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NON-TRADITIONAL PRODUCERS
OF MICROBIAL EXOPOLYSACCHARIDES

T. P. PIROG, A. A. VORONENKO, M. O. IVAKHNIUK

National University of Food Technologies, Kyiv, Ukraine

E-mail: tapirog@nuft.edu.ua

Data on exopolysaccharides synthesis by psychrophilic fungi and bacteriae, halo- and thermophilic archaea and bacteriae, including those isolated from deep-sea hydrothermal vents — sources — were provided. Physiologic significance, physico-chemical properties and possible practical applications of exopolysaccharides from unusual sources were analyzed. Most of them have immunomodulating, antiviral, anticoagulant, antitumor, antioxidant activities promising for medical and pharmaceutical applications.

Meanwhile, based on the literature date, the conclusion follows about the urgent necessity to develop efficient technologies for synthesis of these exopolysaccharides by nontraditional producers, which currently lags far behind common techniques.

Key words: exopolysaccharides, thermophiles, psychrophiles, halophiles, hydrothermal vents.

Microbial exopolysaccharides (EPS) are high molecular hydrocarbonic exogenous products of microbial metabolism [1−3]. They are widely used in industry (food production, chemistry, oil production, etc.) due to their ability to gel, emulsify, flocculate, form suspensions and to change rheological parameters of aqueous systems [3−5].

Most of currently known microbial EPS have similar functional properties that determine their practical significance [2, 4]. Thus, it is not surprising that only a few of many isolated, described and studied polysaccharides of microbial origin (xanthan, gellan, alginate, dextran) are produced industrially [1, 4].

A polysaccharide must now have unique properties to enter the free niches of rapidly developing fields like medicine, pharmacy, cosmetics, and nature conservation.

Since late XX century, scientists actively study microorganisms living in habitats previously overlooked in the search for bioactive compounds-producing microorganisms (permafrost, hot springs, oceanic depths, salt marshes, etc.). Quite possibly, they survive in such places due to specific adaptive mechanisms and synthesis of protective compounds [5], including EPS with new properties.

Such organisms are known as extremophiles, or microorganisms isolated from extreme habitats [6, 7]. We argue that the terms "extremophile" and "extreme" are not quite applicable, since microbiology considers "extreme" conditions in which only specialized microorganisms survive and many other taxa perish. Therefore this review refers to them simply as "nontraditional".

To date, a number of reviews have been published about synthesis of EPS by non-traditional producers [6−17]. However, the reviewers mostly paid attention to habitat description, physico-chemical properties and environmental significance of the synthesized polysaccharides and almost ignored the possibility of practical applications [13]. In addition, the reviews were devoted to a specific group of microorganisms (thermophilic [15], halophylic bacteriae [14, 16] and archaea [18], cryophilic yeast [19], sea microbes [9, 10, 17], and microorganisms isolated from hydrothermal vents [8]). Only a few papers reviewed several unusual producers at once [6, 7, 12]. The listed studies were published in 2010−2012 and include mostly summaries of specifics of EPS biosynthesis and their physico-chemical properties. A recent paper [11] discusses practical applications of several polysaccharides, synthesized by bacteriae isolated from hydrothermal sources.

This review aimed to summarize the available information on EPS synthesis by non-traditional producers (thermo-, cryo, halophilic microorganisms and bacteriae isolated from deep-sea hydrothermal vents), and properties of polysaccharides that support their potential practical application in medicine, pharmaceutical, food industries and nature conservation.

Thermophiles

The studies of thermophilic microorganisms started approximately in 1967 [20]. The paper briefly summarized the available knowledge about the microorganisms. In those days, attention was mostly paid to their environmental niche and the mechanisms enabling their survival at high temperatures.

One of those adaptive mechanisms is synthesis of microbial EPS. It should be noted that, unlike industrial mesophilic producers, using thermophils for the preparation of polysaccharides has a number of technological advantages, in particular, at elevated temperatures, the viscosity of the culture fluid and the possibility of the process infection are reduced, as well as mass exchange processes increase, etc. [21–25].

Archaea. The first reports of EPS synthesis by thermophilic archaea began to appear at the end of XX century [26–29]. In 1993, Nicolaus et al. [26] found out that the thermoacidophilic archaea *Sulfolobus solfataricus* MT4 and MT3, isolated from a hot acidic spring (Agnano volcanic crater, Italy) produced EPS at 75–88 °C.

The main disadvantage of those archaea as well as almost all other thermophilic producers of EPS is the low concentration of the target product (Table 1). This can be caused by low concentrations of the carbon and energy source (2–9 g/l) in the cultivation medium. Special attention was paid to the polysaccharide effect on physiology. Thus, several papers [27, 28, 30] presented data on synthesis of EPS by archaea linked to biofilm formation. Rinker et al. [27, 28] studied the growth of hyperthermophilic anaerobic organism *Thermococcus litoralis* DSM 5473. They established that biofilms formed on hydrophilic surfaces (polycarbonate filters) followed by accumulation of sulfated mannan (over 0.3 g/l EPS). Other researchers [30] studied the biofilm structure in thermoacidophilic archaea of the genus *Sulfolobus*.

Polysaccharides can perform other vital functions other than the formation of biofilms which protect the microorganisms from unfavorable factors and toxins. Thus,

a hypothesis was formulated [29] that EPS of thermophilic methanogenic archaea *Methanosarcina thermophila* TM-1 can be an osmoprotectant.

Notably, researchers [26–30] did not try to intensify EPS synthesis by thermophilic archaea. Due to the low concentration of the target product, this microorganisms are hardly going to be industrially important in the near future. Another complication is the difficulty of culturing most thermophilic archaea that require complex media with a lot of vitamins, amino acids, etc. [27, 28].

Bacteriae. Simultaneously with studying EPS-synthesizing archaea, researchers turned to thermophilic bacteriae. Almost all of them belong to the family *Bacillaceae* (the genera *Bacillus* [31–33], *Geobacillus* [22, 34–36], *Anoxybacillus* [37], *Aeribacillus* [24]) and *Paenibacillaceae* (the genus *Brevibacillus* [23, 25]) with optimal growth temperature of 45–65 °C (Table 1). Notably, the first reports of EPS synthesis by thermophilic bacteriae also included representatives of these families. Thus, Manca et al. [35] in 1996 reported isolation of extremely thermophilic bacteriae *Geobacillus thermoantarcticus*, which at 65 °C synthesized up to 400 mg/l sulfated EPS from soil near the crater of Melbourne volcano (Antarctica).

Besides representatives of *Bacillaceae* and *Paenibacillaceae*, synthesis of polysaccharides is known for hyperthermophilic bacteriae of the genus *Thermotoga* (optimal temperatures 80–85 °C) [27] and thermophiles of the genus *Thermus* (optimal temperature 60 °C) [38].

All thermophilic bacteriae in the literature produce less than 1 g/l EPS [22–25, 32, 33, 35, 36, 38]. Recently, bacteriae *Anoxybacillus* sp. R4-33, able to produce 1.1 g/l polysaccharide and tolerant of high temperature and radiation were isolated from geothermal radon springs (China) [37].

Thermophilic obligate methanotroph *Methylococcus thermophilus* 111п synthesizes up to 5 g/l EPS [2] and thus is a much better choice. Those amounts were achieved after a complex investigation of pH, temperature, diluted oxygen concentration, gaseous methane to oxygen ratio conditions, and the pre-treatment of the inoculum. The exogenous addition of 0.5 g/l aspartic acid (obtained by transferring amino group to oxaloacetic acid) to the culture medium of strain 111п was followed by an almost two-fold increase in the polysaccharide biosynthesis rate [2].

The EPS of several thermophilic and thermotolerant bacteriae were observed to

have antiviral [31, 34, 39] and immuno-modulating [38] activities and to inhibit biofilm formation [40].

Treatment of mononuclear cells of human peripheral blood with polysaccharide solutions (300 µg/ml) of strains *Geobacillus thermodenitrificans* B3-72, *Bacillus licheniformis* B3-15 and T14 stimulated the production of IFN-γ, IFN-α, TFN-α, IL-12 and IL-18 and inhibited the replication of herpes simplex virus type 2 [31, 34, 39]. In the presence of EPS of strains B3-72, T14 and B3-15 the virus was inhibited by 67, 77 and 85%, respectively [39]. Notably, antiviral activity is usually seen in sulfated polysaccharides [41], and the compounds described in [31, 34, 39] did not contain sulfate groups.

Lin et al. [38] isolated from the biofilm of *Thermus aquaticus* YT-1 a polysaccharide that heightened immune response. That EPS was observed to act as an agonist of TLR2 receptor and helped induce synthesis of cytokines IL-6, TNF-α, and nitrogen monoxide (NO) by murine macrophages and human monocytes. That immunoregulatory activity supposedly was caused by galactofuranose in its structure [38].

Several thermophilic representatives of the genus *Bacillus* were also observed to synthesize polysaccharides with anticytostatic activity [22, 33]. Fraction 1 EPS *B. licheniformis* T14, consisting of fructose, fucose and glucose (1:0.75:0.28), at 500 ppm raised LD_{50} of avarol (a cytostatic agent) from 0.18 to 0.99 mg/ml [33], and EPS of *Geobacillus tepidamans* V264 raised it to 2.24 mg/ml [22].

Recently Spanò et al. [40] found that EPS of *B. licheniformis* T14 at 400 µg/ml inhibited biofilm formation by multiresistant strains *Escherichia coli* 463, *Klebsiella pneumoniae* 2659, *Pseudomonas aeruginosa* 445 and *Staphylococcus aureus* 210 by 74, 56, 54 and 60%, respectively. The researchers suggested that due to the emulsifying properties of the polysaccharide it is able to impact the hydrophobicity of bacterial cells and so prevent their primary adhesion to surfaces [40].

A summary of EPS biosynthesis by thermophilic and thermotolerant microbes is given in Table 1. Currently, the microbes are not considered promising due to low EPS synthesis ability. Meanwhile such polysaccharides have properties important for medicine and pharmacy (antiviral, immunomodulating, anticytostatic, etc.),

which can stimulate work on intensifying their synthesis.

EPS-producing microbes from deep-sea hydrothermal vents. Deep-sea hydrothermal vents, characterized by high concentrations of toxic compounds (sulfides and heavy metals), sharp changes in temperature and pressure, are habitats of thermophilic bacteriae with various properties [8, 21, 42–44].

Since the first such vent was discovered in 1977 near the Galapagos, a great many other hydrothermal vents with various unique microorganisms were found [43, 44]. Thus, from the East-Pacific Rise (2600 m deep), EPS-synthesizing strains of bacteriae from the genera *Alteromonas* [45–48] and *Vibrio* [49] were isolated; at Mid-Atlantic Ridge (3500 m deep), bacteriae *Alteromonas macleodidi* subsp. *fijiensis* var. *medioatlantica* were found [50]; at Guaymas Basin and North Fiji Basin (2000 m deep), strains *A. macleodii* [43] and *Alteromonas infernus* [44] were isolated, respectively.

Despite the fact that these EPS-producing bacteriae were isolated from extreme habitats, most of them turned out to be mesophilic neutrophils with optimal growth temperature 25–35 °C and pH 6–8 [43–45, 46–50], and only a few of them were thermophiles (40–45 °C) [49].

The EPS-producing bacteriae isolated from deep-sea hydrothermal vents became a subject of active research in 1990s [42–44, 47–49]. In 1994, Guezennec et al. [42] published results of screening EPS-producing bacteriae isolated from hydrothermal vents. Almost all polysaccharides except for neutral monosaccharides contained sulfate moieties (to 21.5%) and glucuronic acids (to 7.9%), several had amino sugars (to 2.5%).

Interestingly, EPS-producing bacteriae are isolated not only from soil or water near hydrothermal vents [42], but from the surfaces of various organisms living there (shrimps, worms, etc. [45, 46, 48–50]). The strain *Alteromonas macleodii* subsp. *fijiensis* var. *medioatlantica* MS907, producing 9 g/l EPS after 72 hours of culturing was found on carapax of the shrimp *Rimicaris exoculata* [50].

The outer shell of a sea polychaete *Alvinella pompejana* (at the depth of 2600 m) yielded EPS-synthesizing bacteriae *Alteromonas* sp. HYD1545 and *A. macleodii* subsp. *fijiensis* biovar deepsane HYD657 [45, 48]. The strain HYD1545 after 120 hours of culturing produced 11 g/l of polysaccharide [48], and strain HYD657 produced 7 g/l EPS after

Table 1. **Synthesis of exopolysaccharides by thermophilic and thermotolerant microorganisms**

Microorganism	Culture temperature	Carbon source, g/l	EPS concentration, g/l	Physico-chemical properties of EPS		Physiological role, functional properties and prospects of EPS application	References
				content	Molecular mass, kDa		
EPS of thermophilic archaea							
Methanosarcina thermophila TM-1	45–55 °C	Trimethylamine, 4.8	–	Glucuronic acid (over 40%)	–	Osmoprotectant	[29]
Sulfolobus acidocaldarius	76 °C	–	–	Glucose, galactose, mannose, *N*-acetylglucosamine	–	Biofilm formation	[30]
Sulfolobus solfataricus MT3	75 °C	Glucose, 3	7.0 mg/l	Glucose, mannose, glucosamine, galactose (1.2:1.0:0.77:0.73). Sulfates 5–12%	–	–	[26]
Sulfolobus solfataricus MT4	88 °C	Glucose, 3	8.4 mg/l	Glucose, mannose, glucosamine, galactose (1.2:1.0:0.18:0.13). Sulfates 5–12%	–	–	[26]
Sulfolobus tokodaii	76 °C	–	–	Glucose, galactose, mannose, *N*-acetylglucosamine	–	Biofilm formation	[30]
Thermococcus litoralis DSM 5473	88 °C	Maltose, 2	0.18–0.32	Mannan, sulfates 1-2%	41	Biofilm formation	[27, 28]
EPS of thermophilic and thermotolerant bacteriae							
Aeribacillus pallidus 418	55 °C	Maltose, 9	0.17	Fraction 1: mannose, glucose, galactosamine, glucosamine, galactose, ribose (1:0.16:0.1:0.09:0.07:0.06:0.04) Fraction 2: mannose, galactose, glucose, galactosamine, glucosamine, ribose, arabinose (1:0.5:0.46:0.35:0.24:0.16:0.14)	Fraction 1: 700; Fraction 2: 1000	Emulgent	[24]
Anoxybacillus sp. R4-33	55 °C	Glucose, 10	1.1	Fraction 2: mannose, glucose (1:0.45)	1000	Adsorbs heavy metals	[37]
Bacillus licheniformis B3-15	45 °C	Glucose, 6	0.165	Fraction 1: mannose, glucose (1:0.3); Fraction 2: mannose; Fraction 3: glucose	600	Antiviral and immunomodulatory	[31, 32]

Table 1. Continued

Microorganism	Culture temperature	Carbon source, g/l	EPS concentration, g/l	Physico-chemical properties of EPS		Physiological role, functional properties and prospects of EPS application	References
Bacillus licheniformis T14	50 °C	Sucrose, 50	0.366	Fraction 1: fructose, fucose, glucose and traces of galactosamine, mannose (1:0.75:0.28:traces:traces)	1000	Antiviral, immunomodulatory and anticytotoxic. Inhibits biofilm formation	[33, 39, 40]
Brevibacillus thermoruber 423	55 °C	Maltose, 18	0.897	Glucose, galactose, galactosamine, mannose, mannosamine (1:0.3:0.25:0.16:0.04)	–	–	[25]
Brevibacillus thermoruber 438	55 °C	Maltose, 18	78.1 mg/l	–	–	–	[23]
Geobacillus tepidamans V264	60 °C	Maltose, 30	111.4 mg/l	Glucose, galactose, fucose, fructose (1:0.07:0.04:0.02)	>1000	Anticytotoxic	[22]
Geobacillus thermoantarcticus	65 °C	Mannose, 6	0.4	Fraction 1: mannose, glucose (1:0.7); Fraction 2: mannose and traces of glucose Sulfated	Fraction 1: 300; Fraction 2: 300	Emulgent	[35]
Geobacillus thermodenitrificans B3-72	65 °C	Sucrose, 6	70 mg/l	Fraction 1: glucose, mannose (1:0.3); Fraction 2: mannose, glucose (1:0.2)	Fraction 2: 400	Fraction 2: antiviral and immunomodulating	[34, 36]
Methylococcus thermophilus 111п	40 °C	Methan	5	Fraction 1: mannose, galactose, glucose, fucose, xylose, rhamnose, glucuronic acid. Fraction 2: mannose, glucose, xylose, rhamnose	–	Intensification of oil production	[2]
Thermotoga maritima DSM 3109	88 °C	Maltose, 2	0.120	Glucose, ribose, mannose (1:0.06:0.03)	–	Flocculant	[27]
Thermus aquaticus YT-1	60 °C	–	–	Galactofuranose, galactopyranose, *N*-acetylglucosamine (1:1:2)	500	Immunomodulatory activity; adjuvant to vaccines	[38]

Note: «–» — no data available.

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52 hours of culture [45]. Further research [51] of EPS of strain HYD657 established that they efficiently protect keratinocytes from inflammation agents. The protective effect was also found towards Langerhans cells, which are sensitive to the ultraviolet and play an important role in the system of human skin immune protection. Nowadays, cosmetic preparation Abyssine® was developed based on the polysaccharide (deepsane). It is recommended for soothing and protection against irritation of sensitive skin [52].

Notably, the polysaccharide of strain HYD657 has an unusual component, a residue of 3-O-(1-carboxyethyl)-D-glucuronic acid [45]. Currently, the compound was also found in EPS of the strain *Alteromonas* sp. HYD1644, isolated from the epidermis of the polychaete *Alvinella caudata* [46], and in drought-resistant cyanobacteriae *Nostoc commune* DRH-1 [53]. Helm et al. [53] suggested that this and other uronic acids with carboxyethyl moieties play a key part in providing survival in unfavorable conditions. For example, such functional groups can help EPS attach to adjacent chains of the polymer, organic (biofilms) or inorganic surfaces, etc.

The strain *Vibrio diabolicus* HE800[T] was isolated from polychaete *Alvinella pompejana*. The strain produces a polysaccharide similar to hyaluronic acid [49]. The EPS is made up equally from glucuronic acid and hexosamines (*N*-acetylglucosamine and *N*-acetylgalactosamine) [54]. Treating damaged skullcap skin of Wistar rats with the EPS made the wound close sooner, while the trabecular and cortical anatomic structure of the defect fully recovered [55]. Zanchetta et al. [55, 56] suppose that the effect is caused by the ability of EPS to form extracellular matrix that helps direct adhesion of osteoblasts and pericytes, generally protect the damaged site while it heals, and to bind calcium.

Senni et al. [57] suggested that glycoso-aminoglycan polysaccharide of strain HE800[T] is a promising agent for various derivatives (heparan sulfate, chondroitin sulfate, etc.). Such depolymerization of native polysaccharide to molecular mass of 22 kDa with further deacetylation and sulfation (sulfate content 34%) resulted in a polymer similar to heparan sulfate. Those derivatives were observed to stimulate proliferation of dermal and gingival fibroblasts and inhibit secretion of matrix metalloproteinases [57].

The EPS of *Alteromonas infernus* GY785 after sulfation (sulfate content 40%) and controlled depolymerization by free radicals to molecular mass of 24 kDa substantially raised APTT (activated partial thromboplastine time) [58, 59]. The anticoagulant activity of the polysaccharide was on the level of calcium pentosan polysulfate though 2.5–6.5 times lower compared to heparin [58]. Notably, due to the low sulfate content in the native polysaccharide (5.5–10%) it did not have anticoagulant activity [58].

Recently the effect of depolymerized EPS of strains *V. diabolicus* HE800[T] and *A. infernus* GY785 on the complement system was studies [60]. The low molecular (2.9 kDa) derivative of the polysaccharide of strain HE800[T] to a large extent activated the system (60% activation at 50 µg EPS), while the depolymerized (molecular mass 23 kDa) and sulfated (sulfate content 37–42%) EPS of strain GY785, conversely, caused its significant inhibition (78% inhibition at 10 µg EPS). Due to those properties, the polysaccharides are promising for treating diseases caused by deregulation of immune system and over activation of the complement system.

Therefore, EPS of bacteriae isolated from hydrothermal vents can become widely accepted into medical, pharmaceutical and cosmetic industries due to anticoagulant, protectant, immunomodulatory and regenerative activities. Notably, such microorganisms can synthesize up to 11 g/l of the product, and some polysaccharides from hydrothermal-dwelling bacteriae are already mass-produced. For example, EPS of *A. macleodii* subsp. *fijiensis* biovar deepsane HYD657 is used for cosmetics (Abyssine®).

Data on EPS of bacteriae isolated from hydroterms are summarized in Table 2.

Psychrophiles

Cold environments are found from deep seas to snow-laden mountaintops, from Arctic to Antarctica. Temperature of almost 75–80% of the Earth surface is below 5 °C [60–62]. Cold habitats are characterized by frequent sharp changes in temperature (cycles of freezing and thawing, etc.), UV-radiation, nutrient concentration [63, 64]. Oceanic and sea waters also have pressure and salinity oscillations [21]. Evidently, microorganisms would not survive in such conditions without relevant adaptive mechanisms [62, 65, 66].

EPS play a large role in it. Exopolymers, including polysaccharides, take part in

Table 2. Exopolysaccharide synthesis by bacteriae isolated from deep-sea hydrothermal vents

Microbial source*	Carbon source, g/l	EPS content, g/l	Physico-chemical properties of EPS		Physiological effect, functional properties and possible implementations of the EPS	References
			Chemical composition	Molecular mass, kDa		
Alteromonas infernus GY785	Glucose, 30	Fraction 1: 5.5 Fraction 2: 4.3	Fraction 1 (water-soluble): glucose, galactose, glucuronic and galacturonic acid (1.0:0.9:0.7:0.4). Sulfates 5.5–11%	Fraction 1: 1000	Anticoagulant, adsorbent	[44, 58, 59]
Alteromonas macleodii subsp. *fijiensis* ST716	Glucose, 30	6	Galactose, glucose, mannose, glucuronic and galacturonic acid (1.0:0.95:0.4:1.1:0.57). Sulfates 5%	330	Thickener	[43]
Alteromonas macleodii subsp. *fijiensis* biovar deepsane HYD657	Glucose, 30	7	Galactose, glucose, rhamnose, fucose, mannose, glucuronic, galacturonic and 3-O-(1-carboxyethyl)-D-glucuronic acids (1.0:0.43:0.8 6:0.5:0.43:0.5:0.5:0.5). Sulfates 7.5%	1100–1600	Protects keratinocytes and Langerhans cells from inflammation agents	[45, 51]
Alteromonas macleodii subsp. *fijiensis* var. *medioatlantica* MS907	Glucose, 30	9	Galactose, glucose, glucuronic and galacturonic acids (1.0:0.5:0.7:0.26)	1500	Thickener	[50]
Alteromonas sp. HYD1545	Glucose, 30	11	Glucose, galactose, mannose, glucuronic and galacturonic acids (1.0:0.55:0.04:0.24:0.14)	1800	–	[48]
Alteromonas sp. HYD1644	Fructose, 40	Fraction 1: 7.5 Fraction 2: 5.0	Fraction 1 (water-soluble): galactose, glucose, rhamnose, mannose, glucuronic, galacturonic acids and 3-O-(1-carboxyethyl)-D-glucuronic acids (1.0:0.74:0.7:0.13:0.4:0.19:0.23)	Fraction 1: 5000	Thickener	[46, 47]
Vibrio diabolicus HE800^T	Glucose, 40	2.5	Glucuronic acid, N-acetylglucosamine, N-acetylgalactosamine (1:0.5:0.5)	800–850	Raw material to obtain glycosaminoglycan derivatives. Fastens bone fusion	[49, 54–57]

aggregation, adhesion to surfaces and other microorganisms, biofilm formation, nutrient storage, etc. in marine bacterial communities [66–68]. Often aggregates of salty drops remain unfrozen after the sea water freezes, and the microbes are trapped in salt canals [63, 66]. Then, EPS are cryoprotectants and protectants from high salinity [62, 65, 66].

The majority of microorganisms, able to survive at low temperature, are yeasts and bacteriae [8]. Notably, phylogenetic research also registers a lot of representatives of *Archaea* [61], although they have not been cultured.

Fungi. EPS synthesis by fungi at relatively low temperatures is a novel approach. The first report of polysaccharide production by cryotolerant mycelial fungi appeared only at the beginning of XXI century. In 2002, Selbmann et al. [69] established the ability of *Phoma herbarum* CCFEE 5080 cultured on medium containing sorbitol (60 g/l) to produce 13.4 g/l 7412 kDa glucan. Due to cryoprotectant properties of the polysaccharide, strain CCFEE 5080 is able to grow at 0–5 °C (optimal temperature 28 °C) [70].

Another glucan-producing fungus is strain *Thelebolus* sp. IITKGP-BT12 [68]. Unlike the strain CCFEE 5080, at 18 °C it synthesizes only 1.94 g/l EPS. Experiments have shown that the glucan has significant antiproliferative effect on cells of skin cancer in B16-F0 mice. IC_{50} (the concentration at which maximal inhibition occurred) of the EPS was 275.4 µg/ml. The polysaccharide had almost no effect on normal fibroblasts of the L929 line (at the concentration of 187.5–1500 µg/ml cytotoxicity was almost absent) [67].

Recently, isolation of EPS-synthesizing cryotolerant yeasts of the genera *Sporobolomyces* [71] and *Cryptococcus* [72–74] was reported from Livingstone Island. Cultivation in medium with sucrose (40–50 g/l) and ammonium sulfate (0.25%) at 22–24 °C resulted in 4.6–6.4 g/l of polysaccharides (Table 3).

Research of economically valuable properties of EPS of yeasts from the Livingstone Island confirmed their possible use in cosmetics, food industry [73, 75, 76] and medicine [78]. EPS of strain *Cryptococcus laurentii* AL_{100} exhibited high emulgent activity, significantly enhanced by other polysaccharides (xanthan, guar gum, cellulose, etc.) [73].

Other researchers showed that cosmetic emulsions with 2% EPS *Sporobolomyces salmonicolor* AL_1 remained stable for a month at −10 °C and for 3 months at 22 and 45 °C [75, 76]. To achieve similar results, concentration of synthetic emulgent Arlacel 165 or Rofetan N/NS was 5% [75]. Besides that, EPS of *S. salmonicolor* AL_1 has anticytostatic activity. At 5 ppm it changed LD_{50} of (cytostatic) avarol from 0.18 to 0.10 ppm [77].

EPS of cryotolerant fungi can be used as emulgents and thickeners in food and cosmetic practices at low temperatures. They are promising for medicine and pharmacy due to antitumor and anticytostatic activities.

Bacteriae. Reports of EPS synthesis by cryophilic and cryotolerant bacteriae started shortly after the first study about polysaccharides of cryotolerant fungi [69].

Polysaccharides of cryotolerant bacteriae isolated from free ice and marine aggregates in the Antarctic ocean, with *in situ* temperature of 4 °C were described in 2005 [78]. Six of the studied isolates belonged to the genus *Pseudoalteromonas*, three to the genera *Shewanella*, *Polaribacter*, and *Flavobacterium*. A strain $CAM030^T$ represented the family *Flavobacteriaceae*, later it became a new taxon *Olleya marilimosa* [79]. Most cryophilic bacterial producers isolated after 2005 belong to the genera *Pseudoalteromonas*, *Polaribacter* and *Flavobacterium* (Table 3).

By their monosaccharide content, the polysaccharides of cryophilic bacteriae are similar to EPS of marine bacteriae (Table 2).

Lowering the growth temperature from 20 to 10, or to −2 °C caused an almost 30-fold rise in EPS-producing ability of strain *Pseudoalteromonas* sp. CAM025 (up to 99.9 and 97.2 mg EPS/g biomass, respectively), and a changed monosaccharide ratio [80].

Cryoprotectant properties of EPS of *Pseudoaltermonas* sp. SM20310 were studied in [63]. At 30 mg/ml EPS the number of living cells of strain SM20310 and *E. coli* DH5α was 7 to 18 times as high as in the control group (without EPS) after three cycles of freezing-thawing. Other researchers [68] report that adding the polysaccharide of cryotolerant bacteriae *Flavobacterium* sp. ASB 3–3 at 50 mg/ml led to a four times increase in the number of living cells of strains ASB 3–3 and *E. coli* DH5α after two cycles of freezing-thawing compared to the cultures without EPS.

Cryotolerant bacteriae *Pseudoalteromonas elyakovii* ArcPo 15 isolated from Chukchi Sea were observed to synthesize 1.7 MDa EPS with high cryoprotectant activity [81]. Adding the

EPS (0.5%) to a suspension of *E. coli* DH5α resulted in 94.2% survival of the cells after five cycles of freezing-thawing. Adding 20% glycerin resulted in 54.1% survival of the cells.

Due to the cryoprotectant ability of bacterial EPS we suggest using them as alternative cryoprotectant agents for long-term storage of suspended cultures [82, 83].

According to Carrión et al. at 10% EPS of *Pseudomonas* sp. ID1, survival of *E. coli* ATCC 10536 after freezing and storing for seven days at −20 and −80 °C was 36 and 64%, respectively [82]. Cell survival decreased at lower EPS concentrations. After similar freezing of EPS-synthesizing strain ID1, the cell survival rates were 75 and 94%, respectively. Another study [84] showed that EPS of cryophilic *Colwellia psychrerythraea* 34H are a better cryoprotectant agent for freezing cells at −80 °C than 10% glycerin solution.

Notably, cryoprotectant properties of polysaccharides are not limited to merely the protection of microbial cells. Sun et al. [84] reported that, survival rate of human dermal fibroblasts after 20 hours at 4 °C reached 76.1% with 500 µg/mg EPS of *Polaribacter* sp. SM1127, while without the polysaccharide it was only 44.2%.

In the native environment, other physico-chemical factors besides temperature can induce EPS synthesis, such as pressure and salinity [63, 83]. For example, culturing *C. psychrerythraea* 34H at high hydrostatic pressure (up to 400 atm) resulted in EPS content increasing 4.5−7.5 times.

Polysaccharides of cryophilic and cryotolerant bacteriae can also hold moisture [84, 85], emulsify [82, 68, 86], flocculate [68, 86] and adsorb metal [86, 87].

Research of EPS of bacterial strains *Polaribacter* sp. SM1127 and *Zunongwangia profunda* SM-A87 [84, 85] showed that after 72 hours of incubation with silica gel (relative humidity 43%) the polysaccharide of strain SM1127 retained 76% moisture, which is higher than for hyaluronic acid, glycerin, sodium alginate. This is possibly due to not only a lot of glucuronic acid and *N*-acetylglucosamine (components of hyaluronic acid), but also fucose, which has moisturizing properties, in EPS [84]. The polysaccharides also have antioxidant activity [84, 85]. Thus, the level of neutralization of 2,2-diphenyl-1-picrylhydrazyl radical radical (DPPH·), hydroxyl radical (·OH) and superoxide anion

$(O_2 \cdot)$ at 10 mg/ml of EPS of SM1127 and SM-A87 10, was 27.2−55.4%. Further research [87] established the ability of EPS of strain SM-A87 to adsorb Cu^{2+} and Cd^{2+} (48 and 39.75 mg/g EPS, respectively).

After optimization of the culture medium [88] in the fed-batch culture [85], the concentration of EPS of strain *Z. profunda* SM-A87 increased to 17 g/l, which is 1.93 times higher compared to the initial.

Recently Sathiyanarayanan et al. [68, 86] isolated cryotolerant *Flavobacterium* sp. ASB 3−3 and *Pseudomonas* sp. PAMC 28620 (AS-06/29) from the soil of Svalbard Arctic glacier fore-field. The optimal carbon and energy source for those bacteriae, unlike other microbial sources of EPS (Table 3) is glycerin. At the medium with 30 g/l of this substrate, the bacteriae produced 7.25 g/l EPS with flocculant and emulgent properties.

In kaolinite suspension (0.5%), flocculant activity of 40 mg/l EPS for strains PAMC 28620 and ASB 3-3 70 was 71.2 and 91.3%, respectively [68, 86]. The polysaccharide of strain ASB 3-3 emulsified *n*-hexane (emulsification index 66.3%) and *n*-hexadecane (64.3%) just as efficiently as sodium dodecyl sulfate [68]. EPS of strain PAMC 28620 efficiently emulsified toluene (67.2%) and methyl octanoate (66.7%) [86]. Besides that, polysaccharide of strain PAMC 28620 expediently adsorbed Fe^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+} (approximately 99%), and Mn^{2+}, Ca^{2+} (92%) [86].

Unlike thermophilic and thermotolerant sources (Table 1 and Table 2), cryophilic and cryotolerant microorganisms synthesize more EPS (up to 17 g/l; Table 3), and their polysaccharides have cryoprotectant, emulsifying properties, retain moisture and adsorb heavy metals. That, consequently, makes the polysaccharides potentially attractive for various fields from food industry (foodstuffs storage) and cosmetics (production of protective cosmetics) to environment-friendly technology (purification of waste waters).

Halophiles

Halophiles are organisms able to survive in briny habitats, whose development requires salt. The salt in question is generally NaCl, while many researchers in their experiments on halophilic cultures use sea salt which contains not only NaCl but also comparatively small amounts of other salts of two- and monovalent metals [89].

Table 3. EPS synthesis by cryophilic and cryotolerant microorganisms

Microbial source	Incubation temperature	Carbon source, g/l	EPS concentration, g/l	Chemical composition	Molecular mass, kDa	Physiological effect, functional properties and possible avenues of implementation of EPS	References
EPS of cryotolerant fungi							
Cryptococcus flavus AL_{51}	24 °C	Sucrose, 50	5.75	Mannose, glucose, xylose, galactose (1:0.47:0.17:0.03:0.08)	1010	–	[72]
Cryptococcus laurentii AL_{62}	22 °C	Sucrose, 40/50	4.73/4.6	Xylose, mannose, glucose (1:0.74:0.41)	8	–	[74]
Cryptococcus laurentii AL_{100}	22 °C	Sucrose, 40	6.4	Arabinose, mannose, glucose, galactose, rhamnose (1:0.25:0.2:0.1:0.05)	4.2	Emulgent	[73]
Phoma herbarum CCFEE 5080	28 °C	Sorbitol, 60	13.4	Glucan (glucose 100%)	7412	Cryoprotectant	[69]
Sporobolomyces salmonicolor AL_1	22 °C	Sucrose, 50	5.2–5.6	Mannose, glucose, galactose (1:0.1:0.08)	>1000	Thickener, emulgent	[71, 75–77]
Thelebolus sp. IITKGP-BT12	18 °C	Glucose, 50	1.94	Glucan (glucose 100%)	500	Antiproliferative activity	[67]
EPS of cryophilic and cryotolerant bacteriae							
Flavobacterium sp. ASB 3-3	25 °C	Glycerin, 30	7.25	Glucose, galactose (1:0.43). Sulfates were found	–	Emulgent, flocculant, cryoprotectant	[68]
Polaribacter sp. SM1127	15 °C	Glucose, 30	2.11	N-acetylglucosamine, mannose, glucuronic acid, galactose, fucose, glucose, rhamnose (1:0.84:0.76:0.62:0.26:0.06:0.03)	220	Cryoprotectant, moisture-retention agent, antioxidant	[84]
Pseudoalteromonas elyakovii ArcPo 15	15 °C	Glucose, 20	1.64	Mannose, galacturonic acid (3.3:1.0)	17000	Cryoprotectant	[81]
Pseudoalteromonas sp. CAM025	10 °C	Glucose, 30	99.9 mg/g biomass	Glucose, galactose, rhamnose, mannose, fucose, arabinose, ribose, glucuronic acid (1:0.64:0.61:0.31:0.25:0.12:0.05:0.26). Sulfates 5%	5700	Cryoprotectant	[80]

Table 3. End

Microbial source	Incubation temperature	Carbon source, g/l	EPS concentration, g/l	Physico-chemical properties of EPS		Physiological effect, functional properties and possible avenues of implementation of EPS	References
				Chemical composition	Molecular mass, kDa		
Pseudomonas sp. ID1	11 °C	Glucose, 20	–	Glucose, galactose, fucose (1:0.5:0.48), uronic acids are present	2000	Cryoprotectant, emulgent	[82]
Pseudomonas sp. PAMC 28620	25 °C	Glycerine, 30	7.24	Rhamnose, galactose, glucose, fucose, mannose, ribose (1:0.32:0.25:0.07:0.07:0.03), sulfates detected	–	Emulgent, flocculant, adsorbent	[86]
Pseudoaltermonas sp. SM20310	15 °C	Glucose, 30	0.567	Mannose, glucose, galactose, rhamnose, xylose, N-acetylglucosamine and N-acetylgalactosamine (1:0.15:0.13:0.03:0.01:0.06:0.02)	2000	Cryo- and osmoprotectant	[63]
Zunongwangia profunda SM-A87	9.8 °C	Whey (60.9%, v/v)	12–17.2	Glucose, mannose, galactose, xylose, fucose, glucuronic acid, not indentified carbohydrate (1:0.84:0.29:0.29:0.05:0.06:0.21)	3760	Moisture-retention agent, antioxidant, adsorbent	[85, 87]

As to salinity, halophiles can be halotolerant (upper salinity limit 15%), weak (NaCl content of 2–5%), moderate (5–5%) and extreme halophiles (20–30%) [16].

Usually, they can be found in various saline habitats such as salt lakes, salt evaporation ponds, saline soils, mines, food products, etc. [21, 90]. Traditional halophilic sources are salterns, which usually have high salt content, intensive sunlight and low oxygen levels [90–95].

Archaea. Main papers on polysaccharide synthesis by halophilic archaea include research on isolation of new producers [94, 96], EPS structure [97–99], and the possibilities of their practical application [96].

In 1988, Antón et al. [96] established that extremely halophilic archaea *Haloferax mediterranei* ATCC 33500 cultured on a medium with 1% glucose and 25% sea salt produced 3 g/l of sulfated high molecular polysaccharide. Viscosity of EPS solutions was stable in wide ranges of pH, temperature and salinity. Hence EPS of strain ATCC 33500 can be utilized in increasing oil production from wells with high salt content. Later, researchers established the structure of repeating sequences of EPS strain ATCC 33500 [98] and other EPS-synthesizing archaea, in particular *Haloferax gibbonsii* ATCC 33959 [97] and *Haloferax denitrificans* ATCC 35960 [99].

At the end of the twentieth century, for new producers of polyhydroxyalkanoates and EPS, Nicolaus et al. [94] isolated three obligate halophilic strains T5, T6 and T7, which synthesized 35–370 mg/l EPS, from the salt works of Tunisia. The isolates belonged to the genus *Haloarcula*. Among halophilic EPS-synthesizing archaea is strain *Halobacterium volcanii* 1539, which produces 300 mg/l sulfated polysaccharide [100].

There have been no new studies on EPS synthesis by halophilic archaea after that, until a recent

report of EPS-synthesizing archaea *Haloterrigena turkmenica* DSM-5511, isolated from briny soil (Turkmenistan) [101]. The polysaccharide has high emulsifying (emulsification index of sunflower and olive oils are 62.2 and 59.6%, respectively) and antioxidant activity (68.2% neutralization of DPPH· at 10 mg/ml EPS). The EPS also better than hyaluronic acid and sodium alginate retained moisture.

Similar properties were found in certain polysaccharides of cryophilic bacteriae [85, 86] (Table 3). However, the level of target product is too low (at least now) in strain *H. turkmenica* DSM-5511 to consider it a marketable EPS source.

Bacteriae. Polysaccharides of halophilic bacteriae induced scientific interest almost simultaneously with the first reports of EPS synthesis by halophilic archaea. The most studied bacteriae belonged to the genera *Halomonas* [90–92, 95, 102–110], *Idiomarina* [111], *Alteromonas* [111], *Salipiger* [93] and *Halobacillus* [112].

Those bacteriae are moderately halophilic, their optimum salt content is 2.5–13%, usually 7.5% (Table 6). Most of them survive increased salinity (up to 20–25%) [64, 91, 113], and therefore are halotolerant microorganisms.

In early 1990s, reports were published on the synthesis of sulfated polysaccharide (2.8 g/l) by moderately halophilic bacteriae *Volcaniella eurihalina* F2-7 [104, 109] (now *Halomonas eurihalina* [114]).

Soon, wide-scale screening of possibly halophilic producers isolated from solar salterns in Morocco was published [92]. Thirty two isolates of the genus *Halomonas* were selected for a more detailed analysis out of more than 500 isolates. Only four of them accumulated over 2 g/l polysaccharide, and the highest amount (2.8 g/l) was produced by strain S-30. According to phylogenetic analysis, the strain and isolates S-7, S-31[T] and S-36 were combined into a new species *Halomonas maura* [115]. Further optimization of the cultivation medium (reducing sea salt concentration, instead adding 2.5% NaCl and 0.05% $MgCl_2 \cdot 6H_2O$) increased EPS production of strain S-30 to 3.8 g/l [103].

Strain *Halomonas xianhensis* SUR308, isolated from soil of a solar saltern (India) [90, 91], on a medium with glucose (1%) and NaCl (10%) produced 2.56 g/l EPS [91]. Further increase of glucose content to 3% and decrease of NaCl to 2.5% was followed by increased EPS production to 7.87 g/l [90]. The polysaccharide was not toxic for Huh7 human hepatocytes.

Also, the polymer had high antioxidant activity: the level of neutralization of DPPH· was 72% at 1 mg/ml EPS 72% [91].

Poli et al. [95] reported isolating a moderately halophilic bacteria *Halomonas* sp. AAD6[T] from Turkish salterns. Later it was identified as the typical strain of a new species *Halomonas smyrnensis* [113]. It produced levan (a fructose homopolysaccharide). Adding 50 mM boric acid, 0.8 mg/l thiamine and trace quantities of salts of Mn, Zn, Fe and Cu to the culture medium resulted in a five times increase in levan concentration (up to 8.84 g/l) compared with the initial medium [116].

Further studies aimed to lower the production cost of the target product by using various molasses instead of sucrose in the EPS biosynthesis medium [105]. EPS concentration reached 7.56 g/l (12.4 g/l after 210 hours of cultivation) in culture medium with beet pre-treated with calcium phosphate, sulfate acid and activated carbon. In culture medium with likewise pre-treated starch molasses (a side product of manufacturing dextrose from starchy materials) it was 4.38 g/l. Using starch molasses as a substrate resulted in levan with high emulgent activity [117]. Levan of strain AAD6[T] was shown to be useful in targeted delivery of drugs, in particular, of antibiotic vancomicyn [118]. It also increased LD_{50} of avarol from 0.18 ppm to 10 ppm [95]. Anticoagulant activity of artificially sulfated derivatives of that EPS was studied in [119].

Ruiz-Ruiz et al. [110] studied antitumor properties of polysaccharides of halophilic bacteriae *Halomonas stenophila* B100 and N12[T]. Artificially sulfated EPS (sEPS) of strains B100 and N12[T] (sulfate content 23 and 17%, respectively) efficiently decreased proliferation of T-cells of acute lymphoblast leukemia line Jurkat (500 µg/ml sEPS of strain B100 resulted in 100% inhibition of cell proliferation). Only sEPS of strain B100 induced apoptosis of tumour cells (lines CEM, MOLT-4, HPB-ALL, etc.), while healthy T-cells resisted the apoptosis induction [111]. Authors considered that antitumor effect to directly depend on the concentration of sulfates. It was suggested that sulfates change the charge of polymer molecule to negative and affect its structure, increasing the interaction between EPS and the target cell surface [110].

Bacteriae of the genus *Halomonas* are not only moderately halophilic producers of polysaccharides. A strain isolated from the hypersaline soil of solar saltern (Spain), *Salipiger mucosus* A3[T] (sEST 5855[T]) cultured

for 72 hours in a medium with 1% glucose and 7.5% sea salt produced 1.35 g/l EPS [93]. Approximately the same amount of EPS (1–1.5 g/l) was obtained from strains *Idiomarina fontislapidosi* F23[T], *Idiomarina ramblicola* R22[T] and *Alteromonas hispanica* F32[T] isolated similarly from hypersaline habitats [111]. Unlike these bacteriae, strain *Halobacillus trueperi* AJSK produced almost 13 g/l EPS on an optimized medium [112].

Many polysaccharides of moderate halophilic organisms can adsorb cations of various metals [93, 103, 106, 111, 117] (Table 4), emulsify carbohydrates, vegetable and mineral oils [91, 93, 102, 103, 106, 111, 117] (Table 5). Besides that, EPS of *Halomonas*

Table 4. **Adsorption of metal cations by polysaccharides of halophilic bacteriae**

EPS-producing microbe	Adsorption rate, mg/g EPS			References
	Cu^{2+}	Pb^{2+}	Co^{2+}	
Alteromonas hispanica F32[T]	6.95	30	4	[111]
Halomonas almeriensis M8[T]	19.2	24.5	10	[106]
Halomonas anticariensis FP35[T]	26.6	26.3	10.5	[117]
Halomonas anticariensis FP36	28.1	25.15	10.5	[117]
Halomonas maura S-30	4.24	46.4	0.72	[103]
Halomonas ventosae A112[T]	12	24.8	2.5	[117]
Halomonas ventosae A116	27.6	25.7	10	[117]
Idiomarina fontislapidosi F23[T]	16.3	40	8	[111]
Idiomarina ramblicola R22[T]	26.25	44.65	10	[111]
Salipiger mucosus A3[T]	15.7	43.5	8.7	[93]

Table 5. **Emulsifying properties of polysaccharides of halophilic bacteriae**

EPS-producing microbe	Emulsifying index,%						Refe-rences
	Oil			Tetra-decane	Octane	Kero-sene	
	sunflower	olive	mineral				
Alteromonas hispanica F32[T]	55	40	50	50	55	67.5	[111]
Halomonas almeriensis M8[T]	65	67.5	67.5	62.5	65	65	[106]
Halomonas anticariensis FP35[T]	47.5	40	47.5	45	45	–	[117]
Halomonas anticariensis FP36	37.5	42.5	50	55	42.5	–	[117]
Halomonas stenophila HK30	70	85	55.8	41	56.7	80	[102]
Halomonas ventosae A112[T]	51	42.8	35.5	57.5	57.5	–	[117]
Halomonas ventosae A116	60	55	62.5	60	60	–	[117]
Halomonas xianhensis SUR308	–	71.3	–	80.3	76.3	–	[91]
Idiomarina fontislapidosi F23[T]	65	60	62.5	45	60	55	[111]
Idiomarina ramblicola R22[T]	60	65	62.5	55	60	62.5	[111]
Salipiger mucosus A3[T]	70	60.3	71	75	70	70	[93]
Control							
Triton X-100	62.5–67.5	60–62.5	60–67.5	62.5–65	60–62.5	60–62.2	
Tween 80	62	61.5–62.5	60–70	60–62.5	60	60	[91, 93, 102, 106, 111, 117]

Table 6. **Synthesis of exopolysaccharides by halophilic and moderately halophilic microbes**

Microbial source	Salt content	Carbon source, g/l	EPS content g/l	Chemical composition	Molecular mass, kDa	Physiological effect, functional properties, possible avenues of implementation of EPS	References
EPS of halophilic archaea							
Haloarcula sp. T6	NaCl, 200 g/l	Glucose, 6	0.045	Mannose, galactose and glucose (1:0.2:0.2)	–	–	[94]
Haloarcula sp. T7	NaCl, 200 g/l	Glucose, 6	0.035	Mannose, galactose and glucose (1:0.2:0.2)	–	–	[94]
Haloarcula japonica T5	NaCl, 200 g/l	Glucose, 6	0.37	Glucuronic acid, mannose and galactose (3:2:1)	–	–	[94]
Halobacterium volcanii 1539	NaCl, 156 g/l	Galactose, 10	0.3	Mannose. Hexuronic acids present. Sulfates 0.6%	–	–	[100]
Haloferax mediterranei ATCC 33500	Sea salt, 25%	Glucose, 10	3	Mannose, glucose, galactose. Sulfates 6%	>100	Thickener, intensification of oil production	[96]
Haloterrigena turkmenica DSM-5511	NaCl, 200 g/l	Glucose, 10	0.207	Glucose, glucosamine, glucuronic acid, galactose, galactosamine (1:0.65:0.24:0.22:0.02). Sulfates 2.8%	Fractions 1–3: 801.7; 206; 37.6	Emulgent, antioxidant, moisture retention agent	[101]
EPS of moderately halophilic bacteriae							
Alteromonas hispanica F32^T	Sea salt (7.5%)	Galactose, 10	1.25	Mannose, glucose, xylose (1:0.29:0.11). Sulfates 0.25%	19000	Biofilm formation. Emulgent, adsorbent	[111]
Halobacillus trueperi AJSK	NaCl (61.56 g/l)	Glucose, 22,2	12.93	–	–	–	[112]
Halomonas alkaliantarctica CRSS	NaCl (100 g/l)	Sodium citrate, 3	2.9 g EPS/g biomass	Mannose, xylose, glucose, galactosamine, fructose, rhamnose, not indentified component (1:0.7:0.3:0.2:traces:0.3)	–	Thickener	[64, 108]
Halomonas almeriensis M8^T	Sea salt (7.5%)	Glucose, 10	1.7	Fraction 1: mannose, glucose, rhamnose (1:0.38:0.01); Fraction 2: mannose, glucose (1:0.97). Sulfates 1.4%	Fraction 1: 6300; Fraction 2: 15	Emulgent, adsorbent	[106]
Halomonas anticariensis FP35^T	Sea salt (7.5%)	Glucose, 10	345.5 mg/l	Mannose, galacturonic acid, glucose (1:0.82:0.33). Sulfates 0.73%	20	Biofilm formation. Emulgent, adsorbent	[117]
Halomonas anticariensis FP36	Sea salt (7.5%)	Glucose, 10	0.386	Mannose, galacturonic acid, glucose (1:0.87:0.4). Sulfates 1.16%	46	Biofilm formation. Emulgent, adsorbent	[117]
Halomonas eurihalina F2-7	Sea salt (7.5%)	Glucose, 10	2.8	Glucose, mannose, rhamnose (molar ratio 2.9:1.5:1). Sulfates 2.7%	–	Thickener, emulgent, intensification of oil production	[104, 109]

Table 6. End

Microbial source	Salt content	Carbon source, g/l	EPS content g/l	Physico-chemical properties of EPS — Chemical composition	Molecular mass, kDa	Physiological effect, functional properties, possible avenues of implementation of EPS	References
Halomonas maura S-30	Sea salt / NaCl (2.5%)	Glucose, 10	3.8	Mannose, galactose, glucose, glucuronic acid (1:0.4:0.84:0.63). Sulfates 6.5%	4700	Emulgent, thickener; adsorbent	[92, 103]
Halomonas smyrnensis AAD6^T	NaCl (137.2 g/l)	Sucrose, 50	1.84–8.84	Fructose (levan)	>1000	Flocculant [118]; targeted drug delivery [119], anticoagulant [120]; anticytotoxic activity	[95, 116]
	NaCl (137.2 g/l)	Processed beet molasses (30 g/l carbohydrates)	12.4	Fructose, glucose (traces)	>1000		[105]
Halomonas stenophila B100	Sea salt (7.5%)	–	–	Glucose, galactose, mannose (1:0.91:0.34). Sulfates 7.9%	375	Antitumor activity	[110]
Halomonas stenophila HK30	Sea salt (5%)	Glucose, 10	3.89	Glucose, glucuronic acid, mannose, fucose, galactose, rhamnose (1:0.3:5.5:0.23:0.19:0.005:0.002)	Fraction 1: 1400; Fraction 2: 82	Biofilm formation. Thickener, emulgent; flocculant	[102]
Halomonas stenophila N12^T	Sea salt (7.5%)	–	–	Glucose, mannose, fucose (1:0.52:0.53). Sulfates 2.45%	250	Antitumor activity	[110]
Halomonas ventosae A112^T	Sea salt (7.5%)	Glucose, 10	283.5 mg/l	Mannose, glucose, galactose (1:0.43:0.25). Sulfates 1.09%	53	Biofilm formation. Emulgent, adsorbent	[117]
Halomonas ventosae A116	Sea salt (7.5%)	Glucose, 10	289.5 mg/l	Mannose, glucose, galactose (1:0.42:0.22). Sulfates 0.71%	52	Biofilm formation. Emulgent, adsorbent	[117]
Halomonas xianhensis SUR308	NaCl (10% / 2.5%)	Glucose, 10 / 30	2.56 / 7.87	Glucose, galactose, mannose, xylose, ribose (1:0.74:0.39:0.04:0.02)	–	Thickener, emulgent, antioxidant	[90, 91]
Idiomarina fontislapidosi F23^T	Sea salt (7.5%)	Glucose, 10	1.45	Fraction 1: mannose, glucose, galactose, xylose (1:0.61:0.32); Fraction 2: mannose, glucose, galactose, xylose (1:1:0.5:traces). Sulfates 0.65%	Fraction 1: 1500; Fraction 2: 15	Biofilm formation, emulgent, adsorbent	[111]
Idiomarina ramblicola R22^T	Sea salt (7.5%)	Glucose, 10	1.5	Fraction 1: mannose, glucose, rhamnose (1:0.37:0.1); Fraction 2: mannose, glucose, galacturonic acid, rhamnose, xylose (1:0.35:0.47: traces: traces). Sulfates 0.5%	Fraction 1: 550; Fraction 2: 20	Biofilm formation. Emulgent, adsorbent	[111]
Salipiger mucosus A3^T	Sea salt (2.5–7.5%)	Glucose, 10	1.35	Mannose, galactose, glucose, fucose (1:0.97:0.58:0.39). Sulfates 0.9%	250	Emulgent, adsorbent	[93]

stenophila HK30 have high flocculant activity: 72.06% EPS in a suspension of kaolinite (0.5%) at 20 mg/l EPS [102].

Table 6 summarizes information on EPS synthesis by halophilic archaea and moderately halophilic bacteriae.

Thus, studies of EPS from non-traditional sources (cryophilic fungi and bacteriae, halo- and thermophilic archaea and bacteriae, including those from deep-sea hydrothermal vents) is a novel field which began to develop rapidly at the end of the twentieth century. Many of those isolated microorganisms produce polysaccharides. The physiological effect, physico-chemical properties and possibilities of industrial application of those EPS are studied. Those substances due to their immunomodulating, antiviral, anticoagulant, antitumor, antioxidant activities can be widely employed, in medicine and pharmacy, etc.

Meanwhile the practical implementation of polysaccharides is limited by the low efficiency of production. Non-traditional sources produce EPS in much lower concentrations than the traditional ones. In our opinion, solving this problem is only a question of time, because various approaches to metabolic and gene engineering for microbial synthesis intensification are already developed [88, 112, 120−122].

EPS biosynthesis by non-traditional sources currently requires expensive carbohydrate materials (glucose, fructose, sucrose, and maltose) (Tables 1−3, 6). At the same time, many new studies aim to substitute carbohydrate substrates with cheap industrial wastes (whey, crude glycerin, oil-containing wastes, and agricultural wastes) in culturing traditional producers of polysaccharides. Those approaches to microbial polysaccharide production are reviewed in [123]. We demonstrated that it is possible to obtain microbial EPS ethapolan using fried vegetable oil [124] and its mixture with molasses [125].

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НЕТРАДИЦІЙНІ ПРОДУЦЕНТИ МІКРОБНИХ ЕКЗОПОЛІСАХАРИДІВ

Т. П. Пирог
А. А. Вороненко
М. О. Івахнюк

Національний університет
харчових технологій, Київ, Україна

E-mail: tapirog@nuft.edu.ua

Наведено дані літератури щодо синтезу
екзополісахаридів психрофільними гриба-
ми, гало- і термофільними археями та бак-
теріями, зокрема й виділеними з глибоко-
водних гідротермальних вентів — джерел.
Проаналізовано фізіологічну роль, фізико-
хімічні властивості та можливі галузі
практичного використання екзополісаха-
ридів, синтезованих нетрадиційними про-
дуцентами. Більшості з них притаманна
імуномодулювальна, противірусна, антико-
агулянтна, протипухлинна, антиоксидантна
активність, що робить їх перспективними
для застосовування у медицині та фармаце-
втиці.

Водночас аналіз літератури засвідчив не-
обхідність розроблення ефективних техноло-
гій одержання таких полісахаридів, оскіль-
ки показники їх синтезу нетрадиційними
продуцентами є значно нижчими порівняно
з традиційними.

Ключові слова: екзополісахариди, термофіли,
психрофіли, галофіли, гідротермальні венти.

НЕТРАДИЦИОННЫЕ ПРОДУЦЕНТЫ МИКРОБНЫХ ЭКЗОПОЛИСАХАРИДОВ

Т. П. Пирог
А. А. Вороненко
Н. А. Ивахнюк

Национальный университет
пищевых технологий, Киев, Украина

E-mail: tapirog@nuft.edu.ua

Представлены данные литературы о синтезе
экзополисахаридов психрофильными грибами,
гало- и термофильными археями и бактерия-
ми, в частности выделенными с глубоковод-
ных гидротермальных вентов — источников.
Проанализированы физиологическая роль,
физико-химические свойства и возможные
отрасли практического использования экзо-
полисахаридов, синтезированных нетрадици-
онными продуцентами. Большинство из них
обладает иммуностимулирующей, противови-
русной, антикоагулянтной, противоопухоле-
вой, антиоксидантной активностью, что делает
их перспективными для применения в медици-
не и фармацевтике.

В то же время анализ литературы показал
необходимость разработки эффективных тех-
нологий получения таких полисахаридов, по-
скольку показатели их синтеза нетрадицион-
ными продуцентами значительно ниже по
сравнению с традиционными.

Ключевые слова: экзополисахариды, термо-
филы, психрофилы, галофилы, гидротермаль-
ные венты.

ELECTRONIC DATABASES OF ARTHROPODS: METHODS AND APPLICATIONS

O. M. KLYUCHKO[1], Z. F. KLYUCHKO[2]

[1]Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the National Academy of Sciences of Ukraine, Kyiv
[2]Schmalhauzen Institute of Zoology of the National Academy of Sciences of Ukraine, Kyiv

E-mail: kelenaXX@ukr.net

The purpose of the work was the examination of various databases construction as well as examination of possible errors. Peculiarities of biological objects which should be taken into account during databases design were analyzed. The methods for electronic collections with databases of biological organisms elaboration were studied. The appropriate algorithms for environmental conservation were analyzed and compared with some foreign analogs in order to study positive and negative experiences. The requirements for database with information about *Noctuidae* (*Lepidoptera*) and some *Araneidae* were formulated for the development of electronic information system "EcoIS". The description of developed relational database with information about insects with analysis of selected object area were suggested taking into concideration the characteristics of biological objects and characteristics of information systems analogues. Conclusions concerning described new means with bioobjects databases and their application on the example of "EcoIS" system were done as well as some recommendations for the construction of databases with the information about living organisms basing on our experience.

The importance of electronic information systems (IS) with databases (DB) have uprised during the last years for biotechnology and other biological disciplines: systematics, taxonomy, ecology, nature conservation, for their application for monitoring of polluted environment.

Networks with distributed databases for biotechnology, environmental protection of bioobjects. Electronic databases with access to the Internet with information about living organisms are technical information systems (ISs) invented during the last decades due to the progress in information and computer technologies (ICT). They were designed either for academic purposes — to maximize the accumulation of information about the groups of living organisms, or for the needs of the economy, in particular for biotechnology, for monitoring of polluted areas in industrial centers, and etc. [1–9]. Mathematic methods as well as models that we described in our previous articles and those published by other authors also may be used for ISs functioning or to be simulated in result of their functioning [9–81]. A spectrum of mathematic methods were used for the newest biomedical ISs elaboration [1, 11, 75, 77–146, 159]. Content for the databases described in this article was obtained usually from the results of biological and medical observations and experiments [10, 12–17, 24–44, 47–49, 61, 68, 71–74, 82–90, 94, 104, 106, 109, 111–113, 125–159]. All such technical information systems (tIS) are electronic databases (DB) distributed in networks today [1–11, 25–69, 90–109, 112–120, 159]. Present work was done after the analyzis of approximately 250 current publications in fields of biotechnology, other branches of biology and technology, including articles with original authors' works. Priority of authors' works was reflected in [160–162].

Development of electronic databases in biological sciences. Electronic ISs with DB for biological sciences should contain information about the diversity of living organisms

and their evolution — the "Invention of the Nature". Another, alternative kind of diversity that has been invented by human — diversity of the data that appeared and evolved as a result of computer networks development, as well as new computational methods and methods of electronic information recording. The last one exists during only the last 20–25 years. However, there is a large chaos in biological ISs data. For today the most important is the way of data mining, recording and obtaining from DB, in which the data are logically organized and structured. As a result an alternative type of diversity appeared — diversity of DB [1, 112, 114, 159]. In this article we give the examples of how the variety of information and natural diversity could be correlated.

Prerequisites for the development of electronic information systems in Ukraine. In Ukraine, at the time of former USSR, numerical groups of scientists-biologists and other professionals had organized the network of bio-stations for the environment ecological state monitoring and environmental biodiversity protection. These means were enough perfect for those time. Due to the works of biologists of previous generations there were localized and stopped locusts'propagations in South Ukraine as well as some other environmental cataclysms. As continuation of these works we began to develop "EcoIS", a new electronic information system, which is based on bioorganisms DB development. As bioindicators we have chosen insects-moth *Noctuidae (Lepidoptera)* [152–159]. We see them as successful bioindicators for number of reasons, the most important of which are following: 1) there are more than 700 species in Ukraine; 2) these species are enough well studied; 3) these species can be determined enough well; 4) these moth are well visible during material gathering due to their morphological qualities; 5) collection of *Noctuidae* is carried out at night time and moth species dominate usually in collected material; 6) most *Noctuidae* species are pests in agriculture that make their studies necessary for economy [156–159]. Therefore their studying and use as bioindicators are useful for the solution of several problems of Ukrainian economy. Enough important argument is that during the monitoring of changes in *Noctuidae* quantitative and qualitative composition in industrial areas vicinity it is possible to make conclusions about environmental pollution harmful effects on living organisms' population. For the development of electronic

information system "EcoIS" we used some results of Prof. Klyuchko Z.F studies of *Noctuidae* in Ukraine and at territories of border countries since 1961 [1, 156–159] (Fig. 1). Part of these works were carried out jointly by both authors in the extreme conditions of Caucasus Mountains at Elbrus region on 1997–2006 (Cabardin and Balkar region, Russia), and obtained results could be applied to high altitudes' influence studies
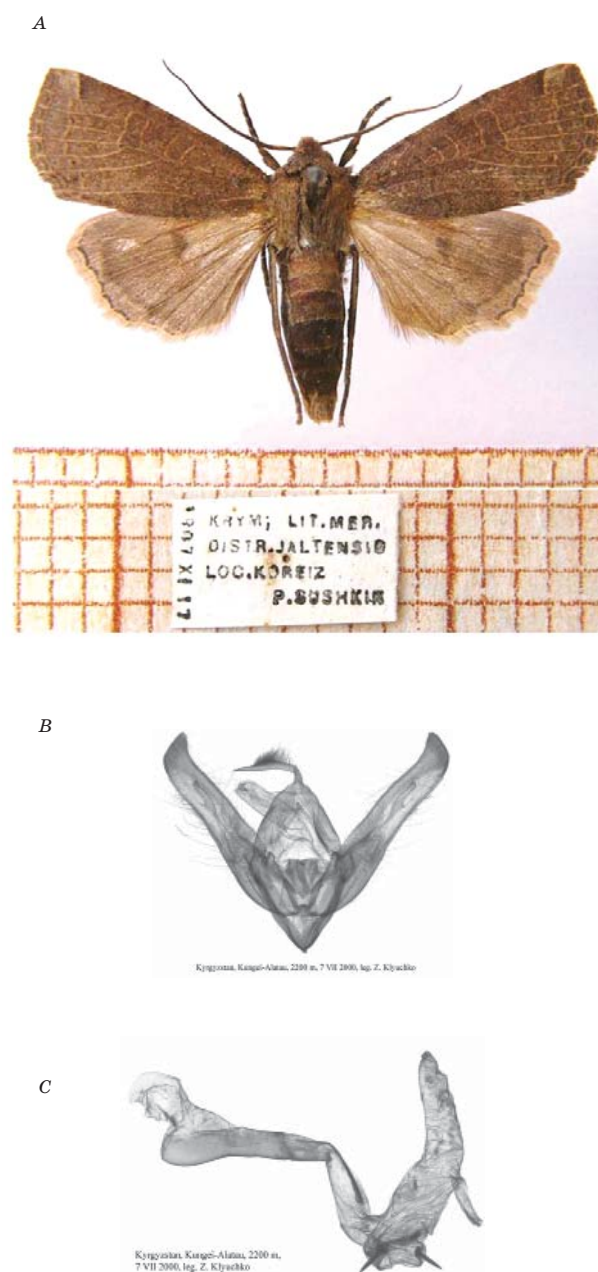
Fig. 1. Materials from Prof. Zoya F. Klyuchko private laboratory:
her preparations and collections that we have used for databases construction. Her notes about places where she collected some materials are seen as well

[1]. It is known that bioorganisms adaptation to extreme conditions occurs according the two strategies that are fundamentally different for organisms like higher mammals (and humans) and insects. Our developed electronic information system "EcoIS" is aimed on the second strategy of insect organism adaptation studies [1, 159].

Application of some mathematical methods for the work with bioobjects databases: methods of cluster analysis, neural networks and other [1–9, 57, 58, 67, 159]. The application of these methods in order to distinguish similar bioorganism characteristics for the development of biological DB and subsequent modeling have demonstrated good results [1–9, 159]. The use of methods of cluster analysis and neural networks for mathematical models development in ecology was used to study the distribution of organisms in different areas, pests penetration and spreading in bioorganisms' population, the influence of environmentally dangerous factors on living organisms' population. An important task is the elaboration of medical and biological DB, which enable to distinguish reliably the groups of objects according to their similar characteristics [1–9, 159].

From the obtained results one could see how it is possible to apply traditional methods of clusterization for the practical analysis of data sets registered on biological samples (for example, for different types of drawings on the wings of insects-moth) during databases construction [1–9, 159]. The similarity of measure was used in combination with one of four types of clustering algorithms: 1) method of agglomeration hierarchical clusterization with single linkages; 2) method of agglomeration hierarchical clusterization with complete linkages; 3) Ward method; 4) rough clustering method [1, 115]. The results demonstrated that clusters were generated usually according to two types of attributes that have a biological meaning; the best match was achieved using Ward method. During the elaboration of such DB, the nominal attributes were not included in the list of meaningful attributes [1, 115].

During the attributes defining for the elaboration of insect DB it was necessary to choose the right attributes [1–9, 159]. Today, for the development of entomological DB, the specially developed digital photographic equipment is used, which makes it possible to express the categories "more reddish", "less gray", and etc. in digital forms. Consequently,

one can use the notion of numerical attributes with appropriate methodological consequences.

Basing on information about butterfly specimens that were ordered in electronic DB for museums or collected for species conservation, the possibility of two mathematical methods that are valid for actual number of species in the region was explored. Some authors suggested following two methods [1, 83, 88]: 1) phenomenological model based on the saturation curve of the number of collected insect specimens; 2) model based on the concept of species number increasing which is described by normal logarithmic law. Using these methods for the analysis of daytime butterflies for some regions, it was found that the location of places with large species diversity (calculated according to the asymptotic approximation) differs from what can be expected if to analyze only the raw data from collection [1, 83, 88]. In addition, it has been shown that there was a difference in the results of two different assessment procedures and these differences become significant for processing of results obtained for a small number of individuals. It was proved that the concept of normal logarithmic distribution of species should be preferred, since it allows to analyse much better the state of fauna, for example, it avoids risky inaccuracies in the estimations [1, 83, 88]. Finally, basing on DB data as well on calculated variables of butterflies' species diversity, it was demonstrated an interesting fact: the places with species-representatives of disappearing and small species are rarely the same as "hot spots" or biodiversity centers [1, 83, 88]. Consequently, some regions with species biodiversity, where the butterflies' protection have been already organized, in reality have not been co-located with those areas where the rare or disappearing butterflies' species have been registered. The Monte Carlo method was also used to analyze the data for some environmental conservation problems solution. Thus, the use of these theoretical methods have made significant corrections in nature conservation practice [1, 83, 88, 156–159].

Problems of network databases design for biotechnology, ecology and systematics. In biotechnology, systematics, taxonomy, during the analysis of characteristics of bioorganisms that need to be ordered in DB, one can reveal the regularities that determine the logical structure of these data in DB (or "data representation model"). According to their peculiarities, such biological objects form an ideal hierarchy, therefore, a hierarchical

model of DB organization developed for technical sciences could be applied there successfully. However, the simple borrowing of this model was unsuccessfull due to the great individual bioorganisms' variability; so, for the development of such databases the special techniques invention was necessary. Let's analyze the suitability of such methods on the example of *Noctuidae (Lepidoptera)* and other insects, dividing these bioobjects into two groups respectively: those that do not contradict to DB construction according to hierarchical model and those that do not fit into such a model [1, 159].

Biological objects and their specificity in DB designing. Designing of the ISs that include the DB, is performed on the physical and logical levels. Solution of design problems at the physical level in many cases depends on the used DB management system (DBMS), which is automated and often is hidden from the user. In some cases, the user can regulate individual parameters of the system. [1, 159]. During the DB construction for systematics and taxonomy, the focus should be primarily on *logical construction.* In course of logical construction it was possible to determine the number and structure of the tables, to form the DB queries, to define types of accounting documents, to develop the algorithms of information processing, to determine forms for input and editing of DB data and to solve the number of other tasks.

The nature of biological objects was those specificity that determine the solution of problem of logical construction. The most important problem here was the data structuring. During the data structures construction it was necessary to distinguish three main approaches.

1 — Collection of information about the objects of solved tasks within the framework of one table (one relation) and its subsequent decomposition into several interconnected tables basing on the procedure of relations normalization.

2 — Formulation of knowledge about the system (definition of output data types and interconnections) and requirements for data processing, obtaining with the help of automation system for construction and development of DB, finished DB schema or even ready-made IS application.

3 — Information structuring for the use of IS in process of system analysis basing on a set of rules and recommendations.

Characterizing bioobjects specificity one could also emphasize the following. 1 — This is the complexity of bioobjects by themselves, in these objects the elements — their components determine often the properties of the whole object, but they are not the arithmetic sum of elements properties. 2 — Complexity and multiplicity of links between objects and between elements in objects. 3 — Hierarchy, which subordinates the largest majority of objects in nature and hierarchical connections. 4 — The presence of many weakly differentiated objects in nature, which, however, are not identical, but have individual characteristics, forming a homologous series of objects. Therefore in process of medical and biological ISs elaboration the problems of differentiation become a topical task.

Specificity of biological objects, collection of information about such objects during expeditions, field observations and their primary analysis play a key role in the design of biological DBs. Respectively, below we would like to analyse some characteristics of insects — moths (*Noctuidae, Lepidoptera*). According to our plans this information about *Noctuidae* systematics and taxonomy has to be basic in course of the development of our electronic information system "EcoIS". Moths (*Noctuidae, Lepidoptera*) are night butterflies, many of which can be seen and collected at summer nights. Their place in Linnaeus hierarchy of living organisms [41] is shown on Fig. 2. The example of hierarchical position in classification of moth specie *Agrotis segetum* and spider specie *Argiope lobata* are represented. In fact, this figure demonstrates the classification data of these two species only from the level "Type — *Arthropoda*", which is the same to both species. At lower hierarchical level — at level of classes — the lines of *Noctuidae* and *Argiope* are divergent. Above the type of "*Arthropods*" the level "Kingdom of animals" has to be placed; and above it the "Domain of eukaryotes" has to be drawn (both one are not shown on Fig. 2) [1].

Such views about hierarchical classification of living organisms naturally fit into modern bioinformatics program methods based on the hierarchy of classes that have become widespread in modern ICTs, for example, in object-oriented programming. It would be necessary to provide the use of several languages in biomedical DB, developed for such industries as ecology, zoology, and etc. For example, for our purposes in Ukraine, in biological DB the information was given in Ukrainian and Latin, since all known bioorganism species have traditionally Latin names, and only a part of them also has the

names at languages of population who lives at this territory.

Characteristics of bioobjects Noctuidae corresponding to DB construction according to hierarchical model. For classification of organisms in living nature, Carl Linnaeus proposed his scheme on 1761 [1, 41]. The information used for the registration of living organisms in biology (ecology, taxonomy, and etc.), at first glance, has been structured quite simply — it is a hierarchical structure of living organisms classification in systematics (Fig. 2) [1].

By opening any biological determinant (for example, for insects) [1], one can find the following information (our examples were written for Slavonic-speaking countries; for other groups of countries the determinant structure is the same):

– the name of species (Latin, Ukrainian or Russian); the names of the people who defined it and the publications in which the information on the species is given; the data of collection; the place of collections: parts of the world, country, region and settlement; the geographical area of its inhabitance; the

Type	*Arthropoda*	*Arthropoda*
Class	*Insecta*	*Spider-like*
Series	*Lepidoptera*	*Spiders*
Family	*Noctuidae*	*Spiders-colopods*
Genus	*Agrotis*	*Argiope*
Species	*Agrotis segetum* Denn&Shiff	*Argiope lobata*
a	*b*	*c*

	Spiders – Latin names
1	*Agelona labyrinthica*
2	*Araneus diadematus*
3	*Argiope lobata*
4	*Argyroneta agnatica*
5	*Atipus piceus*
6	*Dolomedes fimbriatus*
7	*Latrodectus tredecimguttatus*
8	*Lycosa singoriensis*
9	*Misumena vatia*
10	*Nephila clavata*
11	*Segestia senoculata*
12	*Tegenaria domestica*

Fig. 2. **Scheme for classification of Artropods by Linnaeus:**
a — the general hierarchy of classification units; *b* — example of hierarchy classification of insects:
Agrotis segetum Denn & Shiff (Dennis & ShifferMuller); *c* — example of hierarchy classification of spider
Argiope lobata; *d* — an example of the table fragment from the database of spiders from the system "EcoIS"
(explanations see in text) [1, 41]

habitant biocenosis, the plants on which it is fed (including information whether it is a pest in agriculture); biological data on the number of generations during the season, periods of flight, and etc. Being standardized since the time of Linnaeus [41], these data could be structurized in the table easily (researchers — biologists also have organized these data in such a way), and it seems to be ideally adapted for the further transfer to the DB tables [1]. During our DB elaboration the names of species and other classification terms were written in Latin, Ukrainian and/or Russian languages (on Figs. 2−4 we have demonstrated only ones in Latin).

Characteristics of bioobjects, which complicate the traditional methods of database designing. The experience revealed that with each of these simple elements of the future DB contents the certain problems may be associated due to the peculiarities of living organisms [1]. Many of them have an unstable name and an unstable taxonomy. Thus, *Noctuidae* classification, which seemed to be established for decades, is being re-examined now [1]. Consequently, during the hierarchical model constructing, these facts lead to the errors as a result of ambiguity of parent and derivative elements selection. If insects were collected on certain plants, it is often not possible to conclude whether these plants are a source of their nutrition or a place of residence, or both ones at the same time.

Specialists solve a number of problems in connection with the species location registration, for example, during the development of the first electronic plants database in Ukraine by collaborators of Botanic Institute of the National Academy of Sciences of Ukraine named by Cholodny [1]. For our objects during DB construction, we had found the following. The exact location (important for DBs) may change its name: for example, the Kyiv region of Southern Borshchahivka did not exist fifty years ago, as well as the name of district Vyhurivshchyna in Kyiv appeared on the maps, and then disappeared again during XX c. The similar situation was with other regions of Ukraine — during different historical periods the administrative boundaries of individual regions have been changed. Even the names of countries could be changed during the different historical periods. Thus in Europe, some border territories from time to time were the parts of different countries (Friuli-Venezia Giulia province in modern Italy during its history was the part of Yugoslaviia, and before that —

the part of Austrian Empire, and so on). Data of observations were considered unchanged often but… For example, materials from entomological collections of Russian Empire of XIX−early XX c, which were dated according to unformatted calendar require correction before their ordering in modern electronic databases. The names of observers could also be changed (for example, ladies' names after marriage). Consequently, even the simple and factual information that we consider sufficiently suitable for ordering in biologic DBs is characterized by instability, and this instability must necessarily be taken into account [1].

Difficulties in problems' solution during the elaboration of database of insects' adaptation characteristics in mountainous regions (one of the tasks discussed below) could be linked with the fact that during field observations a large number of heterogeneous data were recorded, which complicates the construction of object model. For example, for a few names of *Noctuidae* from the list of a few dozen names it was necessary to take into account many characteristics, and these data are difficult to divide into groups for the selection of objects and attributes [1].

Similar problems appeared also during the conducting of environmental works in the United Kingdom, when it became necessary to analyze the hundreds of thousands of insects' characteristics for different species and plants on which they feed. Such large-scale work was carried out using a large amount of the data that was pre-recorded in electronic databases, which were specially designed to solve this problem, while some data were lost due to the individual variability of studied living organisms. Data analysis was based on the Monte Carlo method [13]. As were noted by authors, even when trying to analyze a small number of insects, for example, only a few *Noctuidae* names from the list of several dozen, these data were difficult to subdivide into the groups for defining of objects and attributes [1]. Some important characteristics, such as changing of colors and shades of the wings, which are of primary importance for biologists, are difficult to express in the form suitable for the table (for example in cases when biologists use usually the expressions like "less dark olive", "reddish", and etc.) Below we will show that in order to take into account these peculiarities a number of special techniques should be used, among which there are the mathematical methods of cluster analysis (by means of which it is possible to distinguish

between objects that differ slightly), the use of special high-quality digital photographic equipment, which allowed quantitative expression of characteristics, which previously were described only qualitatively and so on. The description of the behavior of living organism and its discretization for the purpose of placement in separate table fields is extremely difficult also, since behavioral acts are difficult to formalize.

Development of electronic database without duplications and ambiguity is still a prerequisite for a skilled approach to species conservation [1]. However, the quality of the most already constructed databases does not reach the necessary standards' levels required by the needs of nature conservation. This is true even for such regions, for which numerous records have been done even for easily identifiable butterfly species [14].

Due to the complexity of biological objects and inaccuracies of many records made during the field observations, the problem of duplication appears quite seriously. According to the theory of databases, the dubbing is divided into *simple (non-excessive)* and *excessive duplication*. During the construction of relational tables, it is impossible to avoid *simple (non-excessive)* duplication if we describe, for example, several types of insects that were registered in one of the regions of Ukraine (Fig. 3). The presence of this type of duplication in database is permitted and the quality of information retrieval is not affected during the future use [1].

Excessive duplication of the data in database can lead to problems in data processing. It appears in situations when an information about any specie is replaced by a dash (as it is often done) relatively to the characteristics of above mentioned species (Fig. 4). During information retrieval in this case, the computer automatically begins to search for information that replaces the dash, and computer resources are spent consequently. In addition, computer memory is still allocated under attributes with dashes, so, there is no significant optimization for it. And finally, since the information in attributes with dashes is linked with pre-specified species, then its deletion in the future will result the loss of information for all types that have been unsuccessfully associated with it. Consequently, when making of relational tables in such databases, all episodes of excessive duplication have to be corrected.

Another problem is typical for all regions of the planet. This is the "spot-like" of fauna researches of different territories — in some regions it has been studied better, other regions, in fact, are "white spots" on natural maps. In addition, records for individual species contain many controversies [1].

However, it was difficult to achieve enough representative survey of both geographic and other one subject that reflects fauna changes during certain time. Such works are quite expensive and records' quality varies even for work performing accuratively. However, because of constant worsening of

Insect (Latin name)	Geography of collection	Described
Agrotis segetum	Kyiv region, Crimea	Den.&Schiff.
Autographa gamma	Kyiv region, Crimea	L.
Spudaea pontica	Crimea	Kl.
Periphanes treitschkei	Crimea	Fr.

Fig. 3. **The example of fragment of the table with simple (non-excessive) duplication**

environmental living conditions, constant climate changes, the quality and importance of insect changes studying is increasing (including moths, butterflies and etc.) It whould be remembered that in the past the quality of such research was lower, which may lead to a non-correct imagination about species distribution in the nature in past years.

So, during the elaboration of biological database with information about living organisms, it is necessary to take into account the above-mentioned features and factors, below we give the examples of such databases implementation.

Databases and electronic collections of organisms, bioindicators, representation of data in such databases in form of relational model. Making of collections of biological organisms (CBO) has always played a key role in the construction of monitoring systems because this is ability to record data sets about bioorganisms. In the époque of ICT, the logical development of collecting techniques was the construction of electronic CBO, when organisms data began to be organized into the databases in digital form. In fact, electronic CBOs are the kind of ISs with databases. On other hand, a well-done electronic CBO is a DB that can act by itself as element of IS. Below we will focus on the construction of such electronic CBO, especially we will be interested in what prototype methods could be used in developing of original database of *Noctuidae* for future its introduction into the electronic information system "EcoIS".

Construction of electronic collections of bioorganisms with databases. Some electronic biological databases have already been constructed and connected through the Internet network [1]. Only one of the largest information sources on the distribution of species, CBO [113] contains a unique combination of attributes, which includes:

− a large array of information resources about about 2.5 billion specimens of living organisms, each with information about their collection — the time and the place where this sample was collected;

− records that were considered as standard for a particular sample of specie, according to which one can identify and update the information about the identity of a sample if the nomenclature will be changed;

− records that contain valuable information about the restrictions that should be imposed during models construction using the data from electronic CBOs;

− historical data on the organisms spreading today and in the past (including paleontological data), so that it was possible to restore the dynamics of biodiversity changes before and after the start of anthropogenic influence;

− current taxonomy data, because the scientists who constructed and fill this database had knowledge in taxonomy, biodiversity (and, respectively, in phylogeny, environmental analysis and comparative genomics) [1].

Insect (Latin name)	Geography of collection	Described
Autographa gamma	Kyiv region, Crimea	L.
Spudaea pontica	Crimea	Kl.
Periphanes treitschkei	–	Fr.
Divaena haiwardi	–	Tams

Fig. 4. **The example of fragment of the table with excessive duplication**

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Methods of modeling for CBO. Constructing of electronic CBO is the first among methods in the collection of the latest computer techniques in systematics and taxonomy [1, 113]. If an electronic CBO was elaborated then the data from its database can be used for the next solution of many problems, for example, such actual for industry task as simulation of species distribution [1, 113]. Methods of modeling of species distribution phenomena are very different; they can be distinguished according to the type of tasks for which they were used, to the data used from CBO, and also according to results of these data statistical processing. All applied methods of modeling could be divided into 3 groups: the first group included modeling methods which necessarily need to have data-characteristics of actually collected organisms of different species; in the second group — there were the methods used to solve problems of forecastings. In the third one there were methods used to solve problems that have the characteristics of the first two types and require both real data and solve forecasting problems; for them such methods as linear and additive models, 'trees' of alternatives in decision making, Bayes approaches, and etc. The last ones were filled with new content, since, by applying them, one can access the occurrence of differences between organisms, can apply expert evaluation and other preliminary information, and basing on all it is possible to predict changes of variables, in particular it is possible to predict changes in species distribution. In [1, 113] was propose the following software samples that can be applied for modeling according to these three groups of methods: for the first one — software BIOCLIM or DOMAIN; for the second one — GARP software, for the third one, respectively, the "mixed type" of software.

Model evaluation. For models evaluation it was necessary to select methods depending on the nature of the particular biological task [1, 113]. Some developers evaluate the models designed according to the CBO data, depending on the size of the structural elements that were put in base of the models. Other studies estimated the possibility of forecasting using models based on CBO data. Other ones have studied the effects of data errors and the existence of branching processes during the work with CBO, and if such a branch occurs, then it is necessary to inform the users who work with this CBO data. In the last case, some simulation and evaluation methods could be applied only to specific tasks and certain data sets, or in general, individual data can not be used for development of forecasting models.

Algorithms for development of biological databases distributed in networks. During the construction of biological databases distributed in networks, it is necessary to do numbers of analytically determined steps [1]. Below there are some algorithms that should be followed for the development of medical and biological databases.

The strategy of mathematical methods use for the construction of tIS with databases in medical and biological practice is determined generally by algorithms for specific tIS construction. In order to avoid errors, the data from CBO have to be used in context of the most comprehensive knowledge of taxonomy or history of systematics; in the case of the studied group of organisms it is recommended to study the insect sample from collection (Fig. 1). Spatial errors in simulation may be due to errors in recordings of geographic data of the place where a sample was collected, a visual inaccuracy of the placement of the record by itself, and errors made during the initial placement of this record [1]. These kinds of errors could be detected because they belong to collection samples that spatially droped out of geographic coordinates or natural zones or if there was a discrepancy between the field records of the person who collected the material and the records in collection. Spatial errors can be corrected by studying the collection sample by itself or corresponding field records, by removing or correcting non-correct records and performing a geographic description extremely accurately.

Warnings and limitations for using of the data from CBO collections. In process of spatial modeling in biology it is necessary to take into account three main features of CBO collections: errors, including taxonomic identification errors and geographic; data biases — primarily biases due to the geographic data and the description of the natural environment in special collections, the presence in many cases of the data that look like as "yes" and "no", which, in the case of modeling, determines the type of algorithm in advance.

Representation of the data in bioobjects' database in the form of relational model. One of our tasks was development of the database with information about insects, first of all *Noctuidae* (*Lepidoptera*) [1, 156−159]. Offering our approach to this problem solution, we based on the following considerations.

Elaborated relational database had to satisfy contemporary standards [1]. Primarily we have spoken about the technical standards — the database must have typical and understandable structure, the material should be accessible and clear, it could be added to the material from other tables from World Wide Web, if necessary, not only the developer, but professional-biologist would be able to make new records (but not worsening irreversibly the main content in cases of incorrect entries!), and etc. At the same time, it was important to comply existing biological standards of taxonomy — compliance with the standards of Latin, Ukrainian and Russian names of insects (for Slavonic-speaking countries), the standard division of material, its completeness for each specie, the compliance with high scientific criteria, and etc. We support these standards, comparing our results with published materials of well-known determinants in biology, current scientific publications of high quality [1, 156−159]. The formats in which the data are presented in scientific publications of various authors, often differ one from another: calculations are done according to different methods which complicates the comparison of these data and their ordering in one database. Data for such databases requires standardization, which does not really exist today in finished form. Although electronic databases may contain information of different formats, we must always keep this in mind using database data for comparisons [1].

Recommendations for the construction of DB of evolving organisms. Since database table contains material about living organisms that evolving and changing constantly, the prospect of its further modernization and development should be laid, if possible, in the possibility of adding of new fields, tables, and etc., without deep reorganization of the database by itself. We considered that it is necessary to make fields (or even to leave the the possibility of new tables adding) for the inclusion of observational materials that do not fit into the current biological standards of the database, but may become valuable in the future (analogous to Linnaeus "Chaos" [41]). In our case, there may be the fields "Comments" and "Other". Actually, the very existence of such information in additional fields is the source of further development of the DB. In these fields you can also record following information. For example, some types of insects biologist could collect as unique sample, or he cannot collect them at all — but they may be collected and/

or determined by other authors. Information about them is very important for biological analysis, but in the main fields of database tables this information will not be included — it may be recorded in "Comments" or "Other". But if to record all single cases the base grows too much, successful searches in it became too complicated (because of effects associated with the complexity of living systems). It should be noted that, although the main task of our databases construction was solved successfully, it set a number of questions, which will be answered only by further work in this direction [1].

Relational database on bio-objects of the highlands of the Ukrainian Carpathians and the Caucasus (Russia). Below the results of electronic databases development with observation materials in zoology, ecology and adaptation biology of insects living on highlands were suggested. On the materials of insects observations and changes of some their characteristics according to their adaptation to different altitudes, an electronic relational database has been developed, which contains the relevant data. These databases could be described briefly by following provisions [1].

1. There are two databases were elaborated. 1) One DB for some insect species registered by the authors at Elbrus region (Kabardino-Balkar Autonomous Region, Russia) during the expeditionary seasons on 2002−2005; and 2) similar DB about some types of insects in the Ukrainian Carpathians (materials of many years studyings of Prof. Klyuchko Z. F.) Development of two databases gives a good opportunity to compare results to two geographically remote mountain regions and for appropriate generalizations.

2. The "core" of database — the main table with systematic data about insects that were collected during expeditions. The material for these table records — the standard data published in the corresponding academic determinants of high quality.

3. In the table containing the data about the adaptation characteristics of some types of insects, the data of the authors' observations were recorded. This material is new, relative form has no developed standards, so the table could be modified with time.

4. The author plans the work on supplementing of described DB with other parts with materials of other (as well as ecologically different) regions.

5. The table with the data of altitudes could be supplemented with new records after the future studies at other altitudes with time.

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6. In developing of this database structure, the author foresaw the necessity of its modification over time due to objective reasons (deepening of scientific knowledge in this field, and etc.) In the structure of the database the possibility of its development and further modification is expected.

Taking into account these problems, we propose the following approach. An electronic database with characteristics of insects first of all has to be based on already published information according to established standardized schemes. However, for database constructing, it is necessary to realize the possibility of its supplementing by additional fields for non-standard records that can be used in the future, or even the development of additional tables without a substantial re-design of the entire database. Of course, it is impossible to predict all future non-standard situations, but for a sufficiently high qualification of professionals involved in this construction, it is possible to minimize the amount of future corrections. This method was used in past by Karl Linnaeus [41], who, by creating of the first classification of living organisms, added the section "Chaos" for all organisms that were not included in his system. This his idea survived during centuries and, trying to "organize" in our practice our ever-evolving wildlife, we decided to use such historically proven techniques at the current level of our life, at modern ICT level [1].

Object area analysis and biological material that characterize mechanisms of insects adaptation to high altitude conditions [1]. During several years we studied the problem of living organisms' adaptation to different ecological conditions of highlands. We collected material that characterized the manifestation of insects' adaptation characteristics in the Caucasus Mountains at the region of Elbrus Mountain (Prielbrussie Region (Russia), at Elbrus Medical and Biological Station of the National Academy of Sciences of Ukraine). Field researches Dr. Klyuchko O. M. carried out on summer seasons, 2002–2005; the techniques of electronic databases construction there were applied directly. There were no regular targeted studies on this issue in the Ukrainian Carpathians, but during few decades of Carpathian insect studies by Prof. Klyuchko Z. F. since 1961 [1, 42] there were gathered enough biological material that we could choose to compare with the data from Elbrus [1, 23, 29–37]. Let's characterize briefly this material, and then — let's examine

in details construction of developed DB. The object of research were insects with their variable characteristics (mainly *Noctuidae* (*Lepidoptera*), as well as some other groups of insects), collected in the zone of mountain forests (ZMF) of both regions, and at Elbrus Region — in ZMF and above this band. At Elbrus Mountain slopes the material was collected at three altitudes: 2100 m above sea level (m. s. l., village Terskol, ZMF), 2800 m.s.l. (Cheget mountain, subalpine meadows), 3100 m.s.l. (peak Terskol, the upper part of the subalpine meadow, the boundary of the mountain snow line). In the Carpathians, the insects of ZMF area were colected at altitudes of 1500–1600 m.s.l. (ZMF is located below here), and insects of subalpine meadows ("polonyny") — at heights approximately of 2000 m.s.l. (Chornohirskyi Range, Hoverla Mountain, Petros Mountain). From insects' characteristics that can reflect their adaptation ability to the highlands, we studied (for each of determined altitudes in each region) 1 — insects' species list; 2 — their quantitative co-relations; 3 — differences in insects behavior at different altitudes; 4 — differences in colors or drawings of insect wings at different altitudes [1, 35].

Insect species' composition at Elbrus Region. It has been found that in ZMF band at Elbrus Region some forestrial *Noctuidae* species are the same in both mountainous regions, both in the Caucasus and in the Carpathians; and they were common in most Palearctic regions: *Apamea illyria, Euchalcia variabilis, Xestia ohreago, Diachrysia chrysitis, Syngrapha interrogationis,* and etc. Some species we registered only in the Caucasus, but not in the Carpathians: *Cucullia propingua, Autographa aemula.* There was a small number of steppe species' representatives — some species *Cucullia, Acronicta euphorbiae.* At these altitudes some *Zygaenidae* species (*Z. lonicerae, Z. filipendulae,* and etc.) were well represented. Above 2800 m.s.l. there are only unique samples of *Macrolepidoptera* samples may be collected. At this attitude the number of all species significantly decreased. At the altitude of 3100 m.s.l. only *Muscidae* (including *M. domestica*) and several *Microlepidoptera* specimens were collected, representatives of other insect species disappeared at all [1, 35].

Insect species' composition at Ukrainian Carpathians. According to Prof. Klyuchko Z. F. data [1, 42], and alternatively to Elbrus slopes, the forest *Noctuidae* species dominated here (such as *Euchalcia variabilis,*

Autographa jota, Autographa pulchrina, Apamea illyria, and etc.). From steppe species only *Hyssia cavernosa*, *Heliothis maritima* occurred occasionally. The subalpine zone is less revealed here than in the Caucasus, hence, the differences in fauna here were less visible. For example, *Noctuidae* there were collected both at the height of the ZMF and in the area of sub-alpine meadows. But we could be definitely sure that, like in the Caucasus, certain species of *Microlepidoptera* occur here mainly at the height of the sub-alpine meadows.

Quantitative composition of insects. Analyzing the total number of insects collected at different heights in both regions, it was possible to make preliminary conclusion that the changes in *Noctuidae* number with a height could be described by a curve with a maximum of about 2100 m.s.l. Above this mark the number of *Noctuidae* gradually decreased to zero at altitude of 3100 m.s.l. The curves of number decrease for *Microlepidoptera* and *Muscidae* at the right from the maximum (for the higher altitudes) and these curves were more lenient. It means that the representatives of these insect groups were registered at such altitudes, where *Noctuidae* were not present at all. In the Carpathians, various authors noted the gradual number decrease of insect species below 500 m.s.l. (in the zone of strong anthropogenic influence) [1, 42]. These data are in good correlation with the data of other authors, for other regions of the planet [88].

Differences in the behavior and colors (pattern) of insects. For today, we have registered reliably the behavioral differences only for insects (*Noctuidae*, to the less extent *Muscidae*) with altitude increase at Elbrus slopes. Thus, the samples of insect different species who demonstrated extremely active behavior at altitude 2100 m.s.l. were very passive at altitude 2800 m.s.l.; they stopped their activity even at day time with temperatures higher than 18 °C. (insects look like as "frozen", or "paralysed" at plants). One can assume that oxygen deficiency and adaptation to this was the reason of such behavior. In attempts to collect the insects during the period of activity, they often "imitated a death". Such reaction in the Caucasus above 2800 m.s.l. demonstrated the majority of insects, whereas with altitude decrease up to 2100 m.s.l. such behavior demonstrated only a few individual samples. In the Carpathians the behavior studies in dependence of altitude were not conducted [1, 35].

Development of relational database on bioorganisms' collection materials in the extreme conditions of the Elbrus and Ukrainian Carpathians. Starting the design of relational database, one could see that it can be logically divided into two parts that are almost identical in structure: 1 — DB with materials from the Elbrus and 2 — DB with materials from the Ukrainian Carpathians. So, designing an object model, we will do it initially for one of the mountain regions, for example, for Elbrus, and then we can use this experience for a similar database for the Carpathians insects. Logical DB model was shown on Fig. 5. We propose to distinguish the following main objects: "Insect", "Adaptation characteristics" and "Altitude". On the communication lines between objects the power relations are indicated. For example, the power of the relation "Insect" — "Adaptation characteristics" was 1: 1, since each type of insect has its own set of adaptive characteristics (the reaction of organism is individual). The power of the relation "Insect" — "Atitude" was "many to many", since many different insects are recorded at one altitude, and each insect specie lives in a certain band of altitudes. So, for the prevention of appearance of indefinite relation "many to many" in data model, we suggested to inquire a cross-reference table in the database [1].

Objects in our logical model were characterized by following attributes:

Object "**Insect**"
Attributes:
Primary key
The name of the insect (in Latin, Ukrainian and Russian).
Area of inhabitance.
Biotopes of inhabitance.
Plants of nutrition.
Number of generations during the season.
Periods of fly.
Data of collection.
References to literary sources.
Comments.
Other.

Object "**Altitude**"
Attributes:
Primary key
Altitudes (meters above sea level).
Natural zone, biotope.
Data of collection.
Comments.
Other.

*Object "**Adaptation characteristics**"*
Attributes:
Primary key
Number of insects collected per day.
Number of insects collected per season.
Demonstrates the changes in behavior (if so, then what).
Demonstrates the changes in color (if so, then what).
Demonstrates the changes in the pattern of wings (if so, then what).
Comments.
Other.

Basing on this analysis, we have designed Tables 1, 2, 3. Examples of tables' fragments we presented in our article as Table 1 ("Insects"), Table 2 ("Altitude") and Table 3 ("Adaptation characteristics"). To make tables, we suggested adding the field "Comments" (the biologist or amateur could record some comments) and "Other" (as were noted, the field observations provide often new material that did not fit into standard scheme). These two types of fields were considered as a source of future structural development and database modification [1].

Using this database in working mode, professional-biologist could find out easily what kind of insects live at studied altitude of each mountain region and which adaptive characteristics it reveals at each altitude. Besides of this, he could perform easily all those operations with data that were previously impossible for him: sorting, searching of particular records, data filtering, outputting the data in necessary format, and etc. These opportunities are very important in the daily routine professional work, since the one who works in this sphere almost every day or adds new information to the previous material (recording the data into a table), or makes the alphabet lists of species (by sorting), or output all species of one genus (by filtering), and etc.

Thus, according to the purpose of the work the detailed examination of various ways of ISs with databases design were done, as well as appeared errors were examined. Peculiarities of biological objects which should be taken into account during database design were analyzed as well. Also, the methods for electronic collections with DB of biological

Table 1. **Example of fragment of the Table "Insects"**

P.key	Insect name	Area of inhabitance	Biotopes	Feeding plants	Number of generations per season	Periods of fly	Data of collections	References	Commentary	Other

Table 2. **Example of fragment of the Table "Altitude"**

P.key	Altitude (meters above sea level)	Biotopes	Data of collections	Commentary	Other

Table 3. **Example of fragment of the Table "Adaptation characteristics"**

P.key	Number of insects collected per day	Number of insects collected per season	Changes in behavior	Color changes	Changes in wings' patterns	Commentary	Other

organisms' elaboration were analyzed; as well as appropriate algorithms (Fig. 6) were developed. In our studyings the algorithms for environmental conservation were analyzed and compared with some foreign analogs in order to study positive and negative experiences. The requirements for database with information about the moths were formulated for the development of electronic information system which we called "Ecological Information System" — "EcoIS". The description of the developed relational database with information about insect with the analysis of selected object area were suggested, taking into account the characteristics of biological objects and characteristics of IS analogues.

Recommendations for the construction of DBs with information about living organisms basing on our experience were done as well.

In present article we also wrote briefly the information about our developed electronic network system with database about *Noctuidae* — "EcoIS". We reviewed the theoretical basis of "EcoIS" and did a brief analysis of the process of this system development. After the elaboration of databases with information about *Noctuidae* (*Lepidoptera*), the data from it could be used for many purposes. One of the most important, from the point of view of economy, areas of further application of these data is the development of a network system

| SPECIE | 1:N | Specie/ altitude | N:1 | ALTITUDE | Altitude/adaptation characteristics | ADAPTATION CHARACTERISTICS |
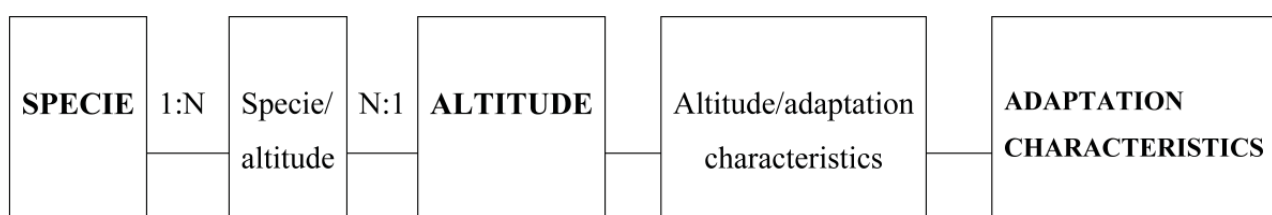
Fig. 5. **Logical model of DB about adaptation characteristics of insects in highlands**

Collection of information about the organism (sample) in field expedition conditions	Creation of mathematical (computer) models using databases
Primary data processing (definition of species, data digitizing, etc.)	Monitoring of changes in populations during defined period of time
Completing of electronic collections about insects with data about them	Monitoring of changes in models during defined period of time
Elaboration of electronic databases	Conclusions about the harmful effects of different factors on populations (urbanization, technogenic influence, etc.)
Development of networks with distributed electronic databases	Recommendations about neutralizing of harmful influence on populations
Stage 1	*Stage 2*

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

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for environmental monitoring of fauna at industrial regions of Ukraine. "EcoIS" there makes it possible to monitor harmful effects of industrial pollution on the population using *Noctuidae (Lepidoptera) as* good bioindicator. Besides of this, there are many pests of forest and crops among *Noctuidae*, and the practice shows that the spreading of such pests has to be monitored in order to prevent their mass reproduction and subsequent destruction

of environment. In general, the use of the developed technical IS with DBs "EcoIS" is aimed on ecological monitoring of insect fauna of Ukraine, first of all, on eco-monitoring of bioindicator fauna (*Noctuidae, Lepidoptera*) for the purpose of better nature conservation and for the prevention of crops losses in agriculture, well as for minimization of risks for people health and lives in industrial polluted areas.

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ЕЛЕКТРОННІ БАЗИ ДАНИХ ЧЛЕНИСТОНОГИХ: МЕТОДИ ТА ЗАСТОСУВАННЯ

О. М. Ключко[1], *З. Ф. Ключко*[2]

[1]Інститут експериментальної патології, онкології та радіобіології ім. Р. Є.Кавецького НАН України, Київ
[2]Інститут зоології ім. І. І. Шмальгаузена НАН України, Київ

E-mail: kelenaXX@ukr.net

Метою роботи був аналіз створення різних інформаційних систем з базами даних, а також помилок, що при цьому виникають. З'ясовано особливості біологічних об'єктів, які слід враховувати під час проектування баз даних. Описано також способи створення електронних колекцій біологічних організмів для вітчизняних потреб на основі баз даних з відповідними алгоритмами для природозбереження. Ці дані зіставлено з деякими зарубіжними аналогами з метою оцінювання позитивного та негативного досвіду. Сформульовано вимоги до баз даних з інформацією щодо нічниць-совок *Noctuidae* (*Lepidoptera*) та деяких павукоподібних *Araneidae* для розробленої нами електронної інформаційної системи «ЕкоІС». Подано опис створюваної реляційної бази даних з інформацією про комах та аналізом обраної об'єктної області, з урахуванням особливостей біологічних об'єктів і характеристик аналогів інформаційних систем. Зроблено висновки щодо нових засобів розроблення баз даних біооб'єктів та застосування їх на прикладі системи «ЕкоІС», а також наведено деякі рекомендації з конструювання баз даних з інформацією про живі організми.

Ключові слова: біоіндикатори, електронні інформаційні системи, бази даних членистоногих, бази даних комах *Noctuidae* (*Lepidoptera*), *Araneidae*.

ЭЛЕКТРОННЫЕ БАЗЫ ДАННЫХ ЧЛЕНИСТОНОГИХ: МЕТОДЫ И ПРИМЕНЕНИЕ

Е. М. Ключко[1], *З. Ф. Ключко*[2]

[1]Институт экспериментальной патологии, онкологии и радиобиологии им. Р. Е. Кавецкого НАН Украины, Киев
[2]Институт зоологии им. И. И. Шмальгаузена НАН Украины, Киев

E-mail: kelenaXX@ukr.net

Целью работы был анализ создания различных информационных систем с базами данных, а также возникающих при этом ошибок. Описаны особенности биологических объектов, которые следует учитывать при проектировании баз данных. Изложены также способы создания электронных коллекций биологических организмов для отечественных потребностей на основе баз данных с соответствующими алгоритмами для природосбережения. Эти данные сопоставлены с некоторыми зарубежными аналогами для оценки положительного и отрицательного опыта. Сформулированы требования к базам данных с информацией о бабочках *Noctuidae* (*Lepidoptera*) и некоторых паукоподобных (*Araneidae*) для разработанной нами электронной информационной системы «ЭкоИС». Приведено описание создаваемой реляционной базы данных с информацией о насекомых и анализом выбранной объектной области, с учетом особенностей биологических объектов и характеристик аналогов информационных систем. Сделаны выводы о новых средствах разработки баз данных биообъектов и их применении на примере системы «ЭкоИС», а также даны некоторые рекомендации по конструированию баз данных с информацией о живых организмах.

Ключевые слова: биоиндикаторы, электронные информационные системы, базы данных членистоногих, базы данных насекомых *Noctuidae* (*Lepidoptera*), *Araneidae*.

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EXPERIMENTAL ARTICLES

EFFECT OF ORGANIC MICROELEMENTS IN LIPOSOMAL FORM ON FERTILIZING ABILITY AND THE LEVEL OF ANTIOXIDANT REACTIONS OF FEMALE RABBITS

O. V. Shtapenko[1]
I. I. Gevkan[1]
Yu. I. Slyvchyk[1]
Ye. O. Dzen[1]
V. Ya. Syrvatka[2]
N. M. Matvienko[3]

[1]Institute of Animal Biology of the National Academy of Agrarian Sciences of Ukraine, Lviv

[2]Ivan Franko National University of Lviv, Ukraine

[3]Institute of Fisheries of the National Academy of Agrarian Sciences of Ukraine, Kyiv

E-mail: shtapenko31@gmail.com

The purpose of this study was to investigate the effect of supplementation with organic zinc, manganese and chromium in the form of liposomal complex on the fertilizing ability and the level of antioxidant responses of female rabbits. Feeding of female rabbits with supplementation of organic forms of trace elements prior to insemination resulted in increase the numbers of corpora lutea, implantation and living fetuses compared to the control group. Moreover, there were the 4,37% and 1,74% decrease in pre- and post-implantation losses in animals receiving the organic microelements prior to insemination, respectively. The level of thiobarbituric-acid-reacting substances in ovary of experimental group was significantly higher ($P \le 0.05$) compare to the control group, while the level of lipid hydroperoxides in experimental group was decreased. In the uterus of rabbits after addition organic compound of trace elements significantly decreasing the thiobarbituric-acid-reacting substances level was by compare to the control animals ($P \le 0.001$). The level of the superoxide dismutase activity in uterus and ovary of female rabbits in the experimental group were significantly higher than in the control group ($P \le 0.01$). Our studies indicated that supplementation organic microelements in liposomal form to the basal diet for 2 weeks before insemination had a beneficial effect on the metabolism intensity and maintaining antioxidant-prooxidant balance in reproductive organs that improve fertilization and embryo implantation.

Key words: organic forms of trace elements, female rabbits, antioxidant reactions.

Modern rabbit breeding is based on high-intensity using of females because the does are being inseminated every 35 or 42 days. In such a system, the females need a large of energy [1]. Reduction in fertility, may occur as a result of poor nutrition, bad feed, incorrect lighting program, suckling a large litter, health problems, moulting, etc. [2]. Deficiency in the nutrient supply during pregnancy has been highlighted as a dominant cause of developmental programming. Manal et al. found that timing of feed restriction is important for reproductive performance in rabbits [3] and also it in late pregnancy have led to increasing mortality of new-born rabbit kits [4]. In accordance with the previous researchers, Goliomytis et al. found that maternal feed restriction during gestation had an adverse effect ($P \le 0.05$) on ratio of stillborn kits and significantly increased mortality rates [5]. Forages are the main source of trace elements for the rabbits, but its contents in forages are very variable; so when the mineral supplementation level is insufficient, reproductive problems may be occur.

Among of all trace minerals, zinc, chromium, and manganese are accumulated in the highest concentration in the conceptus, as compared to other reproductive tissues, which suggests that they play a significant role in fetal development and survival [6]. Furthermore, some research results suggest that feeding proteinated zinc, chromium and manganese to gestating females increases reproductive performance presumably because of their improved bioavailability [6]. However, the maternal-fetal transfer mechanisms of these micronutrients are not well understood. Micronutrient transport mechanisms for the conceptus are developed during the first half of pregnancy and nutrient transport is probably active rather than passive across the maternal-fetal blood barrier with specific receptors on endometrial and conceptus tissues [7].

Maternal zinc deficiency may compromise infant development and lead to poor birth outcomes. The trace elements status in general and zinc in particular of new-born depends on maternal transfer via the placenta, the colostrums or the milk [8]. Zn levels in fetuses are 1.7 to 8.7 times greater than in the endometrium and ovaries at 12 to 30 days of development [9]. Fetal storage of Mn and Cr were dependent on maternal dietary intake. Likely they play a role in fetal bone formation and may initiate estradiol secretion by the conceptus for pregnancy recognition and perhaps in progesterone.

Pregnancy is characterized by physiological changes in antioxidant systems. Oxidative stress play a role in remodeling of uterine tissues, implantation of the embryo, settlement of the villi and development of blood vessels characteristic of gestation [10]. Deficiencies of the vitamin and trace elements can lead to exaggerate oxidative stress and can induce the adverse effects on the health of the mother and on the development and viability of the new-born.

The aim of this study was to evaluate the effect of supplementation with organic form of zinc, manganese and chromium in liposomal to the basal diet on the 14th day prior to insemination on the level of antioxidant responses and reproductive function of females rabbits during the early stage of pregnancy.

Materials and Methods

The study was conducted on females rabbits divided into two groups: the control group and experimental group. The control group was fed the basal diet while the experimental group was supplemented with Zn glutamate (35 µg/kg), Mn glutamate (32 µg/kg), Cr methionate (60 µg/kg), Se (20 µg/kg) with vitamins E (20 mg), A (30 000 IU), D (40 000 IU) two weeks before insemination. All group of animals were artificially inseminated with appropriate hormonal treatment. We used 40 IU PMSG (Pregnant Mare Serum Gonadotropin, Follimag, Intervet, Holland) for synchronized cycle (injected 48 h before AI) and 20 µg/doe GnRH (Gonadotropin-releasing hormone) (Fertagil, Intervet, Holland) for induction of ovulation (injected at the moment of insemination). Rabbits were fertilized intravaginally of 10×10^6 spermatozoa/ doe in 0.5 ml tris-citrate diluents.

On day 14th of gestation rabbits were euthanized with an overdose of sodium pentobarbital, and the ovaries were removed for evaluation the number of corpora lutea. Gravid and non-gravid uterine horns were weighed and the number of dead and live fetuses, number of implantations and resorptions in the uterine horns were recorded. Implantation index [(No of implants/No of corpora lutea) × 100], preimplantation losses [(No of corpora lutea — No of implants)/No of corpora lutea] × 100 and postimplantation losses [(No of implants − No of viable fetuses)/ No — of implants] × 100 were also evaluated.

The procedures and use of the animals in this experiment were approved by the Institution Ethical Committee on the policy statement for care and use of laboratory animals. The rabbits were housed in individual cages, maintained under controlled light/dark cycles (12L:12D) and fed *ad libitum* with a commercial pelleted diet and to water via an automatic water supply system.

The tissues samples (uterus and ovaries) were collected for determining antioxidant enzyme activities and levels of lipid and protein peroxides. Each uterus and ovarian sample was homogenized in cold Tris-HCl buffer (100 mM, pH 7.4) to obtain a 10% (w/v) tissue homogenate. The homogenate was then centrifuged at 5 000 ×g for 15 min. Each supernatant was collected and stored at −20 °C until use. The protein content of each sample was determined using Bradford method (1976) and bovine serum albumin as the standard. An aliquot of the homogenate was used to determine the lipid peroxidation reactions of the sample by measuring the concentration of thiobarbituric-acid-reacting substances (TBARS), carbonyl groups as an indication of oxidative damage to proteins, as well as superoxide dismutase (SOD), catalase

(CAT), lipid hydroperoxides. All enzymatic assays were carried out at 25 ± 0.5 °C using spectrophotometer SF 46 (Carl Zeiss Jena, Germany). Each sample was analyzed in triplicate.

Lipid peroxidation level was determined by quantifying the concentration of TBARS, expressed as µmol of malondialdehyde (MDA) per mg of protein, according to Kamyshnikov [11]. The TBARS level was expressed in µmol MDA per mg protein by using $1.56 \cdot 10^5 \, \text{mM}^{-1} \text{cm}^{-1}$ as molar extinction coefficient.

Lipid hydroperoxides (LHP) assay was developed by modifying the FOX methods described by Wolff (1994) involves the oxidation of Fe^{2-} by peroxides at low pH in the presence of both the ferric-complexing dye xylenol orange and sucrose, the amplifier of the reaction. The method proved to be a convenient, simple and efficient assay for the direct measurement of both water and lipid soluble peroxides. In fact it improves by about 60% the sensitivity of the FOX1 method for water soluble peroxides, and by 7−8 times than of the FOX2 method for lipid soluble peroxides. It allows the detection of 0.1 µM peroxide in the test solution. The method is suitable to measure the lipid hydroperoxides present in phosphatidylcholine liposomes and in human LDL. The data obtained allowed us to define a mathematical expression to calculate the lipid hydroperoxide content of liposomes knowing their oxidation index [12].

Diene conjugates (DC) were evaluated by measuring the optical density of the lipids at 232 nm on the SF-46. Lipids were read in cyclohexane (0.2−0.3 mg/ml) and the optical density (OD). OD/mg lipid was calculated. Lipid content of samples was measured by Chiang [13].

Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was measured with the method by Kostiuk et al. [14]. SOD activity was assessed by its ability to dismutate superoxide produced during quercetin auto-oxidation in an alkaline medium (pH=10.0). Absorbance at 406 nm was measured immediately and after 20 min. Activity is expressed in units of SOD per mg of tissue protein.

Catalase (CAT, E.C. 1.11.1.6) activity was determined by measuring the decrease of H_2O_2 in the reaction mixture using a spectrophotometer at the wavelength of 410 nm by the method of Koroliuk et al., 1988 [15]. The absorbance of solution was measured at 410 nm and was compared with that of the blank. One unit of catalase activity is defined as the amount of enzyme required for decomposition of 1 µmol H_2O_2 per min per mg of protein.

Tests were repeated three times for every type of the samples. The results were presented as mean ± standard deviation. Differences between groups were determined by Student *t*-tests.

Results and Discussion

The different reproductive performance parameters like corpus lutea, number of implantation and fetuses, resorptions sites, fertility rates were detected in the group supplemented with organic trace elements in liposomal form for 2 weeks before insemination and in control (Table).

The data analysis showed that the numbers of corpora lutea, implantation and living fetuses in the experimental group increased in comparison with the control one. The values of resorption in the female rabbits treated with

Effect of liposome preparation on reproductive performance of female rabbits

Parameters	Control group	Experimental group
Number of corpus lutea	10.4±0.4	11.0±0.45
Number of implantation sites	9.4±0.4	10.6±0.74
Total Live Fetuses	8.6±0.4	9.8±0.49
Number of resorptions sites	0.4±0.24	0.2±0.24
Pre-implantation losses (%)	13.46	9.09
Post-implantation loss (%)	4.44	2.70
Total gestational losses	17.31	10.91

Note. The results are represented as $M \pm m$ for 5 rabbits in each group.
For all values $P \geq 0.05$ compared to control, $n = 5$.

liposomal preparation were by 2-times lower as compared to the control group. While the pre- and post-implantation losses decreased in liposomal treated group.

The decrease of gestational losses in this study is the result of supplementation with organic zinc, manganese and chromium in liposomal form before insemination which induces nutrient supply to the fetuses. In a similar study, Stanton et al. [16] observed higher pregnancy rate in cows receiving organic Cu, Zn, and Mn vs. the inorganic forms.

The correlation between nutrition and reproductive performance has been investigated in different animals. Phiri E. C. J. H. et al. [17] found that ewes receiving zinc supplementation had a higher fertility rate and were more prolific (89% vs. 40%). Also, Tang Xiao-lin et al. [18] compared the effects of chitooligosaccharide-zinc (COS-Zn) as a new organic zinc source with zinc sulfate on the growth and reproductive performance of female mice and found that the utero-ovarian index and the number of embryos were significantly increased in COS-Zn group ($P \leq$ 0.05).

In some studies it has been indicated that changes in mineral metabolism could have a harmful effect on fetal growth [19, 20]. For example, zinc element of mothers affects fetal growth, levels of serum Insulin-like Growth Factor I and leptin [21]. In addition, another systematic review showed that zinc supplementation during pregnancy is associated with a 14% reduction in premature deliveries [22].

In Fig. 1, the changes in the intensity of peroxidation processes after supplements of organic forms of trace elements in the reproductive organs of rabbits were determined.
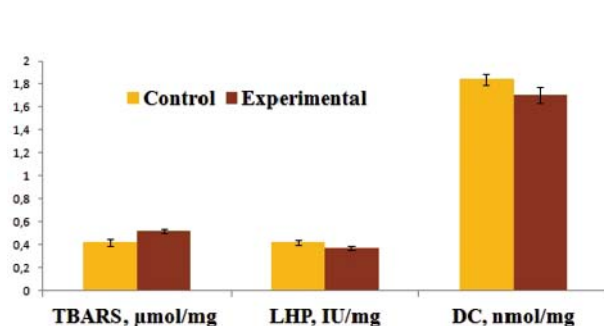
The level of TBARs in ovary of experimental group was significantly higher ($P \leq 0.05$) as compared to the control group, while the lipid hydroperoxides level was decreased in experimental group (Fig. 1).

In the uterus of rabbits after addition of organic compound of trace elements significantly decreasing of TBARs level was compared to that parameter in the control animals ($P \leq 0,001$) (Fig. 2). Content of diene conjugates also was lower in the experimental group. While the level of lipid hydroperoxides in experimental group was significantly increased after adding of preparation, as compared to the control group.

The improvement of reproductive performance in our current results for the group supplemented with organic forms of trace elements as compared to the control were accompanied by using liposomal emulsion with trace elements in organic form during the fertilization period of rabbits. So that, for instance, Mn^{2+} inhibits the free radical chain which follows the formation of hydroperoxides and that lead to the formation of MDA. It has been reported that Mn^{2+} is able to reduse the lipid free radicals (RO and ROO) making them unable to carry on the process of LPO [23].

During the embryo implantation, the increased fluidity in the membranes of endometrial cells is caused by a slight increase in lipid peroxidation, aids the fusion of the trophectoderm with the endometrial cells [24]. It has been suggested that Mn^{2+} supplementation inhibits LPO, thus increasing the membrane integrity and viability, which are required for storage of lipids and phospholipids [25]. It has been reported that Mn^{2+} protects placental membrane from peroxidative damage produced by the superoxide radicals (O_2^{2-}) [26].

Fig. 1. **Effect of organic microelements in liposomal form on the level of oxidative stress biomarkers in the ovarian of rabbits**
* — $P \leq 0,05$ compared to control, $n = 5$.
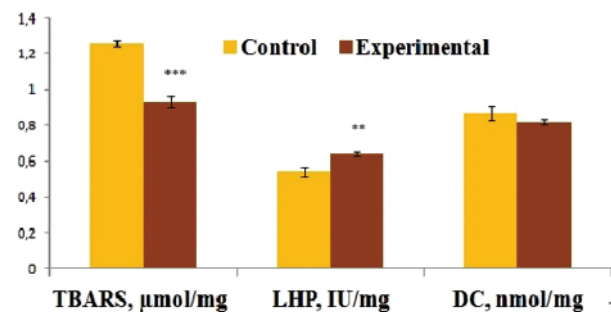
Fig. 2. **Effect of organic microelements in liposomal form in the TBARS, LHP and DC in the uterus of rabbits**
Here and after: *** — $P \leq 0.01$, ** — $P \leq 0.001$ compared to control, $n = 5$.
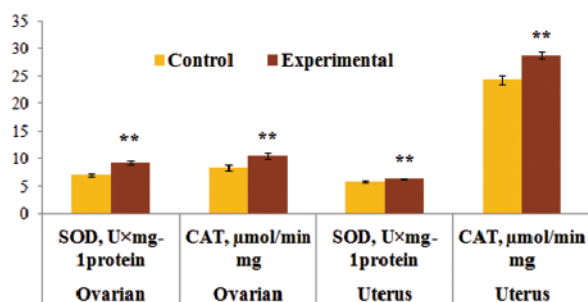
Fig. 3. **Effect of organic microelements
in liposomal form on SOD and CAT activity
in the ovaries and uterus of rabbits**

Several studies have indicated that antioxidative defense is modified during normal pregnancy [27]. It is worthwhile to mention that the decrease of antioxidant enzymes level during pregnancy is dependent on trace elements profile. Accordingly, addition of organic trace elements in liposomal form to the basal diets caused activating effects on antioxidant enzymes superoxide dismutase and catalase.

The level of the SOD activity ($P \leq 0.01$) in uterus and ovary of female rabbits in the experimental group were significantly higher than in the control group (Fig. 3). The activity of catalase in those tissues of experimental groups were also significantly increased, as compared with the control group ($P \leq 0.01$).

Oxidative stress is generated during normal placental development; however, when the supply of antioxidant micronutrients is limited, exaggerated oxidative stress within both the placenta and maternal circulation occurs, resulting in adverse pregnancy outcomes [28]. Manganese and zinc are essential metal acts as a cofactor of various enzymatic systems such as SOD and CAT, and participate in the structure of ceruloplasmin

[29] and, therefore, play important biological functions. The deficiency of these essential trace elements could have devastating effects on the health of the mother, fetus and newborn. Use of organically complexed trace minerals can help prevent these losses, due to their increased stability in the upper gastrointestinal tract of the animal. In current investigation we used liposomal emulsion as a method of direct delivery of trace elements in organic form to the target organs, where the active compounds of our formulation expressed their biological action. Our results have been supported by Tian X. and Diaz F.J. studies [30]. They show that feeding a zinc deficient diet for 3–5 days before ovulation (preconception) dramatically decreases oocyte quality and developmental potential including a decrease in DNA and histone methylation and associated increase in expression of repetitive elements. These epigenetic defects along with previously shown meiotic defects [31] severely compromise fertilization and preimplantation embryonic development.

The result of our studies indicate that supplementation organic microelements in liposomal form to the basal diet on the 14th day prior to insemination provided an increase of reproductive ability — growth and development of embryos and their implantation and improvement in the antioxidant activity, and decrease the oxidative stress in female rabbits during early state of gestation.

In conclusion, supplementation of rabbit does to the basal diet prior to insemination mineral biocomplex improved fertilizing ability in particular it at 4,37% and 1,74% decreased pre- and post-implantation losses. This suggests that organic trace elements in liposomal form able to compensate microelements losses that the usual may occur during gestation.

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ВПЛИВ ОРГАНІЧНОЇ ЛІПОСОМАЛЬНОЇ ФОРМИ МІКРОЕЛЕМЕНТІВ НА ЗАПЛІДНЮВАЛЬНУ ЗДАТНІСТЬ ТА РІВЕНЬ АНТИОКСИДАНТНИХ РЕАКЦІЙ САМИЦЬ КРОЛІВ

О. В. Штапенко[1], І. І. Гевкан[1],
Ю. І. Сливчук[1], Є. О. Дзень[1],
В. Я. Сирватка[2], Н. М. Матвієнко[3]

[1]Інститут біології тварин Національної академії аграрних наук України, Львів
[2]Львівський національний університет імені Івана Франка, Україна
[3]Інститут рибного господарства Національної академії аграрних наук, Київ, Україна

E-mail: shtapenko31@gmail.com

Метою роботи було вивчення впливу органічних сполук цинку, мангану та хрому у формі ліпосомального комплексу на запліднювальну здатність та рівень антиоксидантних реакцій самиць кролів. Згодовування кролицям органічних сполук мікроелементів підвищує кількість жовтих тіл вагітності, імплантацій та живих ембріонів порівняно з контрольною групою. Окрім того, у тварин, які отримували органічні мікроелементи перед осіменінням, відзначено зниження на 4,4% та 1,7% передімплантаційних і постімплантаційних втрат. Рівень ТБК-активних продуктів у яєчниках самиць дослідної групи був вірогідно вищий ($P \leq 0,05$), ніж у контрольній групі, тоді як вміст гідропероксидів ліпідів знижувався. У матці кролиць після додавання органічних сполук мікроелементів істотно знижувався вміст ТБК-активних продуктів порівняно з аналогічним показником у контрольній групі ($P \leq 0,001$). Активність супероксиддисмутази у матці та яєчниках тварин дослідної групи була вірогідно вищою порівняно з контролем ($P \leq 0,01$). Наші дослідження показали, що додавання органічних сполук мікроелементів у ліпосомальній формі до основної дієти впродовж двох тижнів до осіменіння позитивно впливає на інтенсивність обмінних процесів у репродуктивних органах та зберігання антиоксидантно-прооксидантної рівноваги, що покращує запліднюваність та імплантацію ембріонів.

Ключові слова: органічні форми мікроелементів, самиці кролів, антиоксидантні реакції.

APPLICATION OF GLUTAMATE-SENSITIVE BIOSENSOR FOR ANALYSIS OF FOODSTUFF

D. Yu. Kucherenko[1]
I. S. Kucherenko[2]
O. O. Soldatkin[1, 2]
A. P. Soldatkin[1, 2]

[1]Taras Shevchenko Kyiv National University, Ukraine

[2]Institute of Molecular Biology and Genetics
 of the National Academy of Sciences of Ukraine, Kyiv

E-mail: didukh.d@gmail.com

The aim of the work were the optimization of an amperometric glutamate-sensitive biosensor and its utilization for the determination of the glutamate concentrations in food samples. Amperometric method of measurements was used. The biosensor was based on immobilized glutamate oxidase and platinum disc electrode. The biosensor was connected to the working cell with auxiliary (platinum wire) and reference (Ag/AgCl) electrodes. The biosensor exhibited high sensitivity to glutamate, duration of one analysis was about 5 min. An influence of the ionic strength, pH, and buffer capacity on the biosensor operation was investigated. The sensitivity of biosensor to various possible interfering substances, including amino acids, was studied; high selectivity to glutamate was shown. The reproducibility of analysis of food samples and an impact of sample dilution was evaluated. Glutamate concentrations in different sauces and seasonings were measured by the developed biosensor; the results correlated well with those obtained by the spectrophotometric method ($R^2 = 0,988$).

Thus, the amperometric biosensor for glutamate determination was successfully optimized and used for measurement of glutamate concentrations in sauces and seasonings.

Key words: amperometric biosensor, glutamate oxidase, poly(phenylenediamine), glutamate, food samples.

Glutamate (in the form of glutamic acid sodium salt) is a well-known nutritional supplement often used as a flavor enhancer in soups, sauces, chips, seasonings and other types of foods. It can become habit-forming, which makes people consume more and more products with high glutamate level leading to obesity and cardiovascular disease. In some people, excess of glutamate in the body can cause dizziness, headache, numbness, chest pain [1, 2]. Moreover, excessive intake of glutamate can result in disturbance of the endogenous glutamate metabolism, which is associated with the emergence of such diseases as Alzheimer's and Parkinson's, and amyotrophic lateral sclerosis [3, 4].

One more cause of necessity to control the glutamate content is the prevention and detection of falsified products. Considering the glutamate property to give a pleasant taste to almost any product, dishonest manufacturers might add glutamate to inferior foodstuff. Therefore, to avoid the negative consequences of consuming poor-quality products, it is necessary to develop new express methods for glutamate monitoring.

The methods mainly used for glutamate determination are following: high-performance liquid chromatography, capillary electrophoresis, potentiometric and conductometric titration, gas chromatography [5–7]. Combination of enzymatic methods with spectrophotometry can be used too [8]. Additionally, the method of chemiluminiscent determination is reported involving luminol and potassium ferricyanide and using a luminophotometer [9].

However, the above methods involve difficult and time-consuming procedures and complicated sample pretreatment, they are unsuitable for quick analysis of a large number of samples and require complex expensive equipment and skilled personnel [10].

The use of biosensors can be an alternative to these methods. Amperometric biosensors are the most promising and frequently used for glutamate determination. However, high

working potential of amperometric biosensors can cause the oxidation of electrochemically active compounds present in the sample on the transducer surface. To avoid the influence of this effect, different methods are used: modification of the electrode surface, application of various mediators, bienzyme electrodes, combination of several membranes, etc. Several types of glutamate biosensors were reported, including those based on L-glutamate oxidase, L-glutamate dehydrogenase, L-glutamate synthase and L-glutamate decarboxylase [11–15]. L-glutamate oxidase, as compared with the rest of mentioned enzymes, provides a relatively high specificity to the substrate and does not require additional expensive coenzyme NAD^+, thus it is used most frequently for the glutamate determination [15–17].

The glutamate concentration is most often evaluated via detecting hydrogen peroxide or ammonia using electrochemical transducers, occasionally — via detecting oxygen consumption by a fiber optic sensor, which registers the changes in luminescence of a special layer sensitive to the oxygen concentration [18].

An optical biosensor in the test-strip format was created for glutamate analysis in food samples [19]. The peculiarity of this biosensor consists in simultaneous immobilization of several sensitive agents (one indicator dye — 3,3′,5,5′-tetramethylbenzidine, and two enzymes — L-glutamate oxidase and horseradish peroxidase) using a composite membrane system with non-covalent bonding of sensitive components. Several sensors combined with flow-injection systems were created for glutamate determination. For example, a biosensor based on a gold electrode with immobilized glutamate oxidase and a polyionic complex for preventing electrochemical interferents was described [20]; in another work a solid-state electrode based on non-plasticized chitosan was proposed for glutamate determination in food samples (seasoning and soups) [21]. However, the mentioned works do not present reproducibility of biosensor preparation, and have insufficient examination of the biosensors selectivity towards possible interfering substances. Furthermore, the possibility of application of the biosensors for multiple measurements of food samples was not shown in the most cases. We have earlier developed a biosensor for the monitoring glutamate uptake and release from the isolated brain nerve terminals and showed good correlation of the biosensor results with those obtained using radiolabelled glutamate assay, spectrofluorimetric glutamate dehydrogenase assay and amino acid analyzer [22, 23]. In this work we wanted to optimize and apply the biosensor for a food analysis.

The purposes of this work were optimization of the amperometric glutamate-sensitive biosensor based on glutamate oxidase, comprehensive analysis of its selectivity, reproducibility of preparation and other characteristics and application for measurements of the glutamate concentration in food products — sauces and seasonings.

Materials and Methods

Materials. In this work recombinant glutamate oxidase (GluOx, EC 1.4.3.11) from *Streptomyces* sp. with activity of 7 U/mg (Yamasa Corporation, Tokyo, Japan) was used for biosensor creation. For spectrophotometric measurements, horseradish peroxidase (EC 1.11.1.7) with activity of 150 U/mg from Sigma-Aldrich Chemie (USA) was used. Bovine serum albumin (BSA, fraction V), glycerol, HEPES, *m*-phenylenediamine, 4-aminoantipyrine, 3-(N-Ethyl-3-methylanilino)-2-hydroxypropanesulfonic acid sodium salt (TOOS), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA) disodium salt, NaN_3 and 25% aqueous glutaraldehyde solution were obtained from Sigma-Aldrich Chemie (USA). Monosodium L-glutamate (as a substrate of glutamate oxidase) was from Sigma-Aldrich Chemie (USA). Other inorganic compounds were domestically produced and had analytical grade of purity.

Sauces and seasonings were bought in supermarkets in Kyiv (Ukraine).

Preparation of biosensor. In the work, platinum disk electrode was utilized as amperometric transducer. Its scheme and preparation procedure was presented in [23]. The transducers were used repeatedly; their sensitive surfaces were cleaned with ethyl alcohol and cotton wool before every procedure of enzyme immobilization.

First, the transducers were modified with phenylenediamine membrane to improve the selectivity of the biosensor. The membrane was deposited according to the procedure described in [24].

Biorecognition elements of the biosensors were obtained by covalent immobilization of the GluOx in BSA membrane onto the sensitive surface of amperometric transducers. The

initial solution contained 8% of GluOx (hereafter — mass fraction), 4% of BSA, and 10% glycerol in 100 mM phosphate buffer, pH 6.5. Glycerol was added to stabilize the enzyme during its immobilization, to prevent early drying of the solution and to improve the membrane adhesion to the transducer surface. This solution was mixed with 0.8% aqueous solution of glutaraldehyde (crosslinking agent) in a ratio of 1:1 and immediately afterwards deposited (drop-casted) onto the transducer, which were next air dried for 30 min at room temperature. Final deposited volume of the enzyme/glutaraldehyde mixture was approximately 0.1 µl. After immobilization, the biosensors were washed in a working buffer solution from unbound components of the biomembrane and excess of glutaraldehyde.

Measuring procedure. The three-electrode circuit of amperometric analysis was used in this work. Working amperometric electrodes, auxiliary platinum electrode and Ag/AgCl reference electrode were connected to PalmSens potentiostat (Palm Instruments BV, the Netherlands). General view of the biosensor setup is presented in Fig. 1. The measurements were carried out at room temperature in a 3.5 ml open measuring cell at continuous stirring and constant working potential of +0.6 V vs Ag/AgCl reference electrode, which corresponds to the anodic oxidation of hydrogen peroxide. 25 mM HEPES buffer, pH 7.4, served as a working buffer. Concentration of substrates in the working cell was obtained by adding aliquots of the substrate stock solutions. All experiments were performed at least in three replicates. The data shown in the tables and figures are the average of three experiments ± standard deviation.
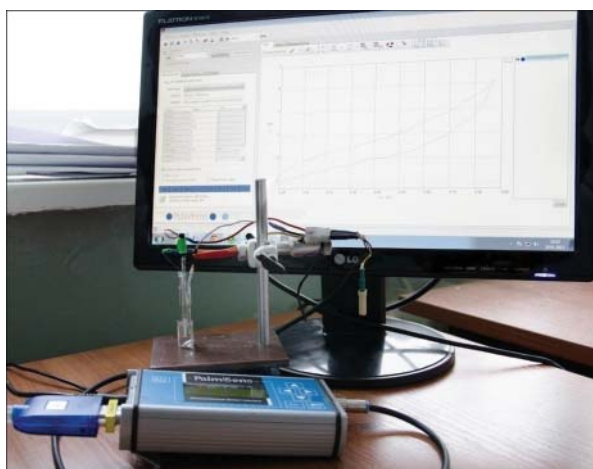
Fig. 1. **General view of the biosensor setup (potentiostat, working cell with three electrodes, and computer)**

Methods of biosensor analysis of glutamate in real samples. Before measurements, the sauces were 10-fold diluted with distilled water. The aliquots of this solution were next added to the measuring cell containing working buffer. The seasonings (dry powder) were dissolved in hot distilled water; the mass fraction of seasoning in solution was 1%. After cooling, the seasoning solutions were filtered through a filter paper for removal of dried vegetables and other insoluble components.

Two methods were utilized for measurement — comparison with the calibration curve and method of standard additions. In the first method, the glutamate concentration was determined by comparing the biosensor response after the sample addition to the measuring cell with the previously obtained calibration graph. In the standard additions method, first a biosensor response to the sample was measured, afterwards the glutamate model solution was added to the working cell three times in turn and responses were measured (without washing between the measurements). Thus, four sequential responses were obtained (in the form of a stairs). Based on the data received, a line chart was plotted — the glutamate concentrations in solution were put on the X-axis, the biosensor responses (in nA) — on the Y-axis; the unknown glutamate concentration (the first response) was taken as a zero X value. Linear extrapolation of this curve crosses the X-axis at a point corresponding to the analyte concentration in the tested sample.

Determination of glutamate in real samples by spectrophotometric method. Spectrophotometric measurements were carried out in 1 ml disposable plastic cuvettes. The composition of solution in the cuvette was as follows: 25 mM HEPES buffer, pH 7.4, 0.3 mM TOOS, 0.1 mM 4-aminoantipyrine, horseradish peroxidase (1.8 U), and an aliquot of glutamate sample (sauce, seasoning or model solution). The reaction started after the addition of GluOx (0.112 U) to the cuvette. In the course of reaction, GluOx oxidized glutamate to α-ketoglutarate and produce hydrogen peroxide. Horseradish peroxidase in the presence of hydrogen peroxide formed a colored (violet) product through oxidation of TOOS and 4-aminoantipyrine. The intensity of the solution color was proportional to the hydrogen peroxide concentration, and hence to the glutamate concentration. The reaction was carried out for 8 min (the time of incubation was determined previously to attain the maximum level of the solution color).
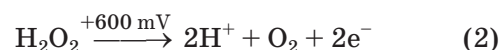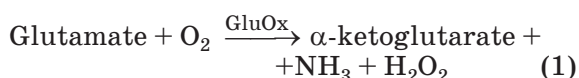
The intensity of light absorption by solution was measured by the Thermo Electron Corporation Bio Mate 5 spectrophotometer. The light wavelength was 555 nm. It was chosen because the highest peak of light absorption by the reaction product was observed at this wavelength. Before the GluOx addition, the blank light absorbance was determined for each solution. This value was subtracted from the value of post-reaction absorbance (in most cases, the basic absorbance was insignificant and could be ignored, except for the most colored sauces).

To obtain a calibration curve, the aliquots of a model glutamate solution were sequentially added to the cuvette with 8-min intervals and the absorbance values were fixed. Totally, six additions were made up to the final glutamate concentration of 120 µM. The calibration curve of dependence of absorbance on the glutamate concentration was then plotted; actually, it was a straight line . The glutamate concentration in sauces and seasonings was determined comparing the samples absorbance with this calibration curve.

The glutamate concentration in the sample was also determined by a proportion (simplified method of standard additions). After measurement of the post-reaction absorbance of the food sample, a model glutamate solution was added to the same cuvette up to the final glutamate concentration of 20 µM; after 8-min interval, the sample absorbance was measured again. The initial (unknown) glutamate concentration in the sample was determined by the ratio between the values of absorbance before and after addition of 20 µM glutamate.

Results and Discussion

Principle of biosensor operation. Determination of glutamate by the amperometric biosensor is based on the enzymatic reaction (1) in the biorecognition membrane, which results in the oxidation of glutamate and the formation of hydrogen peroxide. A positive potential (+0.6 V vs Ag/AgCl) was applied to the transducer, and for this reason hydrogen peroxide was decomposed in reaction (2), resulting in the formation of electrons, which were directly registered by the amperometric transducer:

$$\text{Glutamate} + O_2 \xrightarrow{\text{GluOx}} \alpha\text{-ketoglutarate} + {} + NH_3 + H_2O_2 \qquad (1)$$

$$H_2O_2 \xrightarrow{+600\,\text{mV}} 2H^+ + O_2 + 2e^- \qquad (2)$$

The biosensor response occured immediately after the glutamate addition and the maximum response was observed in 1.5 min.

Influence of the working buffer parameters on the biosensor operation. It is known that the work of any biosensor depends on both its own characteristics and the properties of working buffer solution, in which the measurements are carried out, namely, ionic strength, pH and buffer capacity. Food products, in particular sauces and seasonings, are characterized by a significant ionic strength due to the presence of nutritional additives such as sodium chloride, various acids, preservatives, etc. The ionic strength of solution also depends on the buffer concentration. Therefore, the biosensor operation was studied depending on the value of ionic strength. As a source of ions, different aliquots of 3.3 M NaCl solution were added to the working cell. Next, the biosensor responses to 100 µM glutamate were measured. No significant changes in the biosensor response to glutamate were observed at various NaCl concentrations in the working cell, which is typical for amperometric method of detection.

Optimum pH of enzymes can be changed during immobilization. Therefore, the effect of buffer pH on the work of developed amperometric biosensor was studied. A universal buffer (containing Tris-HCl, KH_2PO_4, citric acid and sodium tetraborate in concentrations of 10 mM) was used. This buffer has the same capacity in a wide pH range. The pH values in experiments ranged from 5 to 10. The highest biosensor responses were observed in the pH range of 7−8.5.

The effect of buffer concentration (buffer capacity) was also investigated. The experiment showed that an increase of buffer concentration from 5 to 100 mM did not affect significantly the sensitivity of biosensor to glutamate, which allows utilization of the developed biosensor to determine glutamate in samples characterized by various buffer capacities.

Response reproducibility and biosensor storage. Response reproducibility is one of the main working characteristics of a biosensor as it is necessary to get accurate results over all the period of measurements. Therefore, the reproducibility of biosensor responses to glutamate was investigated over a prolonged

period of continuous work. One measurement of glutamate took 3−5 min, the intervals between measurements were about 10 min. During intervals, the biosensors were washed from the substrate with the working buffer changing it several times. No noticeable drop in the responses was observed over 10 measurements. The relative standard deviation of responses was 3%.

To determine the optimum conditions of storage, the developed biosensor was tested when storing for a long time. The total period of storage was 65 days. The biosensors were stored under the following conditions: at +25 °C and +4 °C in a dry state and in 25 mM HEPES buffer; in a dry state at −18 °C. Additional substances (1 mM DTT, 1 mM EDTA and 0.1% NaN_3) were added to the buffer during storage to improve stability of GluOx.

When stored at + 25 °C, the biosensors lost their sensitivity during several days. At storage temperatures of +4 °C and −18 °C, the biosensors were stable for a much longer time. After 65-day storage, the highest responses to glutamate demonstrated the biosensors stored in the buffer at +4 °C, the responses decreased by only 15%. However, over the storage period the biosensor was unstable, its sensitivity to glutamate changed to a greater or lesser extent due to the interaction of bioselective membrane with the components of buffer solution.

Significantly worse results were observed when the biosensor was stored in a dry state at +4 °C; by the end of the storage period the responses equaled 35% of their initial values. However, this method of storage can be used if necessary since the response decreases most significantly during the first 20 days of storage, afterwards the biosensor was quite stable. The most predicted was storage at −18 °C since the biosensor sensitivity to glutamate decreased at approximately the same speed, and a decrease in responses at the end of storage was 30%. These results prove the possibility of using biosensor after prolonged storage, but for accurate results recalibration of the biosensor is required before use.

Selectivity of biosensor. The developed GluOx-based biosensor is intended for glutamate determination in food products. Nevertheless, there are many additional substances (e.g. ascorbic acid, cysteine, benzoic acid, etc) in these food products, which can be either oxidized on the electrode upon application of the potential (electroactive substances) or can be substrates for GluOx;

in both cases, presence of such interfering substances can cause non-specific biosensor response and lead to errors in measurements. To avoid impact of electroactive substances on the biosensor response, permselective membrane based on polymerized phenylenediamine (PPD) was deposited on the electrodes before immobilization of GluOx. Effectiveness of the PPD membrane was tested earlier [25, 26].

To test the biosensor selectivity, we tried to obtain biosensor responses to possible interfering substances. It turned out that there was no biosensor response to glucose, citric acid, benzoic acid, sodium azide, α-ketoglutarate, urea, EDTA, NaCl, KCl and $CaCl_2$ in concentration of 1 mM, and the presence of these substances in a working cell did not affect the biosensor response to glutamate.

The biosensor sensitivity to amino acids was also examined (Fig. 2).

The biosensor did not respond to most amino acids. Low sensitivity to asparagine, aspartic acid, glutamine and histidine was observed, but sensitivity to glutamate was 50−100-fold higher; therefore, the presence of amino acids in the sample even in high concentrations could not lead to measurement errors. These results coincide with the data obtained by another team of researchers for a different GluOx-based biosensor [27]. The biosensor sensitivity to some amino acids can be explained by the fact that GlOx is not perfectly selective to glutamate; additionally, trace amounts of other enzymes, such as L-amino acid oxidase, remained after GluOx purification.

According to these results, GluOx-based biosensor is sufficiently selective to glutamate and is suitable for the measurements of real food samples.

Analytical characteristics of developed biosensor. The limit of detection of biosensor for glutamate is defined as the glutamate concentration, the response to which equals three-fold the baseline noise; when using 25 mM HEPES buffer, pH 7.4, it was 0.5−2 μM. The linear working range was from 2 to 700 μM, the sensitivity to glutamate 200−230 nA/mM. The biosensor response time was 5−20 s.

A typical calibration curve of the biosensor for glutamate determination is shown in Fig. 3. The linear part of this calibration curve is described by the equation I = 210*C + 0.3 (R^2 = 0.999), where I is the steady-
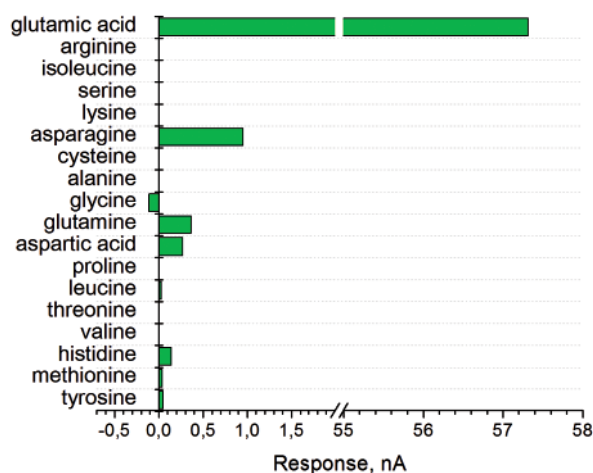
Fig. 2. **Biosensor responses to various amino acids:**
concentration of each amino acid was 1 mM. Measurements were carried out in 25 mM HEPES buffer, pH
7.4, at a constant potential of +0.6 V vs Ag/AgCl reference electrode

state current (nA), C is the concentration of glutamate (mM).

Reproducibility of biosensor preparation. Reproducibility of preparation of biosensors is very important for their large-scale manufacturing. To study this characteristic 11 different biosensors were prepared and the responses of these biosensors to 200 μM glutamate were compared. The relative standard deviation of responses of various biosensors to glutamate was 6%, which is acceptable. The calibration curves of the created biosensors for glutamate determination were studied. They had the same shape and the same linear range. Given that the biosensors were prepared manually, the received reproducibility of characteristics of different biosensors is very good. The difference in the response values did not pose a problem since before use each biosensor was calibrated.

Determination of glutamate concentrations in food samples. To confirm the possibility of practical use of the biosensor, glutamate concentration in real samples was analyzed. The sauces and seasonings were purchased in the supermarket. Before the measurements the sauces were 10-fold diluted with distilled water, and 1% solutions of dry seasonings were prepared. Two methods of glutamate determination (using calibration curve and standard additions) were used, which are described in section 2.5. In total, 6 sauces and 4 seasonings were analyzed. The spectrophotometric method of glutamate determination was used as control. The results are shown in Table 1. The results are expressed

not in moles but in mass fraction since it is not possible to determine molar concentration in dry seasonings. Noteworthy, the method of standard additions showed a better correlation with the spectrophotometric one than using the calibration curve. This is because the method of standard additions takes into account an impact of components of the real sample on the biosensor sensitivity to glutamate. However, use of this method often requires preliminary measurement of the glutamate concentration in the tested sample by the calibration curve. It is necessary in order to determine needed dilution of the tested sample, at which the response to the sample and three responses to the model glutamate solution (according to the method of standard addition) were within the linear range of biosensor analysis. A correlation graph was plotted (Fig. 4). As seen, the results of biosensor measurement well correlated with the control spectrophotometric method (R^2 = 0,988); results are well distributed along the theoretical line.

Reproducibility of analysis of glutamate in a food sample. To study the biosensor accuracy, the reproducibility of glutamate determination in a single sample was evaluated. The aliquots of 10-fold dissolved sauce were added 12 times to the biosensor working cell (with washing of the biosensor between the additions), and the obtained responses were analyzed (Fig. 5).

In the working cell, the sample was 50-fold diluted, thus, the initial sample was diluted in total by 500 times. The relative standard deviation of the biosensor responses was 2.7%, no decrease of responses during

Table 1. **Results of determination of glutamate concentrations in sauces and seasonings**

Measured samples	Biosensor, mass fraction (%)		Spectrophotometry, mass fraction (%)	
	Calibration curve	Standard additions	Calibration curve	Proportion
Sauce #1	0.49	0.52	0.54	0.68
Sauce #2	0.36	0.45	0.39	0.46
Sauce #3	1.08	1.23	1.41	1.57
Sauce #4	1.74	2.11	2.32	2.45
Sauce #5	1.17	1.27	1.14	1.13
Sauce #6	1.92	2.45	2.39	2.22
Seasoning #1	5.39	5.64	5.31	6.65
Seasoning #2	9.68	9.73	9.16	10.18
Seasoning #3	5.62	5.56	5.17	6.33
Seasoning #4	4.61	4.57	4.16	4.74
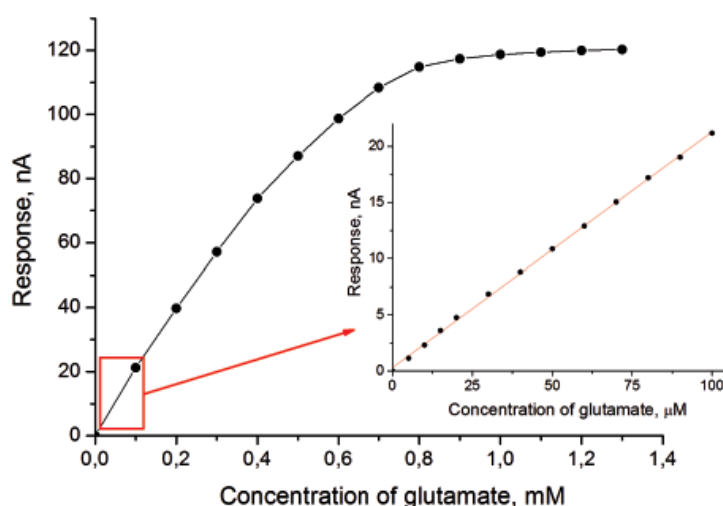
Fig. 3. **Calibration curve of biosensor for glutamate determination:**
measurements were carried out in 25 mM HEPES buffer, pH 7.4, at a constant potential of +0.6 V
vs Ag/AgCl reference electrode

12 measurements was observed. This indicates high accuracy and stability of the biosensor during real samples analysis. However, after a larger number of measurements, the biosensor recalibration is required, since the components of samples likely interact with the bioselective membrane and could change the biosensor sensitivity to glutamate.

Determination of glutamate at different dilutions of food samples. For correct determination of the analyte, its concentration in the working cell should be within the linear range of biosensor analysis. When determining glutamate in food samples, significant dilution of the food samples is commonly required due to high concentration of glutamate in it. Use of different dilutions might affect the results of measurement of glutamate concentration; therefore, an impact of the dilution rate was investigated. In the experiment, different volumes of non-diluted sauce were added to the cell and glutamate concentration in the samples was
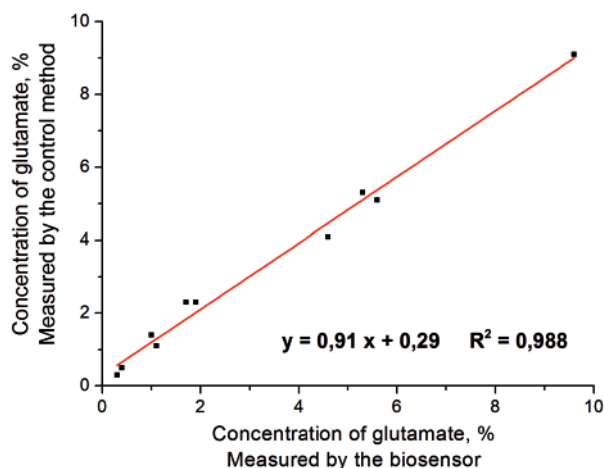
Fig. 4. **Correlation between biosensor and spectrophotometric determination of glutamate concentrations in food samples**
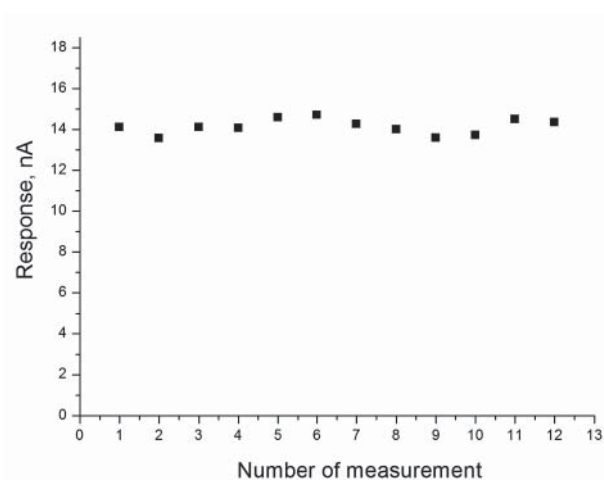
Fig. 5. **Reproducibility of glutamate determination in a single sample of sauce: measurements were carried out in 25 mM HEPES buffer, pH 7.4, at a constant potential of +0.6 V vs Ag/AgCl reference electrode**

determined. Four different rates of sample dilution were further used. Since initially the sauce was not diluted, small volumes of the sample were added to get within the linear range of biosensor analysis.

The results of experiments are presented in Table 2. As seen, the glutamate concentrations in the sauce determined at different dilutions are practically the same. This demonstrates the possibility of using different dilutions of samples. The main condition of accurate results is hitting of the glutamate concentration (in the working cell) in the linear range of biosensor analysis.

Thus, the amperometric biosensor for glutamate determination was optimized and used for measurement of glutamate concentrations in sauces and seasonings.

An influence of the composition of working buffer on the biosensor analytical characteristics was investigated. The highest responses were in the pH range of 7−8.5. Buffer capacity and ionic strength did not affect the biosensor response. The reproducibility of biosensor responses to glutamate over one day was studied; the relative standard deviation of response was 3%. Different conditions of the biosensor storage were evaluated. The storage at −18 °C was preferable, since in this case the biosensor sensitivity to glutamate decreased at approximately the same speed, and the drop in responses by the end of storage period

Table 2. **Results of the determination of glutamate concentration in sauce obtained with different dilutions of the sauce in working cell**

Sample dilution, times	Sample volume, added to the working cell, μL	Response of biosensor, nA	Concentration of glutamate in sauce, mM
2917	1.2	8.6	166.1 ± 10.9
1522	2.3	15.2	167.4 ± 10.7
1000	3.5	23.6	168.7 ± 6.9
700	5	29.1	165.1 ± 2.4

(2 month) was 30%. The results indicate the possibility of using biosensor after prolonged storage, but recalibration is necessary for repeated application.

The reproducibility of biosensor preparation was checked. The calibration curves of all tested biosensors were of the same form and had the same linear range of detection. The relative standard deviation of responses to glutamate for various biosensors was 6%, which is acceptable. The biosensor sensitivity to various interfering substances, including amino acids, was studied and it shown that the biosensor is highly selective to glutamate.

Concentrations of glutamate in 6 sauces and 4 seasonings were measured using the developed biosensor and spectrophotometric method, good correlation ($R^2 = 0,988$) of the results was demonstrated. Duration of analysis of one sample was about 5 min.

Additionally, it was shown that the relative standard deviation of the biosensor responses to the glutamate in food sample was 2.7%; the level of sample dilution did not influence the results of measurement of glutamate concentration.

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ВИКОРИСТАННЯ ГЛУТАМАТ-ЧУТЛИВОГО БІОСЕНСОРА ДЛЯ АНАЛІЗУ ХАРЧОВИХ ПРОДУКТІВ

Д. Ю. Кучеренко[1], І.С. Кучеренко[2],
О. О. Солдаткін[1, 2], О. П. Солдаткін[1, 2]

[1]Київський національний університет
імені Тараса Шевченка, Україна
[2]Інститут молекулярної біології і генетики
Національної академії наук України, Київ

E-mail: didukh.d@gmail.com

Метою роботи були оптимізація амперометричного глутаматчутливого біосенсора і його використання для визначення концентрацій глутамату в зразках їжі. Застосовували амперометричний метод вимірювань. Біосенсор базувався на іммобілізованій глутаматоксидазі та платиновому дисковому електроді. Біосенсор був підключений до робочої комірки з допоміжним електродом (платиновим дротом) і електродом порівняння (Ag/AgCl). Біосенсор мав високу чутливість до глутамату, тривалість одного аналізу становила близько 5 хв. Досліджено вплив іонної сили, рН і буферної ємності на роботу біосенсора. Вивчено чутливість біосенсора до різних можливих інтерферуючих речовин, включаючи амінокислоти; показана висока селективність до глутамату. Було оцінено відтворюваність аналізу зразків харчових продуктів та вплив розведення зразків. Концентрації глутамату в різних соусах і приправах вимірювали за допомогою розробленого біосенсора; результати добре корелювали з результатами, отриманими спектрофотометричним методом ($R^2 = 0,988$).

Таким чином, амперометричний біосенсор для визначення глутамату було успішно оптимізовано і використано для вимірювання концентрації глутамату в соусах і приправах.

Ключові слова: амперометричний біосенсор, глутаматоксидаза, полі(фенілендіамин), глутамат, харчові зразки.

ИСПОЛЬЗОВАНИЕ ГЛУТАМАТ-ЧУВСТВИТЕЛЬНОГО БИОСЕНСОРА ДЛЯ АНАЛИЗА ПРОДУКТОВ ПИТАНИЯ

Д. Ю. Кучеренко[1], И.С. Кучеренко[2],
О. О. Солдаткин[1, 2], А. П. Солдаткин[1, 2]

[1]Киевский национальный университет
имени Тараса Шевченко, Украина
[2]Институт молекулярной биологии и генетики
Национальной академии наук Украины, Киев

E-mail: didukh.d@gmail.com

Целью работы были оптимизация амперометрического глутаматчувствительного биосенсора и его использование для определения концентраций глутамата в образцах пищи. Применяли амперометрический метод измерений. Биосенсор был основан на иммобилизованной глутаматоксидазе и платиновом дисковом электроде. Биосенсор был подключен к рабочей ячейке со вспомогательным электродом (платиновой проволокой) и электродом сравнения (Ag/AgCl). Биосенсор имел высокую чувствительность к глутамату, продолжительность одного анализа составляла около 5 мин. Исследовано влияние ионной силы, рН и буферной емкости на работу биосенсора. Изучена чувствительность биосенсора к различным возможным интерферирующим веществам, включая аминокислоты; показана высокая селективность к глутамату. Была оценена воспроизводимость анализа образцов пищевых продуктов и влияние разведения образцов. Концентрации глутамата в разных соусах и приправах измеряли с помощью разработанного биосенсора; результаты хорошо коррелировали с результатами, полученными спектрофотометрическим методом ($R^2 = 0,988$). Таким образом, амперометрический биосенсор для определения глутамата был успешно оптимизирован и использован для измерения концентрации глутамата в соусах и приправах.

Ключевые слова: амперометрический биосенсор, глутаматоксидаза, поли(фенилендиамин), глутамат, пищевые образцы.

DEVELOPMENT OF RECOMBINANT POSITIVE CONTROL FOR *Francisella tularensis* DETECTION BY qPCR

O. B. Zlenko
A. P. Gerilovych

National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine" of the National Academy of Agrarian Sciences of Ukraine, Kharkiv

E-mail: oksana.ceratium@gmail.com

The aim of the work was to construct and test the recombinant positive control for *F. tularensis* detection by real-time polymerase chain reaction (qPCR). The molecular TA-cloning of pTZ57_F/R plasmid ligated with *tul4* gene PCR product into DH5α *E. coli* was provided. The minimal detection level in a qPCR was one copy number per reaction. The obtained positive control was highly sensitive, specific and safe to be used in the tularemia laboratory diagnostics.

Francisella tularensis (McCoy and Chapin, 1912; Dorofeev, 1947) is a gram-negative non-sporulating zoonotic, intracellular, obligate aerobe pathogen and the causative agent of the illness tularemia. *F. tularensis* is a natural foci disease that occurs in lagomorphs (rabbits and hares), and in rodents, especially microtine rodents (such as voles, vole rats and musk-rats). A wide range of other mammals and several species of birds also can be infected. Among domestic animals, hunting cats and dogs are able to act as a carrier of the bacterium. It can be spread also by insects, were the most important vectors are ticks: 13 species of them that belong to 4 genera *Amblyomma*, *Dermacentor*, *Haemaphysalis,* and *Ixodes* can carry the bacteria [1, 2]. It is transmitted to humans by several ways, including direct contact with infected material or inhalation of infected aerosols, ingestion of contaminated food or water, arthropod bites. Human-to-human transmission is not known to be reported [3].

The inhaled minimum infectious dose is 10−50 colony forming units. *F. tularensis* is considered to be a dangerous potential biological weapon because of its extreme infectivity and ease of dissemination. "Unusual" tularemia outbreaks in war-torn or crisis-afflicted regions which for years had appeared to be free of the disease give rise to speculation that these epidemics may have been artificially triggered [3−5]. Thus, monitoring of *F. tularensis* outbreaks is highly relevant today, especially in high conflict areas as Ukraine. Also, development of native high-quality test systems for tularemia is a question of first priority. The diagnosis of tularemia often relies upon the demonstration of an antibody response to *F. tularensis* or the direct culturing of the pathogen. Established tularemia ELISAs and confirmatory Western blot assays are mostly based on lipopolysaccharide (LPS) -antigen reactions, which are time and cost consuming and give false-positive results with other bacteria, for example *Brucella* genera. As *F. tularensis* is a fastidious, slow-growing organism, culture is often not the preferred diagnostic method for it when a rapid result is required. In addition, handling live *Francisella* poses a risk to laboratory personnel due to the highly infectious nature of this pathogen. PCR is the main method for direct and rapid detection of *F. tularensis* and requires positive controls for valid results. Further, recombinant positive controls are often used to provide additional biosafety and biosecurity measures [1, 6].

Materials and Methods

The plasmid was designed using the Clone Manager 9 (Scientific and Educational Software, USA). For the real-time PCR assay we used the FT-FP tul4 primer system: For: CAGCATACAATAATAACCCACAAGG; Rev: TCAGCATACTTAGTAATTGGGAAGC; Probe: TTACAATGGCAGGCTCCAGAAGGTT [7] with an amplicon product of 103 bp. These primers were checked using BLAST online service for specific annealing. The *F. tularensis* subsp. *holarctica* vaccine strain 15 NIIEG was used as a positive DNA template. The final volume of the reaction mixture was 25 µl and was comprised of AmpliTaq Gold DNA Polymerase (Applied Biosystems, USA) 1,25 U/50 µl, PE-Buffer 1X, dNTP mix 0,2 mM, $MgCl_2$ 1,5 mM, primer F 10 pM, primer R 10 pM, probe 5 pM, with 5 µl of purified template DNA to give the desired genome copy number per reaction volume and 5 µl pf PCR-grade water to the no-template-control reactions. Thermal cycling was performed using the following program: one cycle at 95 °C for 5 min for Hot-Start Tag activation, followed by 40 cycles at 95 °C for 15 s for denaturation, 60 °C for 30 s as annealing, 72 °C for 20 s for extension, and final elongation at 72 °C for 10 min. A fluorescence reading was taken at every 72 °C step.

To ensure inserts of appropriate size we used the M13 F/R primer system. Our reaction mixture contained 12.5 µl of Maxima Hot Start PCR Master Mix (2X) (Thermo Fisher Scietific, USA), 1 µl of 10 pM M13 forward primer, 1 µl of 10 pM M13 reverse primer, 5 µl of template DNA and PCR-grade water up to a total volume of 25 µl. Thermal cycling was performed using the following program: one cycle at 95 °C for 5 min for Hot-Start Tag activation, followed by 30 cycles at 96 °C for 30 s for denaturation, 50 °C for 20 s as annealing, 72 °C for 60 s for extension. Results reading was done in 2% agarose gel.

PCR products were purified using two different kits: The Monarch PCR & DNA Cleanup Kit (NEB, England) and Thermo Scientific GeneJET Gel Extraction Kit (Thermo Fisher Scietific, USA), according to the manufacturers' instruction. The concentration of DNA was determined usind a NanoDrop spectrometer DeNovix DS-11 (Wilmington, USA). The PCR product was subcloned with a vector to insert ratio of 1:5 using the InsTAclone PCR TA cloning Kit (Thermo Scientific, USA). Ligation was carried out overnight at 16 °C. The product was transformed into *E. coli* DH5α chemically competent cells, which were allowed to recover 1 h at 37 °C in Luria broth. Competent cells were produced using the adapted and modified $CaCl_2$ method of Mandel and Higa [8]. Pelleted bacterial strains from 25 ml cultures were resuspended with gentle pipetting in 10 ml of ice-cold 0.1 M $CaCl_2$ (formulated in de-ionized water and autoclaved) and incubated on ice for 20 min. The bacteria suspension was pelleted at 3800 g at 4 °C for 10 min followed by gentle resuspension in 5 ml of 0.1 M $CaCl_2$ + 15% (v/v) glycerol and stored in 100 µl aliquots at −80 °C. Transformed cells were screened for gene insertion using the blue-white method and confirmed by PCR mentioned above. Plasmids were isolated using the Thermo Scientific GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA).

Results and Discussion

It was constructed a plasmid with a 103 bp fragment of the *tul4* gene ligated into the pTZ57R/T vector. The complete sequence of the new plasmid is 2990 bp in length (Fig.1). The vector pTZ57R/T encodes an ampicillin resistance and the *lacZ* gene, which were used as selective markers for *E. coli* DH5α clones. We screened 10 white single *E. coli* colonies by PCR using *tul4* specific primers FT-FP (Fig. 2) and the M13 F/R primer system which generate a PCR product of 103 bp (Fig. 3).

The colonies #1 and #2 showed positive results with PCR products of 257 bp. It was used ligated pTZ57F/R plasmid without insert as positive template.

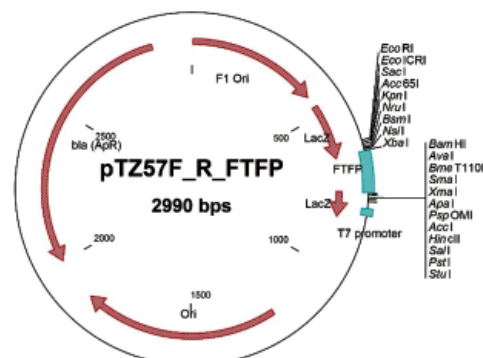Extracted plasmid minipreps from clones #1 and #2 were sequenced (Eurofins,
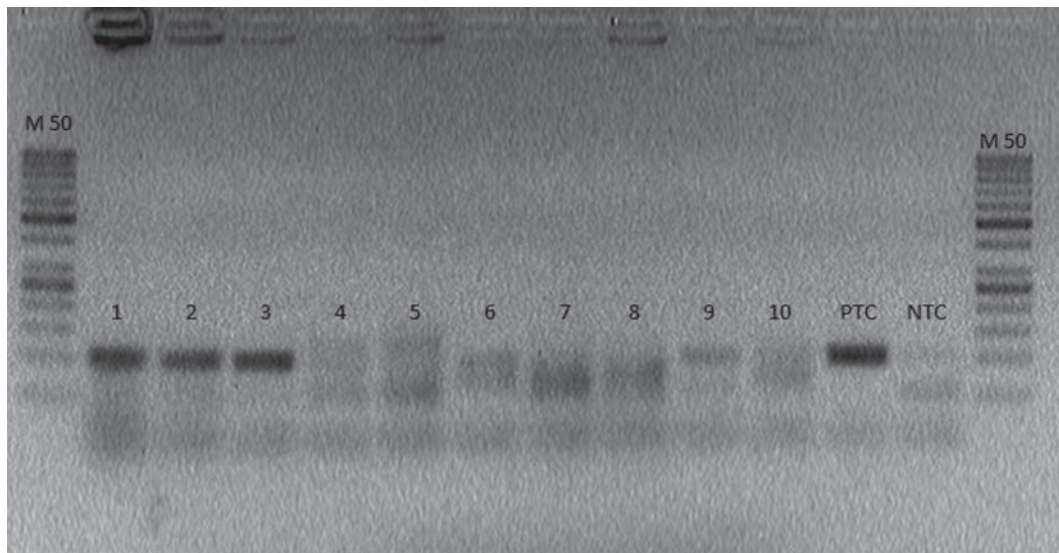

Fig. 1. **Plasmid vector pTZ57F_R_FTFP**

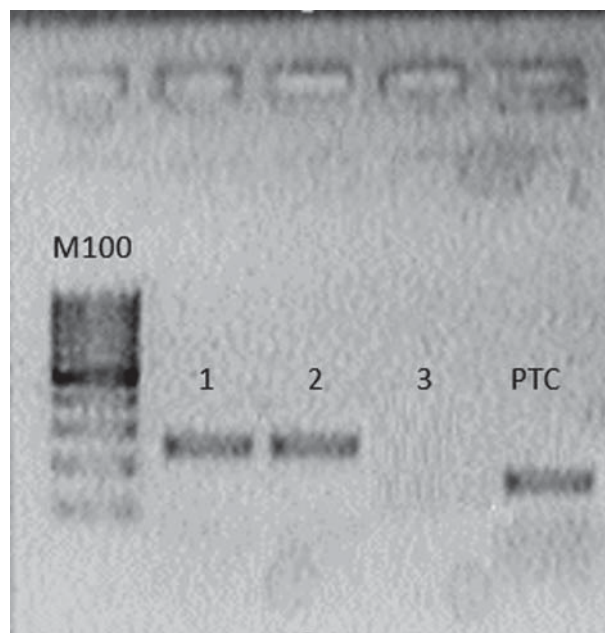*Fig. 2.* **Screening the *tul4* positive clones using FT-FP primers**

Fig. 3. **Screening the *tul4* positive clones using M-13 mers**

Germany) with M-13 primer system. Sequence results has shown that colony #1 sequence is in accordance with *tul4* gene sequences.

Thus, the colony #1 miniprep was chosen for further studies of sensitivity detection. The lowest threshold of detection was 1 copy number per reaction.

It has been developed the highly-sensitive recombinant positive control for detection of *F. tularensis* in conventional and a real-time polymerase chain reaction. The minimum of detection is 1 copy number per reaction.
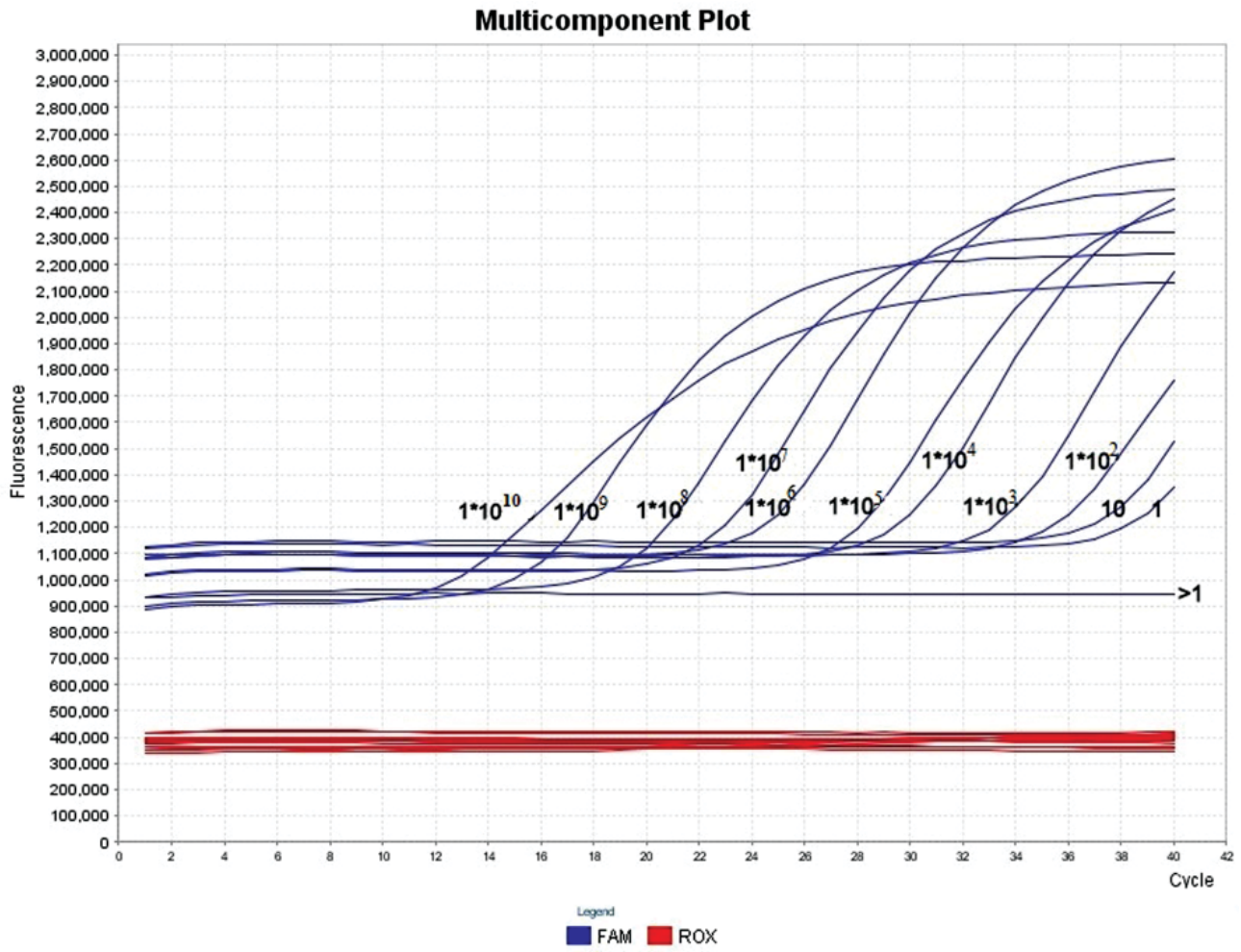
Fig. 4. **The results of pTZ57F_R_FTFP minimal copy number detection through PCR**
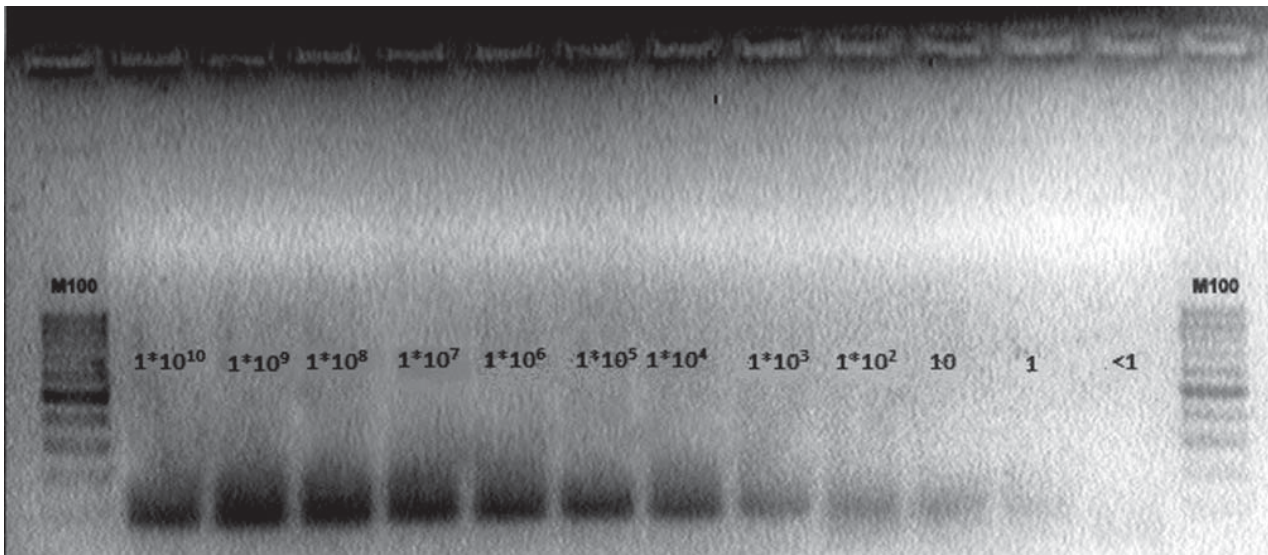
Fig. 5. **The results of pTZ57F_R_FTFP minimal copy number detection (electrophoresis in agarose gel)**

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РОЗРОБЛЕННЯ РЕКОМБІНАНТНОГО ПОЗИТИВНОГО КОНТРОЛЮ ДЛЯ ДЕТЕКЦІЇ *Francisella tularensis* ЗА ДОПОМОГОЮ qPCR

О. Б. Зленко, А. П. Герілович

Національний науковий центр
«Інститут експериментальної та клінічної ветеринарної медицини» НААН України, Харків

E-mail: oksana.ceratium@gmail.com

Метою роботи було конструювання та випробовування рекомбінантного позитивного контролю для виявлення *F. tularensis* під час проведення полімеразної ланцюгової реакції в режимі реального часу (qPCR). Здійснено молекулярне TA-клонування плазміди pTZ57_F/R з ампліконом гена *tul4* і подальшу її трансформацію в компетентні клітини *E. coli* DH5α. Мінімальна діагностична кількість плазміди в реакції становила одну копію. Отриманий позитивний контроль є високочутливим, специфічним і безпечним для використання в лабораторній діагностиці туляремії.

Ключові слова: рекомбінантний позитивний контроль, qPCR, туляремія, молекулярне клонування.

РАЗРАБОТКА РЕКОМБИНАНТНОГО ПОЛОЖИТЕЛЬНОГО КОНТРОЛЯ ДЛЯ ВЫЯВЛЕНИЯ *Francisella tularensis* С ПОМОЩЬЮ qPCR

О. Б. Зленко, А. П. Герилович

Национальный научный центр
«Институт экспериментальной и клинической ветеринарной медицины» НААН Украины, Харьков

E-mail: oksana.ceratium@gmail.com

Целью работы было конструирование и испытание рекомбинантного положительного контроля для выявления *F. tularensis* при проведении полимеразной цепной реакции в режиме реального времени (qPCR). Осуществлено молекулярное TA-клонирование плазмиды pTZ57_F/R с ампликонами гена *tul4* и ее дальнейшая трансформация в компетентные клетки *E. coli* DH5α. Минимальное диагностическое количество плазмиды в реакции составляло одну копию. Полученный положительный контроль является высокочувствительным, специфичным и безопасным для использования в лабораторной диагностике туляремии.

Ключевые слова: рекомбинантный положительный контроль, qPCR, туляремия, молекулярное клонирование.

EFFECT OF MONOCOT INTRONS ON TRANSGENE EXPRESSION IN DICOT *Nicotiana* PLANTS

I. O. Nitovska
M. Yu. Vasylenko   Institute of Cell Biology and Genetic Engineering
B. V. Morgun   of the National Academy of Sciences of Ukraine, Kiyv

E-mail: molgen@icbge.org.ua

The aim of the work was to study the effect of introns of the rice *OsAct1* and the maize *hsp70* genes on transgene expression in *Nicotiana* plants in order to find out their use in the verification of vectors containing these monocot introns. The following methods were used: *Agrobacterium*-mediated transformation of leaves of greenhouse *N. benthamiana* and *N. tabacum* plants by vector pCB271 containing the introns of cereals, light fluorescence microscopy and fluorimetry of Green Fluorescent Protein (GFP). The presence of transgenes was detected by polymerase chain reaction. The transient GFP expression was observed in infiltrated tissue of *N. benthamiana*. Transgenic plants of *N. tabacum* resistant to kanamycin were obtained. Fluorescence of GFP in extracts of some transgenic tobacco lines was shown. The impairment of the transgene expression in some *N. tabacum* transformants has been observed. Eventually, transgenes, containing introns from the *hsp70* corn or from the *OsAct1* rice genes downstream the promotor, were expressed in *Nicotiana* plants. Thus, *N. benthamiana* and *N. tabacum* plants can be used to test vector constructs for cereals transformation. It has been shown that the monocot introns can have negative impact on the transgene expression in *Nicotiana* plants.

Key words: monocot introns, *Nicotiana*, *Agrobacterium*-mediated transformation, GFP, genetically modified organisms.

Genetic transformation of cereals is a powerful tool to improve existing varieties, to increase their economic attractiveness in cultivation [1]. One of the ways to boost the efficiency of transformation of monocotyledonous plants is to create adapted vector designs with special promoters and regulatory elements aimed at enhancing the expression of transgenes. It is shown that the use of regulatory elements that work well in dicotyledonous plants is not sufficiently effective in monocotyledons [2−4]. Therefore, a number of promoters for the expression of transgenes in monocot have been isolated and been adopted, namely: corn ubiquitin gene promoter (*ZmUbi1*) [5], corn sucrose synthase gene promoter (*sh1*) [6], promoters of rice genes of actin 1 (*OsAct1*) [2] and actin 2 (*OsAct2*) [7] and others [8]. Often, for effective transgene expression, promoters of monocotyledonous, as promoters of corn genes *adh1*, *ubi1*, *sh1* and rice *OsAct1*, require

the presence of their first intron in a 5′ non-translated region [5, 6, 9]. The monocot introns themselves, embedded after "non-native" promoters, can also act as separate regulatory elements to increase the expression of transgenes in monocotyledonous plants [10, 11]. This phenomenon known as IME (intron-mediated enhancement) was observed not only for monocots, but also for dicots, and in general for a wide range of organisms, including mammals and invertebrates [12]. Increasing levels of transgene expression due to introns may be more than 100 times [6], but more often it is 2−10 times.

One of the first regulatory elements of the gene expression that was used to create effective vectors for the genetic transformation of monocots was the 5′-region of the rice actin 1 gene (*OsAct1*) [2]. It has been shown that the first intron of the *OsAct1* rice gene can regulate the expression of genes under the control of another promoter in the

absence of the "native" one, even with greater efficiency [9].

Before using genetic constructs to transform target species, it is desirable to test them on model objects, such as *Nicotiana tabacum* or *N. benthamiana*. *Nicotiana* species are dicotyledonous plants, so it is likely that the expression of vectors created for the transformation of monocots will not happen in *Nicotiana* plants. For example, there was a decrease in expression levels of transgenes that were under the control of transcriptional elements of cereals in tobacco cells, as compared to cells once obtained after transformation with vectors that did not contain these elements [6, 10, 11, 13]. Thus, the purpose of our work was to study the possibility to use *Nicotiana* species to test vectors that contain the intron of the maize *hsp70* (heat shock protein 70) gene and the intron 1 from the rice *OsAct1* gene.

In the investigation we utilized the pCB271 binary vector, containing in its T-DNA *npt*II (neomycin phosphotransferase II) gene and *S65Tpgfp* (green fluorescence protein) gene, both under the control of the enhanced e35S promoter of the cauliflower mosaic virus, as well as the regulatory elements between the promoter and the coding part of the genes to elevate their expression in monocotyledons, namely: intron of the corn *hsp70* gene before *npt*II and intron 1 of the rice actin 1 gene (*OsAct1*) before the *S65Tpgfp* gene. To investigate the activity of the vector in *Nicotiana* plants, we have been carried out an agroinfiltration of leaves of *N. benthamiana* greenhouse plants to further study the transient expression of GFP, and have been conducted an *Agrobacterium*-mediated transformation of *N. tabacum in vitro* plants to obtain stable tobacco transformants and to study the presence and expression of *npt*II and *S65Tpgfp* transgenes in them.

Materials and Methods

Plant material. Greenhouse plants of *N. benthamiana* of the wild type 5−6 weeks old, grown under conditions at 20−25 °C and 14-hour photoperiod, were used in the experiments on agroinfiltration. For *Agrobacterium*-mediated transformation, *N. tabacum* plants cv. Petit Havana, grown under aseptic conditions on hormone free MS medium [14] at 25 °C and 16-hour mode of the illumination, were used.

Plasmids and bacterial strains. The binary vectors pCB271, pICH5290 and rICH6692 [15], which are in the collection of the Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine, were used in this work. The test vector pCB271 in its T-DNA region contained a selective neomycin phosphotransferase II (*npt*II) gene, a mutant reporter *S65Tpgfp* gene of green fluorescence protein (GFR) for the screening of transformation events, both under the control of the enhanced e35S promoter of the cauliflower mosaic virus, as well as regulatory elements between the promoter and the coding part of the named genes to enhance their expression in monocotyledons, namely: intron of the corn *hsp70* gene before the *npt*II gene and the intron 1 of the rice actin 1 gene (*OsAct1*) before *S65Tpgfp* (Fig. 1).
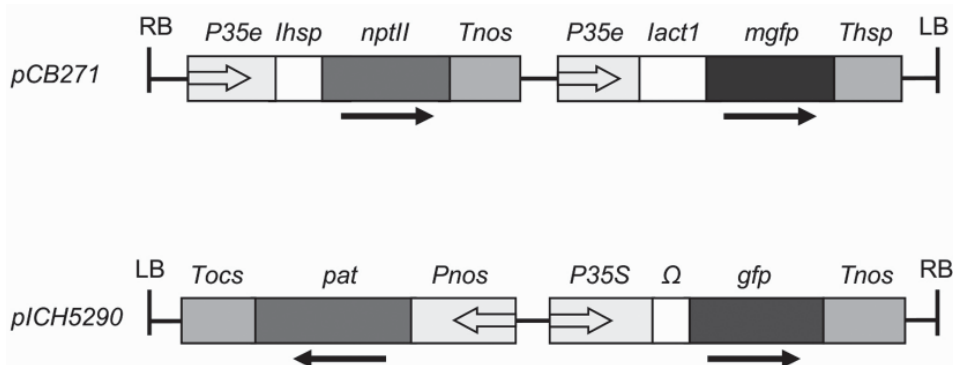

Fig. 1. **T-DNA regions of the plasmids used in the study:**
RB, LB — right and left T-DNA borders, P35e — enhanced 35S RNA gene promoter of the cauliflower mosaic virus, Ihsp — the corn *hsp70* gene intron, *npt*II — gene of neomycin phosphotransferase II, Tnos — the nopaline synthase gene terminator, Iact1 — the intron 1 of the rice *actin 1* gene, *mgfp* — mutant *S65Tpgfp* gene of the green fluorescence protein, Thsp — the wheat *hsp* gene terminator, Tocs — the octopine synthase gene terminator, *pat* — the phosphinothricin acetyltransferase gene, Pnos — the nopaline synthase gene promoter, P35S — 35S RNA gene promoter of the cauliflower mosaic virus, omega (Ω) — leader of TMV virus to enhance the translation, *gfp* — gene of green fluorescent protein wild type

The T-DNA of the control vector, pICH5290 (Fig. 1), had a selective phosphinothricin acetyltransferase gene (*pat*) and a wild type *gfp* reporter gene under the control of the 35S cauliflower mosaic virus promoter (CaMV35S). The vector pICH6692 contained a gene *p19* from tomato bushy virus, which encodes p19 protein, a suppressor of post-transcriptional gene silencing [16]. Plasmids were in *Agrobacterium tumefaciens* strain GV3101, which is derived from the strain C58 [17].

Agroinfiltration. In the experiments on agroinfiltration the night culture *A. tumefaciens* was used. The bacteria were grown on an orbital shaker at 28 °C, 200 rpm in 20 ml with LB medium [18] containing antibiotics, depending on the plasmid: vector pCB271 (50 mg/l kanamycin, 50 mg/l spectinomycin); vectors pICH5290 or pICH6692 (50 mg/l rifampicin, 25 mg/l gentamycin, 50 mg/l carbenicillin). The night culture of the bacteria was precipitated by centrifugation (5000×g, 5 min) and after removal of the supernatant was suspended in an infiltration buffer (10 mM $MgSO_4$, 10 mM MES pH 5.5) to an optical density (OD600) of 0.7–0.8 units. Bacterial suspensions containing the vectors pCB271 or pICH5290 were mixed with the bacterium containing the vector pICH6692 in equal proportions. Bacterial mixtures were injected into different parts of one leaf of *N. benthamiana* plant [15]. The plants continued to grow in greenhouse for 10 days after agroinfiltration.

Fluorescence analysis of GFP. The GFP accumulation in the infiltrated sections of the leaves was visually evaluated under UV illuminations (UVP, Upland, USA) over 3–7 days after infiltration. The fluorescing leaf sectors were marked to facilitate the identification of the infiltrated sections without UV light. Fluorometric measurements of GFP were carried out using a spectrofluorometer Fluorat-02-Panorama of the Lumex Company (St. Petersburg, Russia) both from the leaf surface and in plant extracts obtained from infiltrated areas. The excitation wavelength was 395 nm for the wild type GFP and 485 nm for the mutant GFP S65T [19]. The emission wavelength was 510–515 nm. The fluorescence results were fixed in standard units.

Preparation of plant extracts. The leaf tissue of 300 mg was milled in 3 ml of a cooled buffer (50 mM Tris-HCl, pH 8.0) and centrifuged at 4 °C, 16 000×g for 20 min. The supernatant was used for fluorometric measurements.

Fluorescence microscopy of GFP. Microscopic studies of leaf tissue and protoplasts isolated from infiltrated sectors of the *N. benthamiana* leaf were performed using a microscope Axiophot-35 (Carl Zeiss Microscopy, USA) with an excitation channel of 400–488 nm. Protoplasts for cytological studies were obtained by cultivating leaf strips at a width of 1 mm in a mixture of enzymes: 0.6% Onozuka R-10 (Duchefa, Netherlands), 0.6% Macerozyme R-10 (Duchefa, Netherlands) and 0, 2% Cellulysine (Calbiochem, USA) in 0.5 M sucrose, pH 5.7, at 27 °C overnight.

Transformation. The *Agrobacterium*-mediated genetic transformation of aseptic *N. tabacum* plants was carried out using "leaf discs" method [20], followed by regeneration on selective MS media containing 1 mg/l BAP, 0.1 mg/l NAA and antibiotics: 500 mg/l cefotaxime (Cx) for suppressing bacterial growth and 100 mg/l kanamycin sulfate (Km) for the selection of transgenic plants after transformation with the test pCB172 vector or 5 mg/l of phosphinothricin instead of kanamycin after transformation by the control pICH5290 vector. The cultivation of explants was carried out under aseptic conditions in a 16-hour photoperiod at 25 °C. The explants were transferred to a fresh regenerative medium every three weeks. After 8 weeks culturing, we calculated the frequency of regeneration (RF) in percent, which was equal to the number of explants that regenerated plants on the selective medium, to the total number of explants. The regenerants were placed in jars with the hormone free MS medium containing 500 mg/l Cx and 100 mg/l Km or 5 mg/l of phosphinothricin, depending on the vector used for transformation.

PCR analysis of regenerants for the presence of the transgenes. From the leaves of tobacco regenerants obtained after *Agrobacterium*-mediated transformation, as well as the untransformed plant have been isolated the total DNA using CTAB and PVP-40 [21]. To exclude the contamination of the plant material by *A. tumefaciens*, an amplification of the bacterial *vir*-D1 gene was carried out prior to the PCR assay on the transgenes [22]. The PCR on the *gfp* gene was performed using the following pair of primers: F — 5′-GACGT GAACG GCCAC AAGTT CA-3′ and R — 5′-CGATG CGGTT CACCA GGGTG T-3′, and on the *npt*II gene was used a pair of primers that was described earlier [23]. The amplification product of the part of the *vir*-D1 gene sequence should have been 432 pairs of

nucleotides (bp), gfp — 311 bp, nptII — 700 bp. The reaction mix for PCR (20 µl) contained 0.5 units of FIREPol® DNA polymerase (Solis BioDyne, Estonia), 2 µl of 10× buffer B, 1.6 µl of 25 mM $MgCl_2$, 200 µM of each dNTP, 0.1 µM of each forward and reverse primer and 30 ng of purified total DNA. As a positive control for *gfp* and *npt*II, the total DNA of the tobacco plants transformed by these genes was used, and for the *vir*-D1, the total DNA of *A. tumefaciens* was applied. The amplification program for the detection of the *gfp* gene was given as follows: the first cycle at 94 °C for 4 minutes followed by 34 cycles (denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, elongation at 72 °C for 19 s). The amplification program for detecting the sequence of the *npt*II gene was generated using the descending PCR technique: starting denaturation — 4 min at 94 °C; 9 cycles: 30 s at 94 °C, 45 s at 68 °C, 30 s at 72 °C; 26 cycles: 30 s at 94 °C, 30 seconds at 60 °C, 30 s at 72 °C. The final elongation was 5 min at 72 °C followed by rapid cooling to 22 °C. PCR was performed using the Mastercycler personal (Eppendorf, Germany) amplifier. The amplification products were separated by electrophoresis in 0.8% agarose gel with bromine ethidium (0.5 µg/ml) in LB buffer [24] at 6 U/cm for 90 min.

Statistical processing of the results was carried out according to the standard method [25].

Results and Discussion

The transient GFP expression in N. bentha-miana plants. The effect of the first intron of the rice actin 1 gene (*OsAct1*) on the transient expression of GFP into the leaf blade of *N. benthamiana* after the agroinfiltration was investigated. The upper part of the *N. benthamiana* leaf was infiltrated with the test vector pSV271, which contained this transcriptional element between the promoter e35S and the nucleotide sequence of the mutant S65Tpgfp gene (Fig. 1). The control vector pICH5290, which did not contain the monocot introns before the coding sequence of *gfp* gene was injected into the tip of the leaf blade. After 3 to 6 days following the infiltration, a green fluorescence of GFP protein was observed in infiltrated areas of the leaf under ultraviolet illumination (Fig. 2).

The bright fluorescence of GFP in leaf sections infiltrated with the control vector was observed on the third day after agroinfiltration, whereas for the test vector, the fluorescence peak was on the 5−6 days.

Under UV illumination, the GFP fluorescence in the leaf areas infiltrated by the test vector was less bright compared with zones infiltrated by the control vector (Fig. 2, *B*).

As a result of the fluorescence microscopy GFP, the expression of the vector pCB271 in the tissues (Fig. 3, *A*) and the protoplasts isolated from the *N. benthamiana* leaves infiltrated by the test vector was shown (Fig. 3, *B*).

GFP fluorescence in the leaf tissues agroinfiltrated with pCB271 vector evidenced an expression of the mutant *S65Tpgfp* gene in dicotyledonous *N. benthamiana* plants, despite the presence of the first inron of the rice *OsAct1* gene in its transcriptionally active region. After infiltration of the *N. benthamiana* leaves, we observed a delay of 2−3 days of expression of the test vector compared to the control, possibly due to the presence of a transcriptional element of cereals in the vector pCB271.

The GFP fluorescence of the leaf surface and the plant extracts of infiltrated zones was measured using a spectrofluorimeter. As a result of the fluorimetric measurements from the leaf surface, using the optimal excitation wave for each GFP (395 nm for wild GFP and 485 nm for mutant GFP S65T [19]), it was shown that the wild-type GFP fluorescence was higher compared to the mutant (Fig. 4). When measuring the GFP fluorescence of leaf extracts, we recorded a greater GFP fluorescence levels for extracts obtained from the leaf zones infected *A. tumefaciens* with the
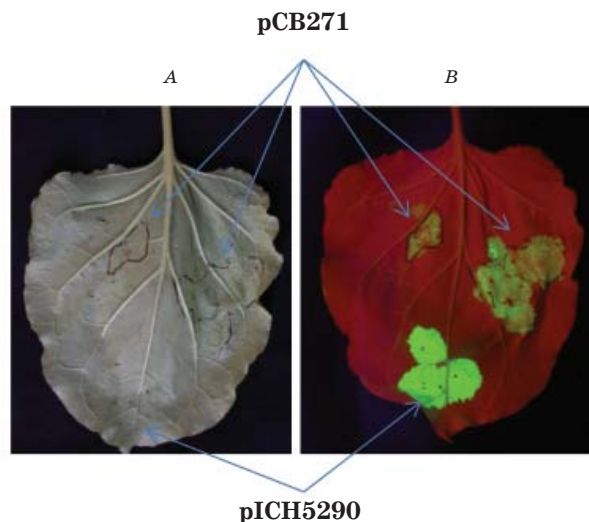

Fig. 2. **The appearance of the *N. benthamiana* lower leaf surface in 6 day after agroinfiltration by vectors pCB271 and pICH5290 in white (*A*) and ultraviolet (*B*) light**
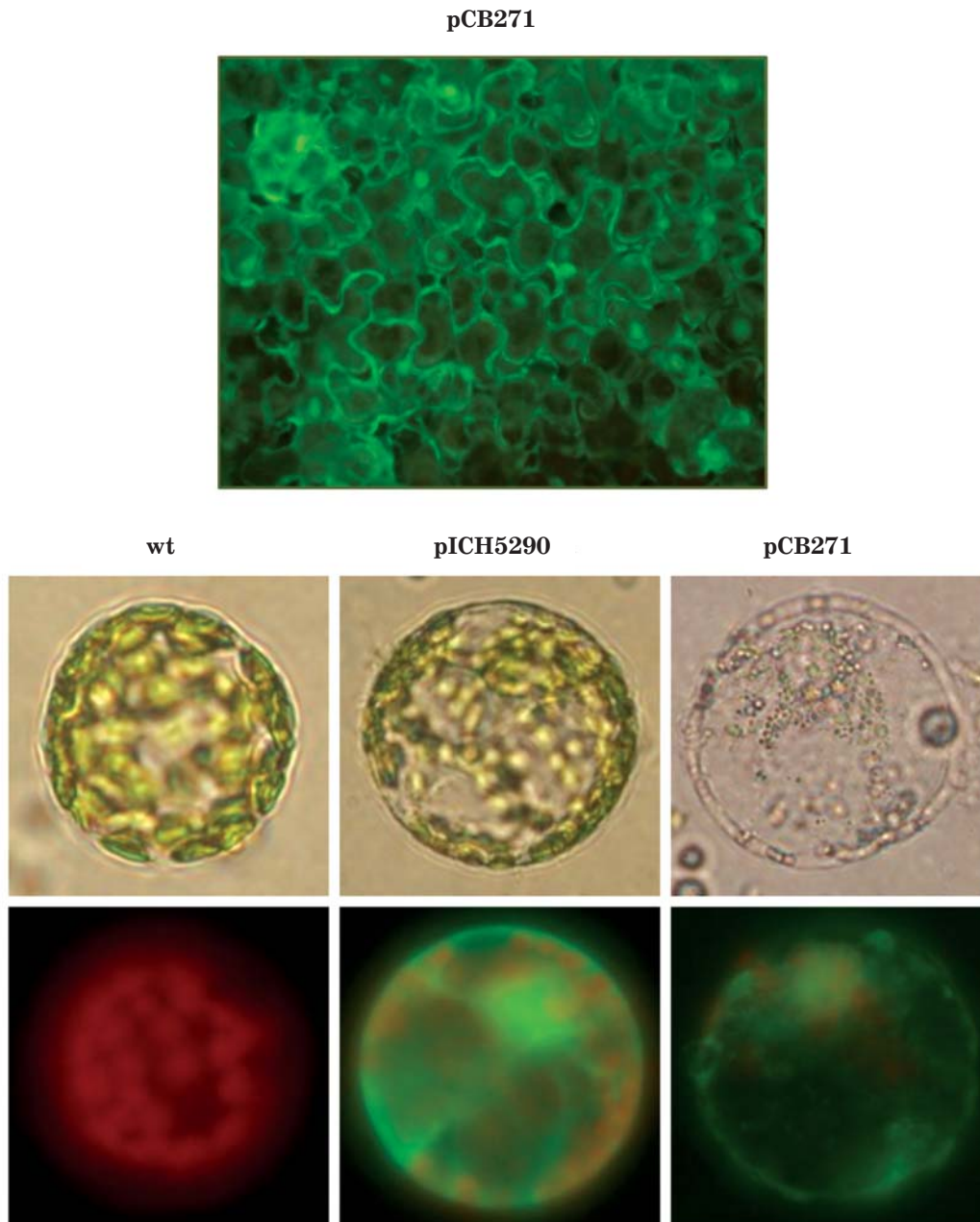
pCB271

Fig. 3. **Microscopic studies of transient expression of GFP in *N. benthamiana* plants:**
A — a leaf tissue after infiltration *A. tumefaciens* with vector pCB271 in UV light; *B* — protoplasts isolated
from non-infiltrated sections of the leaf (wt) and from leaf areas infiltrated by the vector pICH5290 or
pCB271 in ultraviolet (bottom) and white (top) light

test vector relative to the extracts obtained from the zones infiltrated by the control vector (Fig. 4, *A*, *B*).

According to the literature, the mutant form of the GFP S65T protein had 100 times more fluorescence than wild-type GFP when using a wavelength of excitation of 490 nm [19]. In our study, when using the excitation wavelength optimal for a mutant GFP, its fluorescence was only 5–7 times

greater than the wild type GFP (Fig. 4, *B*), possibly due to the presence of rice intron in the transcriptionally active region of the mutant gene. In addition, the *S65Tpgfp* gene is controlled by the enhanced CaMV35S promoter, which should further increase the level of transgene expression. When fluorometric measurements were conducted from the leaf surface, using the excitation wave optimal for each protein, the fluorescence
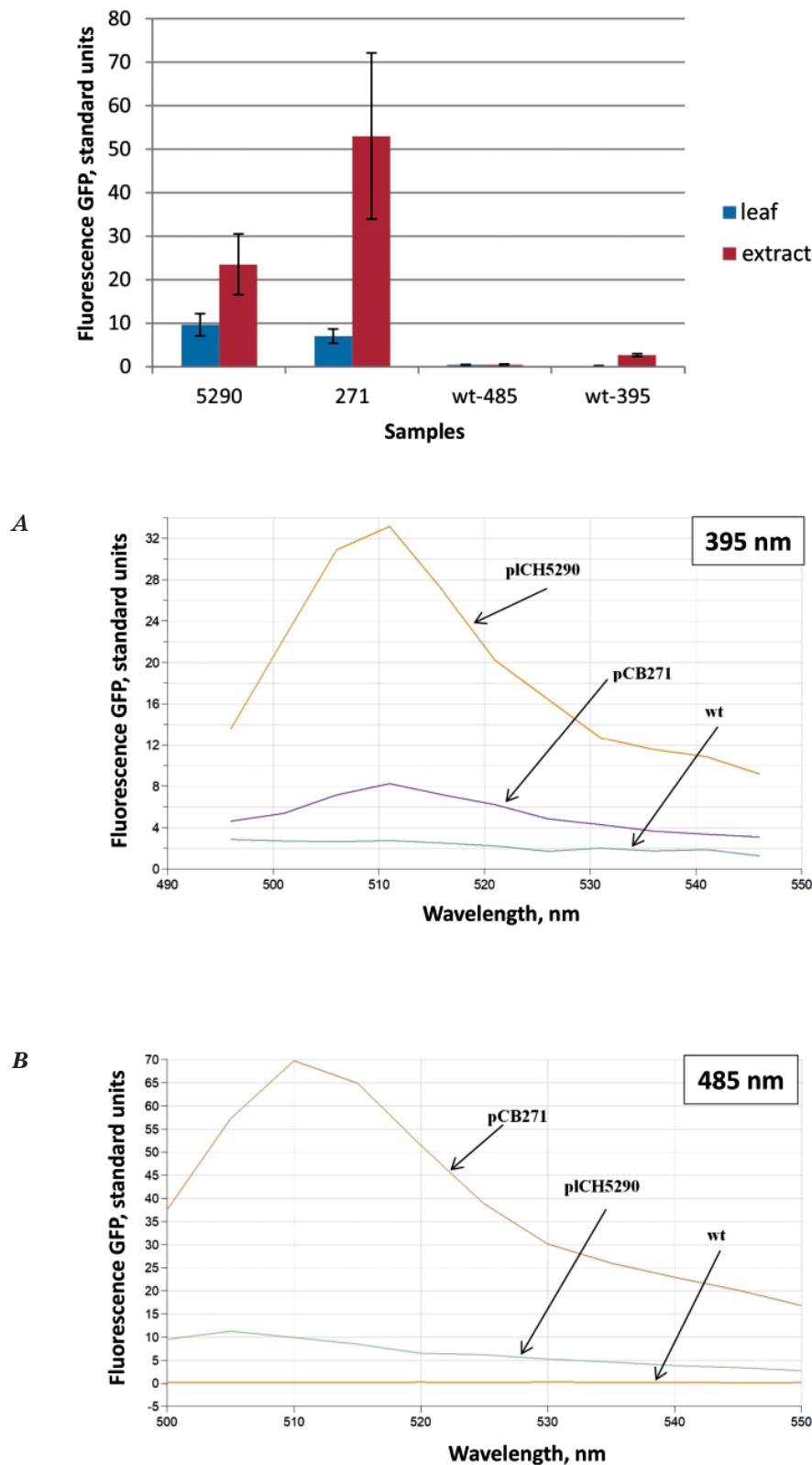
Fig. 4. **Flourimetry of GFP from leaf surface (*A*) and in leaf extracts (*A*, *B*)**
of *N. benthamiana* 6 days after agroinfiltration:
5290 — leaf zones, infiltrated *A. tumefaciens* with vector pICH5290; 271 — leaf areas, infiltrated
A. tumefaciens with vector pCB271; wt-395 — the fluorescence of wild-type non-infiltrated tissue at
a wavelength of excitation of 395 nm; wt-485 — the fluorescence of non-infiltrated tissue at a wavelength
of excitation of 485 nm. Diagram A shows the average values of GFP fluorescence and confidence interval
at $P < 0.05$

of the mutant GFP correlated with such the wild type protein or was smaller, and only in the extracts we observed a slight increase (2–3 times) of the fluorescence of the mutant GFP compared with GFP of the wild type. In our opinion, low expressions of mutant GFP compared to the expected ones are probably due to the presence of a monoclonal transcriptional element above the encoding region of the gene and its negative effect on the expression of GFP in dicotyledonous *N. benthamiana* plants. Consequently, according to our data, the presence of the first intron of the rice *OsAct1* gene in the transcribed region of the vector worsens the expression of the gene in dicot plants of *N. benthamiana*, but it occurs, so this model object may be used for the preliminary testing of vectors that contain the named regulatory element, important for the successful heterologous expression of genes in monocotyledons.

Agrobacterium-mediated genetic transformation of tobacco. The next step of investigation was the obtaining transgenic plants using *A. tumefaciens* containing the vector pCB271 to study the effect of monocot introns on the transgene transcription in tobacco transformants. Two experiments on *Agrobacterium*-mediated transformation of tobacco were carried out (Table). The multiple regeneration of tobacco plants (from 3 to 10 per explant) on the selective media was observed after 4 weeks following transformation. Total DNA of 50 lines of regenerants resistant to kanamycin were tested for the availability of *npt*II and *gfp* genes by PCR method (Table). The presence of fragments of the expected size (311 bp or 700 bp) for both the *gfp* and *npt*II genes was shown (Fig. 5), as an absence of bacterial contamination of the investigated plant material.

In experiments on *Agrobacterium*-mediated transformation, a significant number of regenerated plants became sensitive to kanamycin during further cultivation on the selective medium (Table), which is not typical for tobacco transformation. Sensitive plants were light green or completely discolored, poorly grown on the selective nutrient medium, did not form roots. Sensitive tobacco regenerants, obtained in the second experiment, were investigated for the availability of transgenes (Fig. 5). The presence of the *npt*II gene in 13 of the 16 analyzed plants was shown, and 12 of them contained both genes (Table).

Hence, 81.3% of sensitive regenerants were transgenic. This fact unlikely is due to silencing transgenes, because we did not remove selective pressure after transformation during the plant material cultivation. Therefore, we believe that the emergence of a significant number of transgenic plants that become susceptible to the selective agent during further cultivation is the result of the negative effect of the of the corn *hsp70* gene intron on the expression of the *npt*II gene in tobacco. In the control experiment with pICH5290 vector, which did not contain the monocot nucleotide sequences between the promoter and the coding part of the gene, all obtained transgenic plants did not lose resistance to the selective agent during further cultivation and contained both transgenes. The obtained results correlate with the literature data regarding the reduction of the level of gene expression in tobacco cells

Table. **Tobacco transgenic plants obtaining using *A. tumefaciens* containing the vector pCB271**

Experiment, N	RF, %	Planted regenerants				PCR-analysis of regenerants											
						nptII						*gfp*					
				S		R			S			R			S		
		T, pcs	R, pcs			T, pcs	"+"		T, pcs	"+"		T, pcs	"+"		T, pcs	"+"	
				pcs	%		pcs	%		pcs	%		pcs	%		pcs	%
1	78.5	329	231	97	29.6	33	25	75.8	ni	ni	ni	33	18	54.5	ni	ni	ni
2	55.6	45	25	20	44.4	17	15	88.2	16	13	81.3	15	8	53.3	13	12	92.3

Notes: RF — regeneration frequency; T — total; R — resistant to the kanamycin; S — sensitive to the kanamycin; "+" — the presence of a positive signal, ni — not investigated

after transformation with vectors containing monocot introns as regulatory elements of gene expression in comparison with constructions that do not contain them [6, 9, 11, 13]. Thus, the embedding of the *OsAct1* first intron into the transcription region between the promoter CaMV35S and the *uidA* gene sequence resulted in a decrease in GUS activity after transformation of 4-fold compared with the use of a vector without an intron and no activity of GUS was detected in tobacco cells after transformation by vector containing

the promoter of the rice gene *Act1* with and without the first intron [9]. This may be due to the fact that dicot cells often do not recognize monocot introns and, accordingly, their splicing is not always correct [11].

The expression of the S65Tpgfp gene in transgenic N. tabacum plants obtained by *A. tumefaciens* with the vector pCB271 examined via flourimetric measurements from leaf surface and leaf extracts. Fluorescence of GFP during measurements from the leaf surface was not detected for
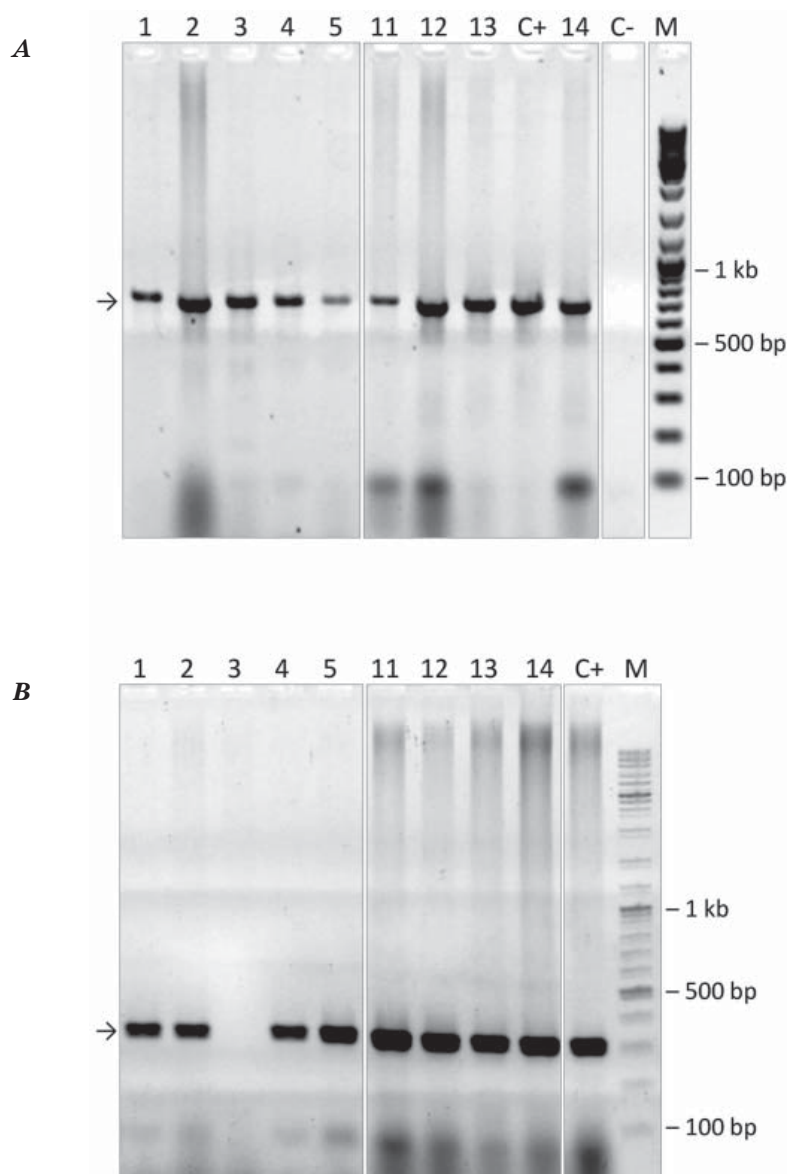
Fig. 5. **Electrophoregrams of the PCR analysis of tobacco regenerants obtained after transformation of *A. tumefaciens* containing pCB271 vector on the presence of *npt*II (*A*) and *gfp* (*B*) genes:**
Tracks 1−5 — DNA samples of the sensitive to the antibiotic tobacco regenerants; 11−14 — DNA samples of the resistant to the antibiotic tobacco regenerants; C — negative control; C+ — positive control; M — the Ladder Mix DNA molecular weight marker. The lengths of the PCR fragments were 311 bp for the *gfp* gene and 700 bp — for *npt*II

transformants and was at the fluorescence level of the leaf of a non-transformed plant (Fig. 6). High peaks of GFP fluorescence were observed for some transgenic lines due to fluorimetry of extracts. The intensity of the fluorescence of GFP in the extracts was up to 25 times larger than those measured from the leaf surface (Fig. 6). We analyzed leaf extracts of 22 lines of transgenic plants obtained as a result of two experiments on the *Agrobacterium*-mediated transformation by the vector pCB271 and the GFP fluorescence was observed in extracts of 7 lines. The lines of transformants differed in the intensity of the GFP fluorescence. The detecting GFP fluorescence in the extracts unlike leaf surface may be associated with a higher content of GFP protein in the extracts compared with a small leaf area taken for measurement in the surface study.

Thus, as a result of the *Agrobacterium*-mediated transformation by the pCB271 vector containing monocot introns between the promoter and the coding region of the genes, we showed that their expression occurs in dicotyledons of *N. benthamiana* and *N. tabacum*. However, there is a negative effect of the introns on transgenic expression exists. It was manifested in lower levels of GFP fluorescence than expected and the presence of a significant amount of kanamycin-sensitive tobacco regenerants containing the *npt*II gene

in their DNA that provides resistance to the antibiotic.

So, the transgenes, which after the promoter contain an intron of the corn *hsp70* gene or the rice *OsAct1* gene, are expressed in *Nicotiana* plants. Consequently, *N. benthamiana* and *N. tabacum* plants can be used to test vectors carrying the mentioned regulatory elements for the gene expression in monocotyledonous plants. However, it has been shown that monocot introns can have a negative effect on the expression of transgenes in dicot *Nicotina* plants, which was found to be lower than expected the fluorescence level of GFP, and the presence of a significant amount of sensitive to kanamycin tobacco regenerants containing *npt*II in their DNA.
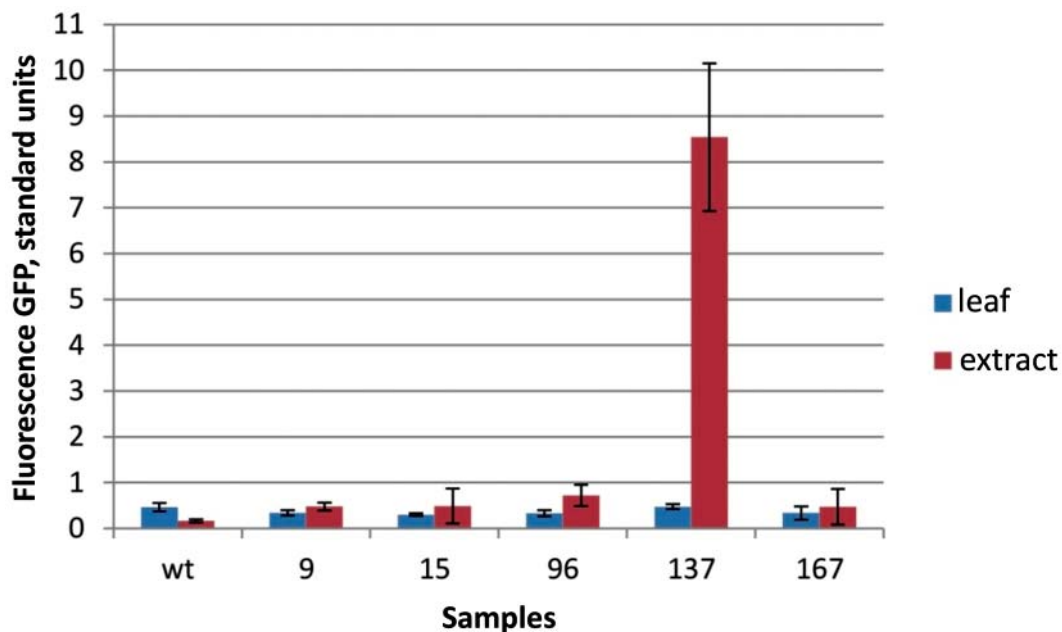
Fig. 6. **Flourimetry of GFP in the leaves of transgenic tobacco lines obtained after *Agrobacterium*-mediated transformation by the vector pCB271:**
wt — non-transformed plants; 9, 15, 96, 137, 167 — lines of transgenic plants

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ВПЛИВ ІНТРОНІВ ОДНОДОЛЬНИХ НА ЕКСПРЕСІЮ ТРАНСГЕНІВ У РОСЛИНАХ *Nicotiana*

І. О. Нітовська
М. Ю. Василенко
Б. В. Моргун

Інститут клітинної біології та генетичної інженерії НАН України, Київ

E-mail: molgen@icbge.org.ua

Метою роботи було дослідити вплив інтронів генів рису *OsAct1* та кукурудзи *hsp70* на експресію трансгенів у рослинах *Nicotiana* для їх застосування при тестуванні векторів, що містять зазначені інтрони однодольних. Використовували методи *Agrobacterium*-опосередкованої трансформації листків тепличних рослин *N. benthamiana* та асептичних рослин *N. tabacum* вектором pCB271, що містить інтрони однодольних, світлову флуоресцентну мікроскопію і флуорометрію зеленого флуоресцентного протеїну (GFP), детекцію трансгенів за допомогою полімеразної ланцюгової реакції. Спостерігали транзієнтну експресію GFP в інфільтрованих тканинах *N. benthamiana*. Отримали трансгенні рослини *N. tabacum,* що є стійкими до канаміцину. Показано флуоресценцію GFP в екстрактах трансгенних ліній тютюну. У частини трансформантів *N. tabacum* спостерігали припинення експресії трансгенів. Таким чином, трансгени, що містять інтрони генів кукурудзи *hsp70* або рису *OsAct1* після промотору, експресуються в рослинах *Nicotiana*. Отже, рослини *N. benthamiana* та *N. tabacum* можна використовувати для тестування векторів, сконструйованих для трансформації злакових. Виявлено, що зазначені інтрони однодольних можуть справляти негативний вплив на експресію трансгенів у рослинах *Nicotiana*.

Ключові слова: інтрони однодольних, *Nicotiana*, *Agrobacterium*-опосередкована трансформація, GFP.

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EFFECT OF INTRACRANIAL CATHETER PLACEMENT ON MICROGLIA METABOLIC PROFILE IN RATS

Y. Hurmach
M. Rudyk
V. Svyatetskaya
L. Skivka

Taras Shevchenko National University of Kyiv, Ukraine

E-mail: jhurmach@gmail.com

The aim of the work was to investigate the effect of the intracranial catheter placement on the metabolic profile of rat microglia. Microglial cells were isolated by the centrifugation in Percoll gradient. Oxidative metabolism and phagocytic activity were investigated by flow cytometry. Arginase activity was examined by colorimetric method. Nitrite level was assayed in Griess reaction. It was found that intracranial catheter placement causes down-regulation of nitrite synthesis by 3 times, augmentation of the reactive oxygen species generation by 1.5 times, and slightly decreases microglia phagocytic activity. Thus, intracranial catheter placement was associated with anti-inflammatory shift of microglia metabolism in rats in distant time period after the device implantation.

Effective drug delivery to the brain structures for the treatment of neurodegenerative diseases, oncopathology, etc. is a serious problem for medical and biological science. The most popular approaches include systemic introduction of therapeutic agents orally or intravenously. However, many of the systemically introduced drugs have very limited ability to penetrate the blood-brain barrier, that severely affects their therapeutic efficiency. In addition, the systemic drug administration stimulates increasing their single and total doses to optimize the dose load of the target tissue in the brain. Increasing the dose of therapeutic agents, especially chemotherapeutic drugs, is associated with their significant systemic toxicity and the development of side effects [1–3]. Currently, numerous biotechnological strategies have been developed and investigated to facilitate the delivery of therapeutic agents to the brain [4–6]. However, the use of targeted intravascular methods of delivery of drugs is a rather traumatic procedure and can cause non-specific inflammatory processes in the brain tissues, which, in turn, can exacerbate the course of neurodegenerative diseases and the tumor process, the basis of which pathogenesis is inflammation [7, 8].

Key effector cells of inflammatory processes in the brain are the microglia – resident brain phagocytes. The functions of microglia in the brain tissue are similar to those of other resident tissue phagocytes. These cells are characterized by high plasticity of metabolism. On the one hand, microglial cells can exert cytotoxic (microbicidal, tumoricidal, etc.) effects, and on the other — have an ability to participate in reparative processes and promote the remodelling of brain tissue and stimulate neuro- and angiogenesis. These characteristics underlie the theory of microglia metabolic polarization, inherent for all resident and circulating phagocytes, to classical proinflammatory (M1) and alternative anti-inflammatory (M2) activation states [9–11].

This study was aimed to investigate the effect of the placement of intracranial catheter on the metabolic profile of rat microglia.
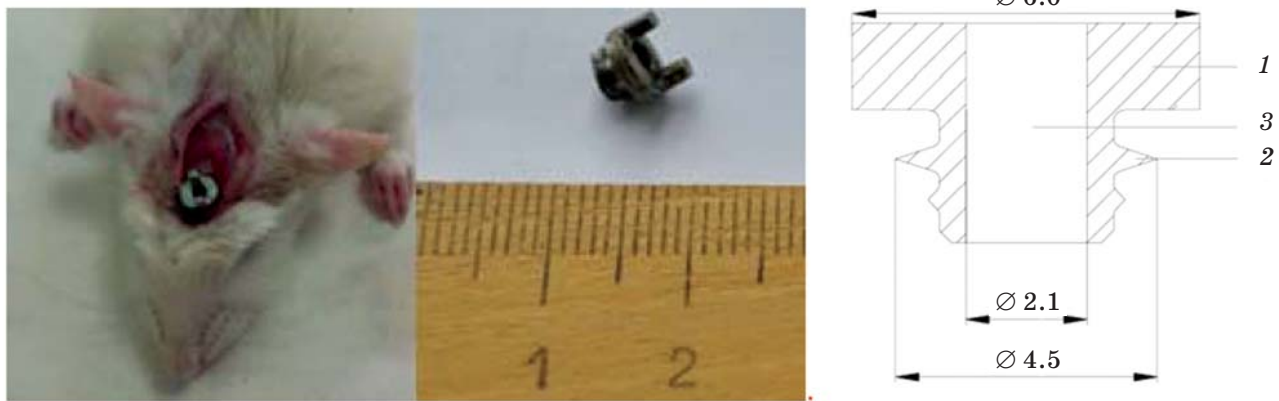
Fig. 1. **Intracranial catheter for multiple intracerebral experimental studies:**
1 — outer sleeve; *2* — self-tapping double-start thread; *3* — inner opening

Materials and Methods

The research was conducted on white outbred adult male rats aged 2–3 months, weighting 200–250 g. Animals were bred at animal facility of Educational and Scientific Center "Institute of biology and medicine" of Taras Shevchenko National University of Kyiv. The animals were kept in the vivarium with free access to water and food. Animal protocol was approved by the Taras Shevchenko National University animal welfare committee according to the Animal Welfare Act guidelines. All animal studies were carried out in accordance with the norms of the Convention on Bioethics of the Council of Europe 'Europe Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes' (1997), the general ethical principles of animal experiments, approved by the First National Congress on Bioethics in Ukraine (September 2001), and national law No. 3447-IV "On the Protection of Animals from Cruel Treatment" [12]. Animals were randomized by weight and divided in two groups ($n = 8$): group 1 — intact animals, group 2 — animals with intracranial catheter.

For these experiments, we developed the device for multiple intracerebral experimental studies (Fig. 1), containing an intracranial catheter (patent of Ukraine for invention No. 114580 dated 26/06/2017 [13].

Developed intracranial catheter is a boss with an outer sleeve with a diameter of 6 mm and a self-tapping double-start thread with a diameter of 4.5 mm, which allows the boss to be screwed and fastened to the scull. The inner diameter of the catheter, through which all diagnostic and therapeutic manipulations are carried out, is 2.4 mm. The inner opening of the catheter is closed with a cap. Trephine opening in the given coordinates is performed by a drill in the form of a trident. The working diameter of the drill is 3.4 mm. It corresponds to the diameter of the trephine opening, and ensures reliable fixation of the threaded part of the boss in the skull.

1–2 days before the device was used, head skin was depilated. Immediately before the surgery, 0.25% of Marcaine ("Recipharm Monts", France) was administered at a dose of 0.04 ml/g to relieve pain for 6–8 hours. Before the device was placed, animals were anesthetized with a Calipsovetum ("Brovafarta", Ukraine). After this, the scalp incision was performed in the projection of the device. Scalp incision was then expanded, washed with hydrogen peroxide, and connective tissue was removed from the skull. Skull surface was then degreased and dried, and trephine opening with the diameter of 3.4 mm was made using a drill, that is completed with the device. The catheter was placed and fastened in the trephine opening with a special screwdriver. The skin was sutured up in such a way that the catheter remained outside. The control animals were treated similarly, but the catheter was not inserted.

The animals were observed for 21 days. The animal behavior, weight, and survival was analyzed. On the twenty second day, their microglia was isolated and the cell functional characteristics were examined.

For the microglial cell isolation, rats were euthanatized by cervical dislocation, the brain was rapidly extracted on ice in a Petri dish, hippocampus was dissected and perfused using 0.9% NaCl with 0.2% glucose ("Darnytsia", Ukraine), and then was homogenized in Potter homogenizer for 15 min at room temperature. The obtained homogenate was filtered through a 40 nm cell strainer (BD Biosciences Discovery) to remove cell conglomerates. The homogenate was transferred into a test tube and centrifuged at 350 g for 10 min at room temperature". The sediment was suspended in 1 ml 70% isotonic percoll solution ("GE Healthcare", USA) and transferred into another test tube. Two ml of 50% isotonic percoll solution were carefully layered on the top of 70% percoll solution. Over the 50% percoll layer, 1 ml phosphate buffer was gently added. Then, density gradient was centrifuged at 1200 g for 40 min. After centrifugation, the layer at the interface between the 70% and 50% Percoll phases contained highly enriched microglia was aspirated. Microglia cells were washed in PBS by centrifugation for 5 min at room temperature and re-suspended in the RPMI-1640 medium (Sigma-Aldrich, USA) for further examination of functional characteristics. The purity of isolated microglia population was estimated by flow cytometry with the use of anti-CD11b antibodies (BD Pharmingen™). The proportion of CD11b+ cells was ≥ 92%. Cell viability, determined by Trypan blue exclusion test, was 96%.

The phagocytic activity was examined as described by Cantinieaux B. et al. with small modifications [14]. FITC-labeled (Sigma Aldrich, USA) thermally inactivated cells of a one-day culture of *S. aureus* Cowan I (collection of the Dept. of Microbiology and Immunology of ESC "Institute of Biology and Medicine") at a concentration of 1×10^7 cells/ml were used as a phagocytosis object. 2×10^5 microglial cells at the volume of 70 µl were placed in the cytometric test tubes, and 30 µl suspension of FITC-labeled *S. aureus* were added and mixed with pipettes. To obtain FITC-labeled *S. aureus,* we dissolved FITC in DMSO (Sigma Aldrich, USA) and mixed with bacteria at the ratio of 0.05 g FITC per 10^8 bacteria. Then the mixture was incubated for an hour in a dark place at room temperature and labelled bacteria were washed thrice by centrifugation. The negative control sample contained 30 µl PBS instead of labeled microorganisms. The cells mixture was incubated for 30 min in CO_2-incubator at 37 °C. After that, the cells

were washed twice by centrifugation in PBS and re-suspended in 400 µl of 0.4% formalin solution ("Enamine", Ukraine). The samples were analyzed by flow cytometry. The results were registered as phagocytosis index that represents the mean fluorescence per one phagocytic cell (that is proportional to the number of ingested bacteria).

Reactive oxygen species (ROS) generation was determined using dichlorodihydro-fluorescein diacetate (DCF-DA) (Sigma Aldrich, USA) [15]. The cells were incubated in a phosphate buffer with 10 µM DCF-DA for 30 min at 37 °C. Fluorescence of the DCF-DA-labeled cells was studied by flow cytometry.

The arginase activity of the microglia was evaluated in colorimetric assay described by Classen et al. [16]. 100 µl 0.1% Triton X-100 (Sigma-Aldrich, USA), 100 µl 50 mmol Tris-HCl (pH 7.5; Sigma Aldrich, USA) with 10 mmol $MnCl_2$ were sequentially added to the cell suspension. The mixture was kept at 56 °C for 7 min to activate arginase activity. 100 µl L-arginine (0.5 mol; pH 9.7; Sigma-Aldrich, USA) was then added to the cells with pre-activated arginase for 2 hours. To stop the reaction of L-arginine hydrolysis, 800 µl of acid mixture (H_2SO_4:H_3PO_4:H_2O = 1:3:7) was prepared immediately before the experiment and were added to the samples. For colorymetric evaluation of urea, α-isonitrosopropiophenon (40 µl, 6 % in ethanol, Sigma-Aldrich, USA) was added to the samples, and the mixture was incubed at 95 °C for 30 min, and than at 4 °C for 30 min. Urea concentration was measured spectrophometrically at $\lambda = 545$ nm. The urea µg was calculated using a calibration curve, that was created using standard urea solutions of known concentration. The data were analyzed using the next formula: µg urea / 60 (MM urea) × 50 (dilution factor)/t (min incubation with arginine) = arginase units per 1×10^6 cells; 1 unit = amount of enzyme necessary to hydrolyze 1 µM arginine per min.

Nitrite production level was measured in cell supernatant by the Griess reaction [16]. To prepare Griess reagent equal volumes of 2% sulfanilamide in 10% phosphate acid and 0.2% naphthylethylenediamine hydrochloride (Sigma-Aldrich, USA) were mixed. 100 µl Griess reagent were added to 100 µl suspension of microglial cells. The mixture was incubated for 30 min at room temperature in the dark. Results were measured spectrophotometrically on a plate photometer Ascent ("Labsystems", Finland) at 540 nm excitation wave length.

Nitrite level was determined using a calibration curve that was created using standard solutions of sodium nitrite. The extinction value was divided by the number of living cells in the sample. Nitrite concentration was presented for 10^6 cells. Each sample was assayed in triplicate, and results are presented as mean ± SD. Statistical significance of the results was determined by Student's *t*-test [17]. For all analyses, $P < 0.05$ was accepted as a significant probability level.

Results and Discussion

Placement of intracranial catheter did not influence significantly animal behavior and did not cause statistically significant changes in their survival or weight throughout the observation period (Fig. 2).

However, on the twenty second day of observation significant alterations in the microglia metabolic profile of catheterized rats were registered. There are several criteria to evaluate metabolic activation profile of phagocytes, including microglia. Arginine metabolism is key characteristics of phagocyte (including microglia) functional polarization. Classic (M1) and alternative (M2) activation of phagocytes is accompanied by different direction of arginine metabolism. Under classic activation, iNOS metabolizes arginine and cytotoxic reactive nitrogen species are formed, while under the alternative activation arginase Arg-1 is induced and metabolizes arginine to urea and ornithine, precursor for polyamines and proline. Biogenic amines (putrescine, spermine and spermidine) are involved in cell growth and can inhibit T-cell immune responses. Increased proline synthesis by phagocytes is characteristic for tumor microenvironment [18, 19]. Proline is a key component of collagen synthesis. Its synthesis is increased during reparation tissue remodelling [20]. Reactive nitrogen species, especially NO, exerts cytotoxic effects and are important signal molecules. In particular, NO activates a number of transcription factors, including NFκB, involved in the synthesis of pro-inflammatory cytokines and several other inflammatory mediators [21, 22]. According to our results, intracranial catheter placement did not cause statistically significant changes in arginase activity of microglia cells (Fig. 3, *A*) while causing statistically significant (almost by three times) decrease in NO synthesis at day 21 of observation period (Fig. 3, *B*).

Arginine metabolism shift to decreased activity of NO-synthase is a sign of alternative or anti-inflammatory metabolic activation of phagocytes, including microglia. In the case of catheter placement, such microglia metabolic skew can result from the activation of prolonged reparation processes.
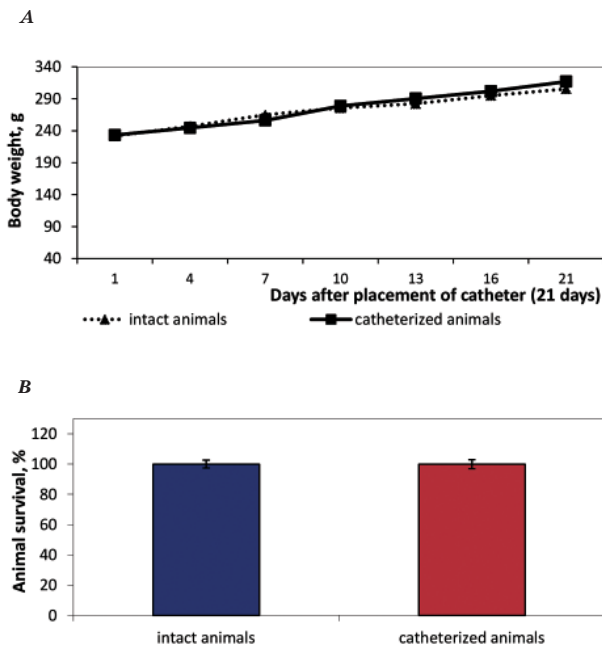

Fig. 2. **The effect of intracranial catheter placement on survival (*B*) and weight of rats (*A*)** (*n* = 8 in all groups)
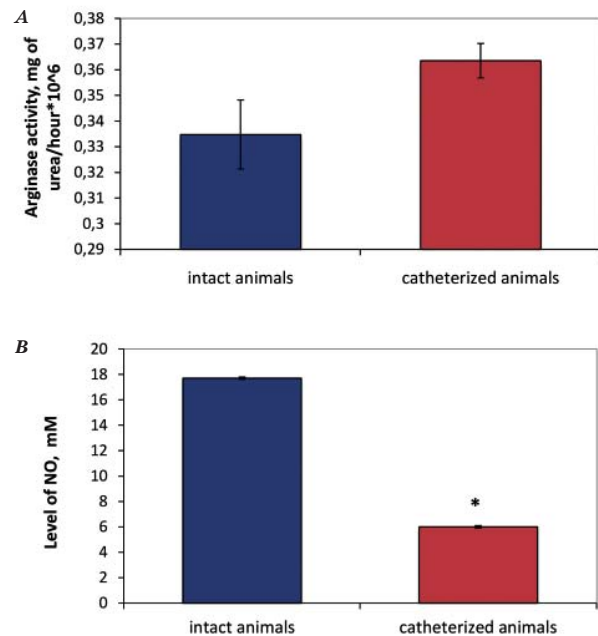

Fig. 3. **The effect of intracranial catheter placement on the arginase activity (*A*) and NO synthesis (*B*) inmicroglia cells** (*n* = 8 in all groups)
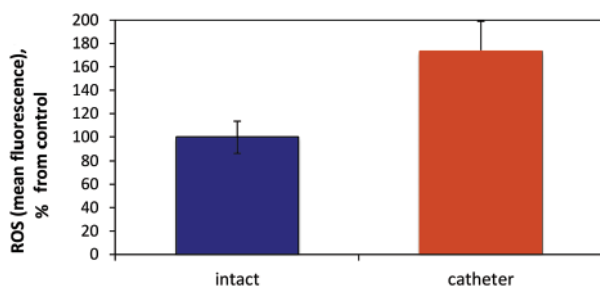* — $P < 0.05$ as compared to intact animal value

Fig. 4. The effect of intracranial catheter placement on reactive oxygen species generation by microglia cells ($n = 8$ in all groups)
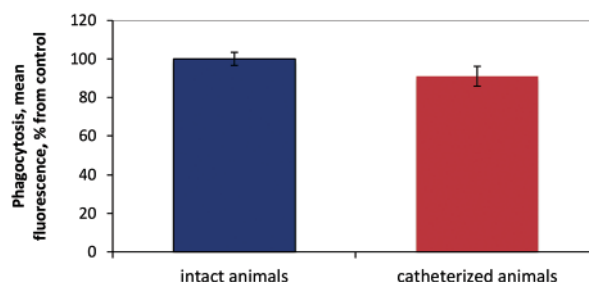* — $P < 0.05$ as compared to intact animal value

Fig. 5. The effect of intracranial catheter placement on phagocytic activity of microglia cells
($n = 8$ in all groups)

ROS synthesis in characteristic for both pro- and in anti-inflammatory metabolic activation of phagocytes, since it is necessary for the regenerative and destructive inflammatory processes [23–25]. Intracranial catheter placement caused 1.5-fold increase in ROS production (Fig. 4). To characterize the functional polarization of phagocytes, the change in their oxidative metabolism is commonly considered in the context of arginine metabolism shift. In our case, increased ROS production along with decrease in reactive nitrogen species synthesis might indicate anti-inflammatory polarization of microglia metabolism.

Phagocytic activity is associated with increased expression of a number of receptors, including scavenger receptors. Generally, up-regulation of this function is considered as a sign of the alternative (M2) cell polarization [26]. In our experiments, phagocytic activity in microglia of experimental animals did not differ from that in control animals (Fig. 5).

Pro-inflammatory phagocyte activation is presumably followed by the decrease of their endocytosis function. Unchanged phagocytic activity along with up-regulated ROS generation and skewed arginine metabolism towards increased arginase activity indicate anti-inflammatory metabolic shift in microglial cells.

Therefore, intracranial catheter placement was associated with anti-inflammatory shift of microglia metabolism in rats in a distant time period after the device implantation. Catheters are devices used for intracerebral drug delivery as well as for modeling of a number of brain diseases such as neurodegenerative disease or tumors. The ability of the device to cause anti-inflammatory metabolic activation of microglia might obscure true results of disease modeling or treatment, and should be taken into account in such experiments.

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ВПЛИВ ІМПЛАНТАЦІЇ ІНТРАКРАНІАЛЬНОГО КАТЕТЕРА НА МЕТАБОЛІЧНИЙ ПРОФІЛЬ МІКРОГЛІЇ ЩУРІВ

Є. Гурмач
М. Рудик
В. Святецька
Л. Скївка

Київський національний університет
імені Тараса Шевченка, Україна

jhurmach@gmail.com

Метою роботи було дослідити впливу встановлення інтракраніального катетера на метаболічний профіль мікроглії щурів. Клітини мікроглії виділяли методом центрифугування в градієнті щільності перколу. Оксидативний метаболізм і фагоцитарну активність досліджували методом проточної цитометрії. Аргіназну активність вивчали колориметричним методом. Синтез нітритів визначали в реакції Грісса. Було виявлено, що встановлення інтракраніального катетера спричинювало зниження синтезу нітритів клітинами мікроглії в 3 рази, посилення синтезу реактивних форм кисню в 1,5 раза і неістотно знижувало фагоцитарну активність. Імплантація катетера не впливала на масу і життєздатність тварин. Таким чином, встановлення інтракраніального катетера було асоційовано з протизапальною метаболічною активацією мікроглії щурів у віддалені терміни після імплантації пристрою.

Ключові слова: фагоцити, мікроглія, метаболічний профіль, інтракраніальний катетер.