)/Abs_{control}$

The IC_{50} of the extracts was determined as the drug concentrations that reduced cell number by 50% in treated compared to untreated cultures.

Antimicrobial activity
Microbial strain

The antibacterial activity was determined using clinical referenced strains bacteria, including Gram (+) and Gram (−) bacteria from the American Type Culture Collection (ATCC) standards. The strains were *Escherichia coli* (ATCC25922), *Salmonella thyiphimurium* (ATCC13311)*, Proteus mirabilis* (ATCC35659), *Staphylococcus aureus* (ATCC25923), *Bacillus cereus* (ATCC10876) and *Micrococcus luteus* (ATCC469). All the strains were obtained from the Laboratory of Bacteriology at Setif hospital, Algeria. Plant pathogenic fungus: *Fusarium culmorum, Aspergillus carbonarius*

(M333), *Aspergillus flavus* (NRRL 3251), *Penicillium glabrum* were obtained from the Laboratory of Applied Microbiology at the Ferhat Abbas University of Sétif, Algeria.

Antibacterial activity

The agar well diffusion method was employed for the determination of antibacterial activities of extract [30]. The extracts were dissolved in DMSO to obtain the doses of 9, 6 and 3 mg/well and sterilized by filtration trough 0.22 µm Nylon membrane filter. The bacterial strains were cultured in sterile distilled physiological water for 24 h. Petri dishes of sterile Mueller-Hinton agar were inoculated by the method of streak with the appropriate bacterial suspension (0.5 McFarland). Wells were cut into the agar and were loaded with 10 µL of extract.

Additionally, and for comparative purposes, standard gentamicin (10 mg/disc) and chloramphenicol (30 mg/disc) is used in the test as positive controls. All the plates were incubated at 37°C for 24 hours. Antibacterial activity was evaluated by measuring the zone of inhibition in millimeters.

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The values of MIC, which represent the lowest extract concentration that completely inhibits the growth of microorganisms, were determined by a micro-well dilution method [31]. Briefly, 100 µL of Mueller Hinton broth (MHB) containing 0.02 g/L phenol red was dispensed into each well of a -96 well microplate. 100 µL of extract is then added to each well in the first column of the microplate except that of the second row. Serial twofold dilutions were carried out until the 12th well of each row. Then, 100 µL of MHB without red phenol were added in the first well of the second row before diluting (positive controls). All the wells were inoculated, except those in the first row (negative controls), by introducing 100 µL of bacterial suspension (10^6 CFU)/mL). On the other hand, MHB without red phenol was put in the first row's wells. The microplate was wrapped loosely and then incubated at 37 °C for 24 hours. The appearance of a yellow color indicates a bacterial multiplication, while the persistence of the initial red color means the contrary.

To determine MBC, the broth was taken from each well without visible microbial growths from the MIC are re-isolated on Muller-Hinton agar. The wells from the positive and negative control rows were also seeded on the same agar to ensure no bacteria growth in the wells. After 24 h of incubation in an oven at 37 °C, the number of colonies on the streaks was compared to those of the control dish seeded with streaks of dilutions of the bacterial inoculum. On reading, the lowest concentration of extract for which no microbial colony is observed (99.99% destruction) corresponds to MBC.

Antifungal susceptibility test

Antifungal activity was tested against four fungal strains using the agar diffusion method. The fungal suspension was obtained from spores after seven days at 28 °C and prepared in sterile distilled water (OD 0.12–0.15 at 525–530 nm). An aliquot of 0.1 mL of this fungal suspension was spread over the surface of the agar plate. Wells (7 mm diameter) were cut into the agar and impregnated with extracts (9, 6 and 3 mg/disk) or with positive controls like Rifampicin (5µg/well). The petri dishes were placed in an oven at a temperature of 28 ± 2 °C for a period of incubation of 10 days. For the determination of the effect of *P. harmala* L. extracts on radial growth against the tested fungus, the diameter of the disc growth was measured every 48 hours of incubation for 10 days.

Statistical analysis

The results are expressed as mean ± SD of triplicate measurements. The GraphPad Prism Software (version 8.00) was used for statistical analysis. Data were analyzed using Student's t-test and one-way analysis of variance (ANOVA), followed by Dunnett test for multiple comparison. The differences were considered significant at $P < 0.05$.

Results and Discussion

Quantitative analysis of phytochemicals

The extraction was performed using ethanol 80%, and water. The yield of extraction of the *P. harmala* L. extracts was significantly different ($P < 0.05$) (Table 1). The yield of extraction of the AqE and the EtE was similar to that found by Zainab et al. [32]. The extraction yield appears to be influenced by the several factors such as the polarity of the solvent [33].

Polyphenols, flavonoids and flavonols are very important compounds in plants because their therapeutic interest and their antioxidant activity have attracted most attention. The results obtained in this study (Table 1) showed a high level of phenolic, flavonoids and flavonols compounds in EtE. The DcE and AqE have average amounts of these compounds.

Table 1. **Yield and secondary metabolites content from *P. harmala* L. extracts**

Extract	Yield (%)	Total polyphenols (µg GAE/mg)	Total flavonoids (µg QE/mg)	Total flavonols (µg QE/mg)
Ethanolic	12.8 ± 0.40^c	102.68 ± 1.61^c	30.33 ± 1.06^c	108.05 ± 1.88^c
Aqueous	12.56 ± 0.67^c	65.92 ± 1.23^c	15.51 ± 0.50^c	46.39 ± 0.23^c
Decoction	10.62 ± 0.70^c	80.62 ± 0.59^c	20.45 ± 1.06^c	52.81 ± 0.87^c

Values were expressed as means $\pm$ SD ($n = 3$). Different letters in the same columns indicate significant difference (c: $P < 0.0001$). GA: Galic acid equivalents; QE: Quercetin equivalents.

Concerning the content of polyphenols in EtE and AqE it was almost similar that found by Zainab et al. [32]. It can be concluded that the phenolic or flavonoid compounds contained in the extracts were influenced by their solubility in the solvent used for extraction.

Antioxidant activity

The antioxidant properties of the extract of *P. harmala* was assayed *in vitro* using different antioxidant methods covering these mechanisms. The TAC of various extracts was estimated using phosphomolybdate assay. The present study reveals that the Ethanolic extract (EtE) showed higher antioxidant activity than aqueous extract (AqE and DcE) (Table 2).

Results showed that the extracts of *P. harmala* L. were able to decolorize the stable, purple-colored DPPH radical into yellow-colored DPPH-H. From the IC_{50} values obtained, the DcE and EtEwas found to exhibit the greatest scavenging activity compared to AqE. All extracts showed lower DPPH scavenging activity than BHT, BHA and quercetin used as standards antioxidants ($P < 0.01$). The comparison of our results with other works, shows that the EtE of the seeds of Algerian *P. harmala* showed a significant scavenging effect to what is obtained by the EtE from the Libyan *P. harmala* (IC_{50}: 179.62 ± 7.32 g/mL) [34]. The results are also compared with the IC_{50} of the ethanolic extract of *P. harmala* reported in Morocco by Khadhr et al. [35] (53.64 ± 0.5 g/mL).

Similarly, to DPPH assay, ABTS assay revealed that EtE exerted a significantly higher antioxidant capacity ($P < 0.05$). In contrast, Wang et al. [36] found that some compounds, which have ABTS scavenging activity, did not show DPPH scavenging activity as in the case of the DcE.

The reducing power is associated with antioxidant activity; it may serve as a significant indicator of electron donating ability of phenolic compounds. *P. harmala* L.

extracts showed considerable reducing power, indicating that they can act as electron donors and could react with free radicals to convert them into more stable products. The result showed that the EtE exhibited the highest reducing capacity followed by AqE and DcE. The reducing power of all extracts was lower than that of ascorbic acid, BHA and quercetin ($P < 0.01$) (Table 2). The chelating capacity of our extracts is important and probably due to the presence of antioxidant molecules capable of complexing with ferrous ions, it helps inhibit peroxidation. From the IC_{50} values obtained (Table 2), the potency of ferrous iron-chelating ability of DcE is statistically lower than those of the EtE and AqE extracts. IC_{50} values show that DcE exhibited potent chelating activities, but remained inferior to that of EDTA ($P < 0.01$).

Furthermore, the extracted phenolic compounds presented also the capacity to inhibit lipid peroxidation in the β-carotene/linoleic acid system. Results showed that all extracts gave a high inhibition and exhibited a high capacity to prevent the bleaching of β-carotene after 24 h of incubation as compared to the positive control (Fig. 1). In the present work, it was found that the EtE ($73.98 \pm 0.21\%$) were more powerful in inhibiting the β-carotene bleaching than AqE and DcE ($38.61 \pm 0.19\%$ and $43.34 \pm 0.89\%$, respectively). Ehanolic extracts of *P. harmala* L. containing higher amount of compounds which have a role in lipid peroxidation, compared with aqueous and decoction extracts. This results are in accordance with that found by Baghiani et al. [37]. The strong antioxidant effect of these compounds may be explained by the "polar paradox" phenomenon [38].

Antiproliferative activity

The effect of different concentrations of the extracts on HeLa cells survival was studied using the MTT reagent. The results obtained suggest that EtE potently suppressed

Table 2. **The *in vitro* antioxidant activity of *P. harmala* L. extracts, BHA, BHT, Gallic acid, ascorbic acid and EDTA**

Sample	DPPH· IC_{50} (µg/mL)	ABTS·+ (µmol g^{-1} TEAC)	Iron chelating IC_{50} (mg/mL)	Total antioxidant activity (µg AAE/mg)	Reducing power EC_{50} (µg/mL)
Ethanolic	61.16 ± 0.82^d	2.107 ± 0.01^c	1.127 ± 0.06^b	123 ± 0.96^b	828 ± 0.13^d
Aqueous	178 ± 0.30^d	1.26 ± 0.01^c	1.478 ± 0.05^b	65.47 ± 2.19^a	848 ± 0.32^d
Decoction	108 ± 0.74^d	1.37 ± 0.018^c	0.74 ± 0.05^b	37.31 ± 1.05^b	969 ± 0.80^d
BHA	5.14 ± 0.17^d	/	/	/	34.01 ± 1.33^d
BHT	28.10 ± 0.17	/	/	/	/
Quercetin	1.13 ± 0.01^d	/	/	/	45.85 ± 0.41^d
EDTA	/	/	0.023 ± 0.05^b	/	/
Ascorbic acid	/	/		/	22.71 ± 0.2^d

Values were expressed as means $\pm$ SD ($n = 3$). Different letters in the same columns indicate significant difference (c: $P < 0.0001$). GA: Galic acid equivalents; QE: Quercetin equivalents.
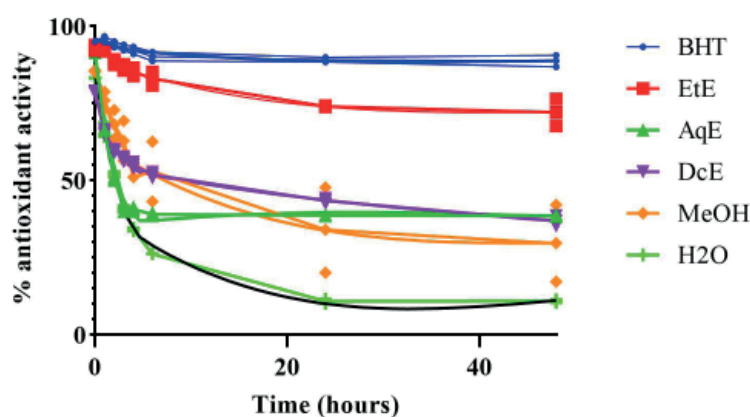

Fig 1. **Kinetics of β-carotene bleaching in the presence of AqE, EtE, DcE, water, methanol and BHT during 24 h**
AqE: aqueous extract, EtE: ethanolic extract, DcE: decoction extract,
BHT: butylatedhydroxytoluene, MeOH: methanol

the proliferation of HeLa cells: 36.48% at 0.015 mg/mL and to 97.99% at 2 mg/mL (Fig. 2), this shows that they are potent inhibitors of cancer cell growth. While the growth of the same cells exposed to aqueous and decoction extracts was not significantly reduced. The IC_{50} values of EtE, DcE and AqE were evaluated to 0.028 ± 0.004 mg/mL, 0.242 ± 0.040 mg/mL and 0.230 ± 0.014 mg/mL, respectively.

The EtE induced a significant decrease in cell viability and presented lower IC_{50} (< 30 µg/mL) for HeLa cell compared to AqE and DcE, so we can consider them as active and potential source of cytotoxic molecules toward HeLa cancer cells. While, the aqueous and decoction extracts can be considered as poorly cytotoxic for HeLa cells since the IC_{50} values are in the range (100–1 000 µg/mL). Cytotoxic effects of the crude seed extract of this plant, such as aqueous and hydro-alcoholic extracts have been previously reported, indicating considerable cytotoxic potential [39]. These cytotoxic natural products may be able to play a vital role in treating selected cancers by working in synergy with conventional chemotherapeutic drugs [40].

Antibacterial activity
Faced with the problems of bacterial resistance to synthetic antibiotics, much work has been done on the antimicrobial
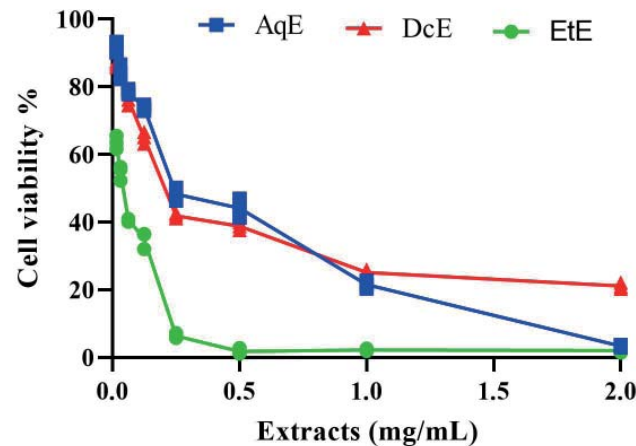
*Fig. 2. **P. harmala** L. extracts inhibited the viability of HeLa cell line in a dose-dependent manner*
Cell viability was determined using MTT assay and expressed as means ± SD.
AqE: aqueous extract, EtE: ethanolic extract, DcE: decoction extract

power of natural plant extracts. The *in vitro* antibacterial activity of AqE, EtE and DcE was evaluated using the agar diffusion method. Based on the results obtained, it is observed that the zones of inhibitions increase with the increase in the dose of the extracts. In addition, all our extracts were effective against all bacterial strains examined compared to the commercial antibiotics (gentamicin and ciprofloxacin) (Fig. 3). These results are in agreement with the bibliographic data of Guergour [41]. According to Biyiti et al. [42], an extract is considered active if its inhibition zone is greater than or equal to 10 mm. From these results, it can be seen that the Gram– bacteria possess strong resistance compared to Gram+ bacteria, which can be attributed to the difference in the morphology of the cell wall [43]. The hydroalcoolic extract was found to be the most effective of the tested solvents, with maximum activity (zone of inhibition) against all strains tested compared to the aqueous extracts. Similar results were observed by Djarmouni et al. [44], they also used different solvent extracts against a wide-range of microorganisms. The same results was also reported by Arif et al. [45], and the antimicrobial activity was tested on four different bacteria using a disc diffusion assay.

The results of MIC and MBC of *P. harmala* L. extracts are shown in Table 3. As can be noted in this finding, EtE showed a higher antibacterial activity against *S. thyiphimurium* and *M. luteus* with a MIC value of 1.5625 mg/mL. The results indicate that the CMB / MIC activity ratio is less than or equal to 4 for each given extract, when the CMB/MIC ratio of a substance is less than or equal to 4, this substance is

considered to be bactericidal, while it is said to be bacteriostatic if this ratio is greater than four [46]. These results states that all the extracts tested are bactericidal against the bacterial strains tested. With the exception of AqE appear to have a bacteriostatic effect on *Bacillus aureus* and bactericidal on *S. thyiphimurium* and *M. luteus*.

Antifungal susceptibility test
Fungi and yeasts may cause serious pathologies that affect humans, among which we can mention mycotoxicosis. The results obtained, show that after 48 h of incubation at 28 °C, all extracts inhibited fungal growth except that of *A. flavus*. The incubation time of fungal cultures treated with *P. harmala* L. extracts is calculated during a period of 10 days. During this period, it appears that the zones of inhibition produced by extracts against filamentous fungi tested remained clear during the 10 days of incubation. EtE manifested a strong activity by diameters of inhibition varying between 20 to 9 mm. However, the DcE and AqE only inhibited *A. carbonarius* during the entire incubation period by diameters of inhibition varying between 10 to 20 mm and 9 to 13 mm, respectively. On the other hand, absence of inhibition on the 6th day and for the entire remaining incubation period against *F. culmorum* and *P. glabrum* fungi who developed resistance against DcE and AqE with the disappearance of the inhibition zones due to sporulation of the fungus. It should be noted that the positive control (Rifampicin) is not active against all fungi tested with a dose of 5 μg/disc. Our results are in agreement with

Table 3. **The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values (mg/mL) of the *P. harmala* L. extracts against three human pathogen bacteria strains tested in microdilution assay**

Extracts	CMI and CMB	*B. cereus*	*S. thyiphimurium*	*M. luteus*
Ethanolic	CMI CMB CMB/CMI	3.125 6.25 2	1.5625 6.25 4	1.5625 1.5625 1
Aqueous	CMI CMB CMB/CMI	3.125 200 64	12.5 3.125 0.25	3.125 12.5 4
Decoction	CMI CMB CMB/CMI	3.125 12.5 4	12.5 12.5 1	12.5 12.5 1
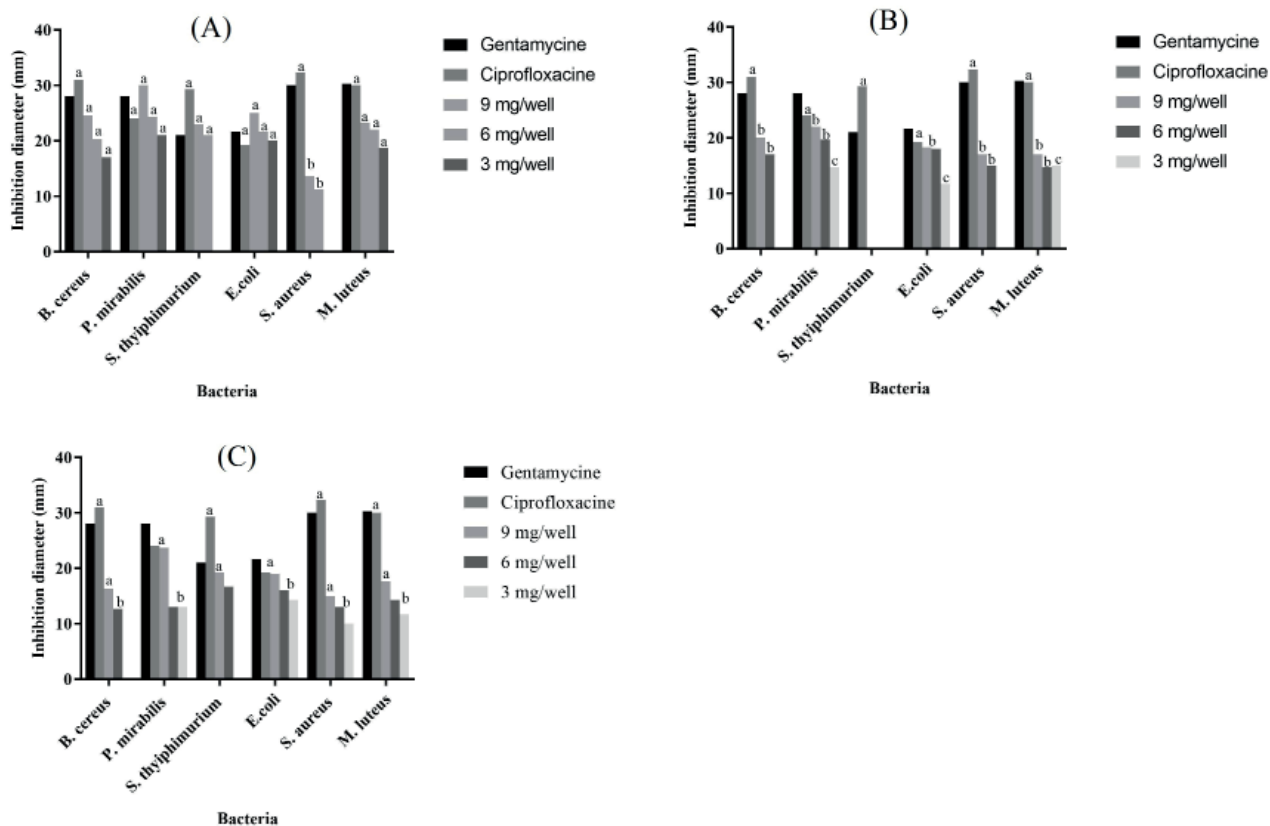
Fig 3. **Zone of inhibition of the *P. harmala* L. seeds extracts and control (ciprofloxacin, gentamycin) against six bacterial strains**
Values were expressed as means ± SD ($n = 3$). Analysis of variance (ANOVA) revealed significant effect ($P < 0.05$). Different letters (b, c and d) significant difference while 'a' indicate a non-significant difference ($P > 0.05$)

those of Diba et al. [47] and Guergour [41] who showed a greater effect of the alcoholic extract of *P. harmala* L. on the sporulation of the yeast Candida and most species of the genus *Aspergillus*. Decreases in inhibition diameter, by prolonging the incubation time, may indicate that the fungi have adapted to the constituents of the *P. harmala* L. extracts and began to develop resistance to the extracts.

Conclusion

In conclusion, *Peganum harmala* L. seeds were extracted with various solvents including water and ethanol. From the present work, it could be concluded that the solvent play an important role in the extraction of the plant constituents. *P. harmala* L. extracts were found to be rich in phenolic compounds and

flavonoids. In terms of antioxidant activity, it is concluded that *P. harmala* L. extracts possessed potent antioxidant activity and could be utilized as new natural antioxidant in food and therapeutics. The cytotoxic and antiproliferative effect show that the extracts have significant *in vitro* cytotoxic activity on HeLa cell line. The results of the present investigation suggest that *P. harmala* L. extracts have a significant antitumor activity, and could be an important basis for the design and synthesis of new antitumor drugs. This study showed that *P. harmala* L. may be a potential source of antimicrobial drug against the strains tested. This is particularly

important in the fight against the recent multi-resistant organisms with drugs.

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ЦИТОТОКСИЧНА, АНТИОКСИДАНТНА ТА АНТИМІКРОБНА ДІЯ ЕКСТРАКТІВ *Peganum harmala* L.

Dehiri Mounira[1], Diafat Abdelouahab[1], Fatmi Widad[2], Ben Mansour Riadh[3], Bouaziz Farid[1], Bahloul Ahmed[2]

[1]Laboratory of Characterization and Valorization of Natural Products, Faculty of Nature and Life Sciences, University El-Bachir El-Ibrahimi, Bord jBou Arreridj
[2]Laboratory of Health and Environment, Faculty of Nature and Life Sciences, University El-Bachir El-Ibrahimi, Bord jBou Arreridj
[3]Laboratory Analysis, Valuation of Food Securities, Biotechnology Research Group and Pathologies, National School of Engineering of Sfax (ENIS), Tunisia

Peganum harmala L., відома як «Harmel», є рослиною, що її широко використовують у традиційній алжирській медицині.

Мета. Вивчити антиоксидантний, антипроліферативний та антимікробний потенціал екстрактів пегану гармали.

Методи. Для кількісного визначення фенольних сполук використовували колориметричні методи, тоді як антиоксидантну активність оцінювали *in vitro* за допомогою аналізу поглинання радикалів DPPH/ABTS, відновлювальної сили заліза, аналізу відбілювання β-каротину, загальної антиоксидантної здатності та аналізу хелатування двовалентного заліза. Для оцінювання антибактеріальної активності використовували дифузію з лунки з агаром і метод мікророзведення бульйону, а для перевірки цитотоксичності екстрактів застосовувадт MTT-аналіз.

Результати. Етанольні екстракти *Peganum harmala* L. показали найвищий вміст поліфенолів і потужний антиоксидант, високу активність проти Грам-позитивних і Грам-негативних бактерій і хороший протигрибковий ефект і були більш цитотоксичними для лінії клітин HeLa.

Висновки. Обрані рослини можуть бути потенційним джерелом біоактивних сполук з антиоксидантним, антимікробним та антипроліферативним потенціалом. Таким чином, показано подальше дослідження цієї рослини *in vitro*, а також *in vivo* для відкриття нових ліків.

Ключові слова: Peganum harmala, поліфеноли, антиоксидантна активність, антимікробна дія, цитотоксичний ефект.

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

T. S. Todosiichuk[1]
O. V. Pokas[2]
S. O. Soloviov[3]
T. S. Ryzhkova[1]

[1]Igor Sikorsky Kyiv Polytechnic Institute, Kyiv
[2]SI "Gromashevsky Institute of Epidemiology and Infectious Diseases of NAMS of Ukraine", Kyiv
[3]Shupyk National Healthcare University of Ukraine, Kyiv

E-mail: todosiichuk.ts@gmail.com

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

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

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

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

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

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

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

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

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

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

Materials and Methods

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

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

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

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

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

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

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

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

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

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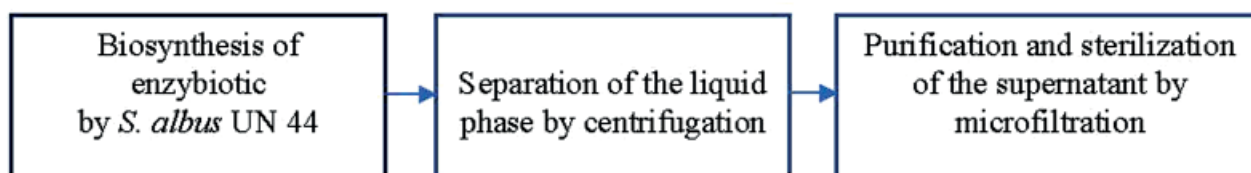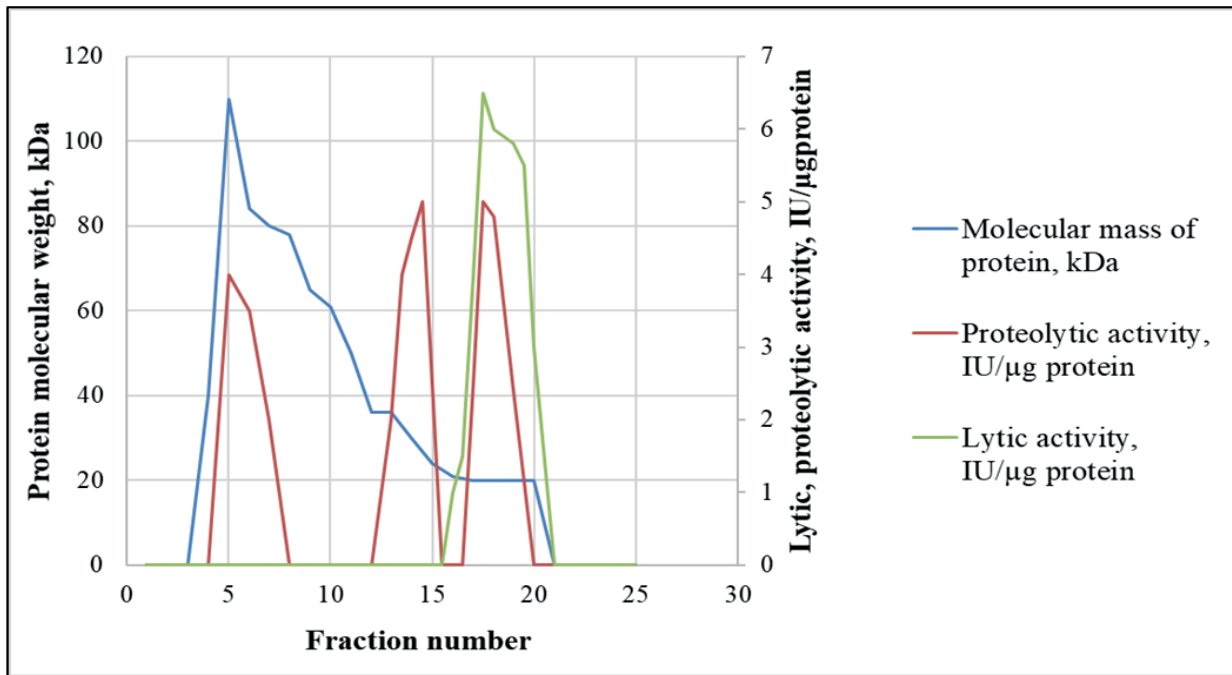

Fig. 1. **Stages of the producing the prototype liquid enzybiotic formulation from *S. albus* UN 44**

45.0 kDa

29.0 kDa

14.0 kDa

Markers   Fraction 13
Example of electrophore

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

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

Results and Discussion

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

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

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

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

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

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

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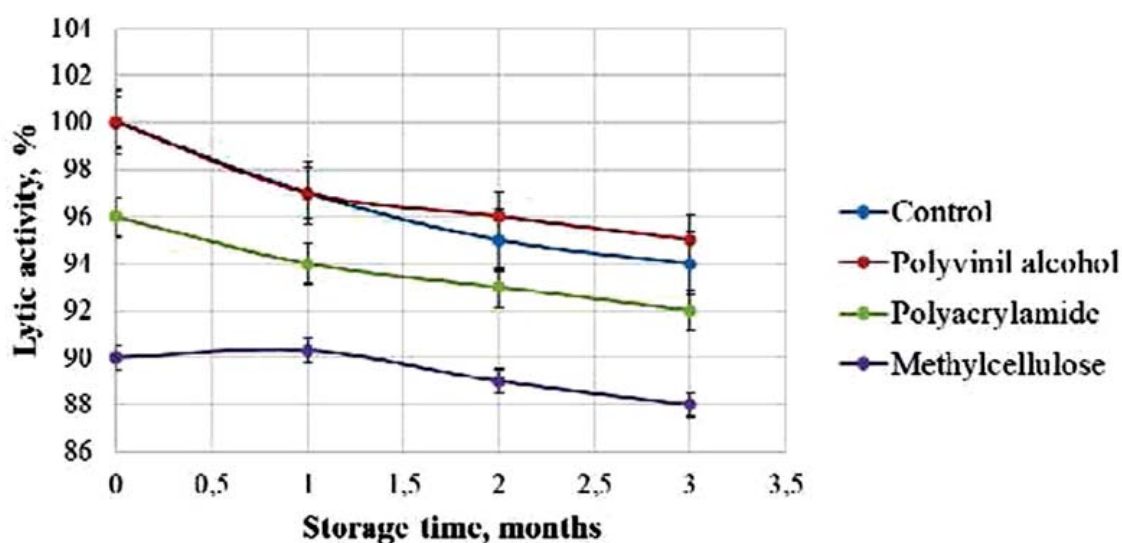

Fig. 3. **Dynamics of staphylolytic activity of liquid enzybioticformulations with various excipients**

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

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

The ability to destroy *P. aeruginosa* biofilm and prevent its formation had previously been shown by us for other formulations of the enzybioticproduced by the studied culture (dry native Cytorecifen and immobilized Cytorecifen-M) [22]. This was the basis for determining such activity of the prototype enzybioticliquid formulation, which also contains a complex of antibiotics, and has primarilyantifungal effects (therefore referred to as
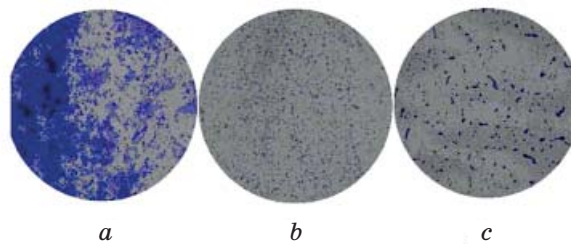
Fig. 4. **Effectof various enzybiotic formulations on the *P. aeruginosa* 278 biofilm formation:**
a — control; *b* — with Cytorecifen-M; *c* — with Streptofungin
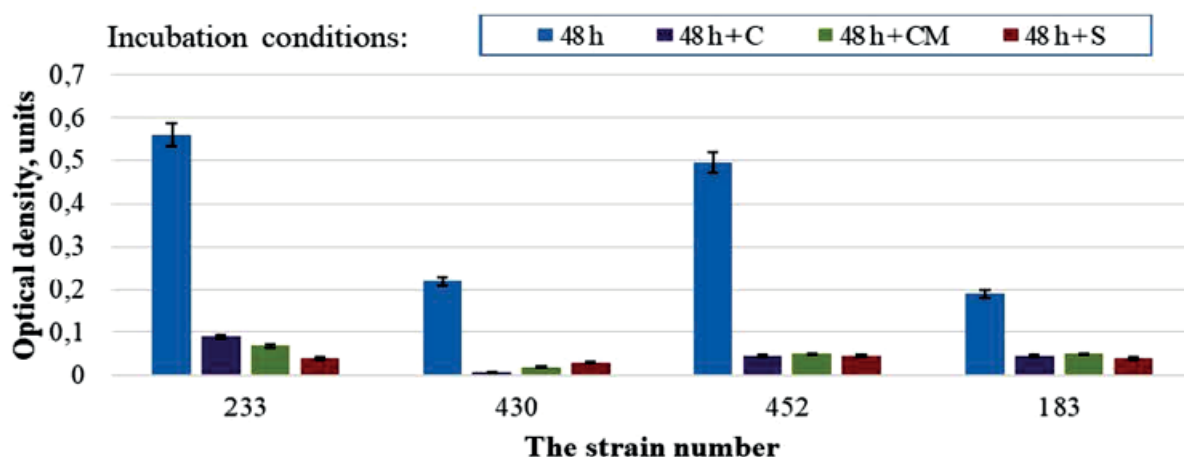
Fig. 5. **Quantitative assessment of the formation and destruction of biofilms formed
by different *P. aeruginosa* strains:**
for 48 h incubation without test enzybiotic formulations (48 h), and with addition of Cytorecifen (C),
Cytorecifen-M (CM), or Streptofungin (S) after 48 h and further incubation during 24 h

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

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

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

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

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

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

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

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

Conclusions

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

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

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БІОТЕХНОЛОГІЧНІ АСПЕКТИ РОЗРОБКИ РІДКОЇ ФОРМИ ПОЛІФУНКЦІОНАЛЬНОГО ПРЕПАРАТУ ЕНЗИБІОТИКА

Т. С. Тодосійчук[1], *О. В. Покас*[2], *С. О. Соловйов*[3], *Т. С. Рижкова*[1]

[1]КПІ ім. Ігоря Сікорського, Київ
[2]ДУ «Інститут епідеміології та інфекційних хвороб ім. Л. В. Громашевського НАМН України», Київ
[3]Національний університет охорони здоров'я України імені П. Л. Шупика, Київ

E-mail: todosiichuk.ts@gmail.com

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

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

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

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

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

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· names of all authors;
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· keywords (not more than ten).
In addition, an abstract should:
· explain how the study was done which includes any model and organisms that were used, without getting into methodological specifics;
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- citations;
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Experimental articles should have the following sections (headlines):
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- materials and methods,
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Electronic journal articles:
Hindorff, L. A. (all authors) A Catalog of Published Genome-Wide Association Studies. Available at http://www.genome.gov/gwastudies (accessed, September, 2012).
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Description of the electronic encyclopedia articles:
Containerization. (2008). In Encyclopedia Britanica. Retrieved May 6, 2008, from http://search. eb.com
Books:
Aralova N. I. Mathematical models of functional respiratory system for solving the applied problems in occupational medicine and sports. *Saarbrücken: LAP LAMBERT Academic Publishing GmbH&Co, KG.* 2019, 368 p. (In Russian). ISBN 978-613-4-97998-6

Extended abstract of dissertation:

Williams J. O. Narrow-band analyzer. Ph.D. dissertation, Dept. Elect. Eng., *Harvard Univ., Cambridge. MA,* 2010.

Dissertation:

Young R. F. Crossing boundaries in urban ecology: Pathways to sustainable cities (Doctoral dissertation). *Available from ProQuest Dissertations & Theses database.* 2007. (UMI No. 327681).

Encyclopaedia, dictionary:

Sadie S., Tyrrell J. (Eds.). The new Grove dictionary of music and musicians (2nd ed., Vols. 1 29). *New York, NY: Grove.*

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Wilkinson J. P. (all authors). Nonlinear resonant circuit devices. *U.S. Patent 3 624 125. July 16, 1990.* NOTE: Use "issued date" if several dates are given.

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Обсяг рукопису (у тому числі таблиці, список цитованої літератури, рисунки, підписи до рисунків) не повинен перевищувати: для оглядових статей — 30 сторінок (60 тис. знаків), для експериментальних — 20 стор (40 тис. знаків), для хроніки і рецензій — 5-6 стор; обсяг рисунків — 1/4 обсягу статті. Сторінки повинні мати суцільну нумерацію, включаючи таблиці, список літератури, підписи до рисунків, абстракт тощо. Таблиці, рисунки і підписи до них слід розміщувати в порядку згадування їх у тексті. Вони повинні мати заголовок і порядковий номер. Примітки до таблиць розміщуються безпосередньо під ними. Не можна дублювати одні й ті самі результати в таблицях і на рисунках. Якщо у статті використовуються ілюстрації, опубліковані іншими авторами, автор рукопису зобов'язаний надати редакції документальне підтвердження дозволу на використання цих ілюстрацій від власника авторських прав.

Допустимі формати зображень: *.tiff, *.bmp, *.jpg (300 dpi). Для оглядів і експериментальних статей допустимо не більше 6 рисунків; для коротких повідомлень не більше 4. Підписи до рисунків не включаються в сам рисунок і подаються окремо.

Для рівнянь використовуйте MathType або Microsoft Equation Editor 3.0 (рекомендований розмір шрифту 10,5, 8,5, 7,5, 14, 10).

Структура статті

При оформленні статей необхідно дотримуватися такої послідовності:
· Титульна сторінка;
· Структуровані резюме (трьома мовами);
· Текст статті
· Подяки;
· Дані про фінансову підтримку;
· Список літератури

Титульна сторінка:
· УДК;
· заголовок статті;
· автори: прізвища та ініціали всіх співавторів;
· назва та поштова адреса установи, в якій працює кожен автор;
· e-mail автора для листування.

Зоголовок статті має бути коротким (не більше 8–10 значущих слів), інформативним, містити ключові слова й точно відображати зміст статті. У перекладі назв статей на англійську мову не повинно бути ніяких транслітерацій з української або російської мов, крім назв власних імен, що не перекладаються, приладів, найменувань фірм, що мають власні назви. Це також стосується абстрактів і ключових слів.

Прізвища авторів статей подаються в одній з прийнятих міжнародних систем транслітерації. Потрібно до опису статті вносити всіх авторів. Якщо авторів декілька і вони працюють у різних установах, слід позначити арабськими цифрами (індексами) установи, в яких вони працюють.

Назва установи англійською мовою: всі значущі слова (крім артиклів і прийменників) повинні починатися з великої літери. Назви, що не перекладаються на англійську мову, даються в транслітерованому варіанті.

Структуроване резюме

Резюме об'ємом до 1500 знаків з пробілами містить:
· заголовки статті;
· прізвища та ініціали всіх співавторів;
· текст абстракту експериментальних статей повинен бути структурованим і обов'язково містити слова Мета, Методи, Результати, Висновки;
· ключові слова (не більше десяти).

Особливу увагу слід приділяти написанню резюме статті англійською мовою. Для цього доцільно користуватися послугами кваліфікованих фахівців-лінгвістів з подальшим науковим редагуванням тексту автором (авторами).

Текст статті

В експериментальних статтях мають бути такі розділи (заголовки): вступ (без назви); матеріали і методи; результати та обговорення; висновки.

У вступі обґрунтовується актуальність та формулюється мета дослідження.

Метод або методологію проведення роботи доцільно описувати в тому випадку, якщо вони відрізняються новизною або являють інтерес з точки зору цієї роботи. На загальновідомі методи досить дати посилання на публікації.

На загальновідомі методи достатньо дати посилання. Одиниці фізичних величин наводяться за Міжнародною системою СІ. Амінокислоти позначаються скорочено символами з трьох латинських букв або в однобуквеному форматі FASTA. Назви ензимів і їхні шифри наводяться згідно з рекомендаціями Міжнародного біохімічного товариства (Enzyme Nomenclature. — San DiegoNew York: Acad. Press., 1992; Supplement, 1993–2005. Інформація на сайті http://www. chem.qmul. ac.uk/iubmb/enzyme/). Необхідно наводити назви фірм-виробників реактивів і матеріалів, що використовувались у дослідах, з посиланням на країну. Обов'язково вказуються вид, кількість тварин і методи знеболювання та евтаназії.

Усі кількісні дані потребують статистичної обробки. Їхні результати слід подавати у відповідних таблицях і рисунках. Достовірність даних відзначається наступним чином: * — $P < 0,05$.

Латинські назви роду і виду організмів потрібно виділяти курсивом. При першому згадуванні наводиться повна видова назва (бажано із зазначенням автора таксонів), а при повторному – найменування роду можна привести скорочено однією буквою, якщо не йдеться про види, що відносяться до різних родів з однаковою першою літерою. Тоді використовують скорочення з декількох літер, наприклад *Staph. aureus*, *Str. lactis*. Латинські назви таксонів наводяться відповідно до сучасної систематики. Необхідно використовувати тільки загальноприйняті скорочення назв заходів, фізичних, хімічних і математичних величин і термінів, усі інші скорочення потрібно розшифровувати. Не варто наводити скорочення (крім загальновідомих) у таблицях і в підписах до рисунків.

Обговорення завершується формулюванням основного висновку, в якому потрібно дати конкретну відповідь на питання, поставлене у вступі. При використанні ілюстрацій з цитованих публікацій обов'язковим є не тільки посилання на джерело ілюстрації, а й дозвіл їх авторів на публікацію (зазвичай такий запит робиться Інтернетом). У кінці статті висловлюється подяка приватним особам, співробітникам установ і фондів, які сприяли проведенню досліджень і підготовці статті, а також вказуються джерела фінансування роботи.

Результати роботи слід описувати гранично точно та інформативно з приведенням основних фактичних даних, виявлених взаємозв'язків і закономірностей, уникаючи їх повторення у таблицях. Слід вказати межі точності й надійності даних, а також ступінь їх обґрунтування.

Висновки (без назви) можуть супроводжуватися рекомендаціями, оцінками, пропозиціями, описаними в статті.

Дані про фінансову підтримку

Дати коротку інформацію про джерело фінансування роботи, щоб уникнути конфлікту інтересів.

Список літератури

Література (References) наводиться в порядку цитування джерел у тексті і позначається цифрами у квадратних дужках. Використання гіперпосилань у списку літератури не допускається. Оскільки журнал прийнято для індексування міжнародними базами даних, список літератури подається до редакції двома окремими блоками:

Блок 1 (Література) — список літератури мовою оригіналу.

Блок 2 (References) – той самий список літератури, в якому повторюються посилання на іноземні джерела, а посилання, написані кирилицею, наводяться в романському (латинському) алфавіті. Прізвища авторів, назви журналів подаються згідно з однією з міжнародних систем транслітерації. Назви статей в посиланнях обов'язково перекладаються на англійську мову.

Автори несуть повну відповідальність за коректність наведення першоджерел. Допускається цитування тільки опублікованих і легко доступних для читачів матеріалів: статті, книги, матеріали конференцій, дисертації, патенти.

Рекомендації щодо складання посилань в романському алфавіті в англомовній частині статті та пристатейній бібліографії, призначеної для зарубіжних БД:

1. Слідувати правилам, що дозволяють легко ідентифікувати 2 основних елементи описів — авторів і джерело.

2. Не перевантажувати посилання транслітерацією назв статей, слід давати тільки їх переклад.

3. Додержуватися однієї з поширених систем транслітерації прізвищ авторів і назв джерел.

4. При посиланні на статті з російських журналів, що мають перекладну версію, краще давати посилання на перекладну версію статті.

На сайті http://www.translit.ru/ можна безкоштовно скористатися програмою транслітерації тексту, написаного кирилицею, в латиницю.

Приклади оформлення посилання у списку літератури

Для журналу «Biotechnologia Acta» рекомендується така структура бібліографічного посилання:

Статті

Загальний формат: прізвища та ініціали авторів (транслітерація, виділяється курсивом), переклад назви статті на англійську мову прямим шрифтом, крапка, (для іноземних джерел після назви статті англійською мовою в квадратних дужках наводиться назва статті мовою оригіналу тим самим шрифтом), крапка, назва джерела (транслітерація виділяється курсивом), ставиться крапка, послідовно вихідні дані статті — рік, том, номер (у дужках), сторінки, які відокремлюються комами. Обов'язково зазначається мова оригіналу.

Це посилання є зрозумілим і зручним для пошуку в базах даних. Це — найбільш повний варіант опису. У зарубіжній БД проста транслітерація заголовка статті без його перекладу на англійську мову не має сенсу.

Приклади:

Статті:

Aralova N. I., Klyuchko O. M., Mashkin V. I., Mashkina I. V., Semchyk T. A. Integrated mathematical model for imitation of the course of viral disease and correction of induced hypoxic stat. *Biotechnol. acta.* 2020, 13 (3), 30–39. https://doi.org/10.15407/biotech13.03.030

Приклад опису статті з електронного журналу

Hindorff L. A. (всі автори). A Catalog of Published Genome-Wide Association Studies. Available at http://www.genome.gov/gwastudies (accessed, September, 2012).

Матеріали конференцій

Riabinina A. A., Berezina E. V., Usoltseva N. V. Surface Tension and Lyotropic Mesomorphism in Systems Consisting of Nonionogenic Surfactant and Water. *Lyotropic Liquid Crystals and Nanomaterials: Proceedings of the Seventh Intern. Conf.* Ivanovo: Ivanovskii Gos. Univ., 2009. (In Russian).

Приклад опису статті з електронної енциклопедії

Containerization. In Encyclopedia Britanica. Retrieved May 6, 2008, from http://search.eb.com

Посилання на книгу:

прізвища та ініціали авторів (транслітерація, виділяється курсивом, крапка, пробіл), повна назва книги в перекладі на англійську мову прямим шрифтом, крапка, пробіл, прізвище відповідального редактора, назва видавництва (транслітерація, виділяється курсивом, наводиться без скорочень, крапка, пробіл), рік видання і номера першої та останньої сторінки цитованої роботи, мова видання.

Посилання на автореферати

Williams J. O. Narrow-band analyzer. Ph.D. dissertation, Dept. Elect. Eng., *Harvard Univ., Cambridge.* MA, 2010.

Дисертації

Young R. F. Crossing boundaries in urban ecology: Pathways to sustainable cities (Doctoral dissertation). Available from ProQuest Dissertations & Theses database. 2007. (UMI No. 327681).

Енциклопедії, словники:

Sadie S., Tyrrell J. (Eds.). The new Grove dictionary of music and musicians (2nd ed., Vols. 1–29). New York, NY: Grove.

Патенти

Загальний формат:

Автор (*усі автори*), Назва патенту. Держава. *Патент* x xxx xxx (номер). Місяць день, рік.

Приклад:

Wilkinson J. P. Nonlinear resonant circuit devices. U.S. Patent 3 624 125, July 16, 1990.

Короткі повідомлення

Журнал публікує менші за обсягом статті, які мають безумовну новизну і значущість для біотехнології. Ці статті проходять прискорене рецензування і публікуються в короткі терміни. Загальний обсяг короткого повідомлення обмежений 10 машинописними сторінками, кількість рисунків і/або таблиць — не більше 3, а список використаних літературних джерел не повинен перевищувати 15.

Розділи короткого повідомлення аналогічні розділам оригінальної статті, але не виділяються заголовками і підзаголовками; результати можуть бути викладені разом з обговоренням.

Доцільність такої позачергової публікації має бути обґрунтована в листі, що надсилається Головому редакторові автором для кореспонденції. У разі прийняття така робота може бути опублікована протягом 3–4 місяців.

Примітка

У зверстаному варіанті статті авторам можна виправляти тільки помічені помилки без внесення структурних змін і доповнень.

Редакційна рада має право відмовити у публікації рукописів, що містять раніше опубліковані дані, а також матеріали, які не відповідають фаху журналу, або матеріали досліджень, що були проведені з порушенням етичних норм.

Рукописи надсилайте до редакції за електронною адресою — *biotech@biochem.kiev.ua*
Тел. + 380 44 235 14 72.

Подаючи статтю до журналу, автор тим самим передає видавцеві (© Інституту біохімії ім. О. В. Палладіна НАН України) виключне право редагувати, перекладати іншою мовою, репродукувати та видавати роботу в обраному видавцем журналу вигляді й форматі.