Research Article

The Development of a Technique for Sensitizing Cells of Various Origins for Biotechnological Purposes

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Abstract The functional activity stimulation of cell cultures was tested in MDBK cell culture, photobacteria *Aliivibriofischeri*and*Halobacteriumhalobium*. Theaim of the investigation was to increase the "yield" of the cells using an environmentallysafe stimulant and membrane-tropic agent that isalso safe for the experimenter. Ultrasonicwaves were used.Experimental ultrasonic exposure varied within the following limits: time from 1 to 300 sec, SATA-intensity of 0.01–2.0 W/cm2, generation frequency of 0.88 or 2.64 MHz, standing or traveling wave. The modulation frequency range was within 0.1–150 Hz. The devices used were: UST-1-01F, UST-5 and UST-1.02C. The modulating generators were G3–112 and CP–110.Stimulation of MDBK cell growth was initiated by US-intensity of 0.03–0.05 W/cm^2, with an exposure of 5–30 sec.Exposure to ultrasound with an intensity of 0.2–0.4 W/cm^2(for 3 min) had a stimulating effect on bioluminescence and was associated with an increase in the growth rate of*A. fischeri*. The findings indicated that 0.4 W/cm^{2}ultrasonic intensity and modulation frequencies from 0.25 to 0.7 Hz can stimulate the growth of archaea.It was revealed that the maximum proliferation index in all cases of stimulant application was noted in cultures with minimal initial proliferative activity in the control.The authors expect thatthese results on the possibilities of acoustic continuous and modulated waves can be applied for biotechnological purposes to develop a new biotechnological method.

Keywords: cell culture, ultrasound, proliferation, stimulation

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1. Introduction

The method of the functional activity stimulation of cell cultures used in biotechnology was tested by us in several objects:transplantablebovine kidney cell culture MDBK, photobacteria*Aliivibriofischeri*and*Halobacteriumhalobium*. In the production of therapeutic, prophylactic and diagnostic drugs, MDBK cell culture is used to cumulateand to cultivate the virus. The membrane of MDBK cells is thin; these cells are very sensitive to changes in cultivation conditions. For the cultivation of animal cells and tissues, including the MDBK cell culture, the usual method of inoculation of the cell suspension in a synthetic nutrient medium, balanced in chemical composition and containing all the necessary components for cell growth, proliferation and reproduction, is used. In

the case of a conventional cell culture method, the proliferation index is 3.5–4.0. The disadvantages of this technique are a slight increase ofthe yield of cells during the cultivation process and incomplete use of the growth resources of the nutrient medium.

When choosing microbial objects for our work, we considered taxonomic differences, the originality of their physiological characteristics and attractive properties for researchers.Halotolerant culture *A. fischeri* belongs to the domain*Eubacteria*, the extremely halophilic culture *H. halobium* belongs to other domain — *Archaea*. The structure of the cell wall of these organisms is significantly different from each other.A characteristic feature of gram-negative bacteria *A. fischeri*is the multilayer structure of the cell wall, which provides significant stability to external influences. The cell integument of bacteria consists of the cytoplasmic membrane, the peptidoglycan layer that forms the inner layer of the cell wall, the periplasmic space and the outer membrane.The latter is an additional layer of the cell wall, consisting of phospholipids, proteins, lipoprotein, small amount of mureinand lipopolysaccharide complex. Bacterial cells can survive even in freezing and thawing conditions.

Archaea*H. halobium*lacks a peptidoglycan layer in its cell integument. Membranes consist of a lipid monolayer surrounded by an S-layer of a glycoprotein, which contains about 50% of the proteins of the cell integument. Conventional lipoprotein membranes are unable to maintain cell shape at high salt concentrations in the medium. The archaeal bilayer membrane is built of polar lipids (isoprenylphosphoglycerol diesters) with a low content of non-polar lipids (c30 isoprenoids) and the indicated high protein content. These are sensory proteins, especially bacteriorhodopsin, which acts as an ATP-dependent translocase[1, 2]. Bacteriorhodopsin is a light-sensitive protein that supplies the cell with chemical energy, using sunlight to pump proton pumps out of the cell. As a result, the archaea use a proton gradient across the membrane to control the synthesis of the ATP-energy transporter. Thus, the structure and composition of the cell integuments of the archaea, as well as in the luminescent bacteria, indicate the resistance of their cells to changes in the surrounding conditions.

Both experimental microbial cultures are chemoorganotrophs and have unique biochemical functions: *A. fischeri* — the function of luminescence, *H. halobium* — the function of bacterial photosynthesis. Bacteria are capable of detoxifying reactive oxygen species, have a high affinity for an oxygen substrate that activates luminescence even at low 0_2concentrations [3]. Archaea, depending on the conditions (light or darkness, the presence of low concentrations of oxygen and assimilable organic compounds), can be phototrophs as an energy source;they also possess protective mechanisms of

active transport of ions, preventing osmotic stress in conditions of high salinity of the environment.

Due to their unique properties, *A. fischeri* and *H. halobium* are widely used as model organisms in biochemical, biotechnological, genetic research, as well as in research on functional genomics.

Working with biological objects of different nature, we set anaim to increase the ”yield” of cells using an environmentally safely stimulant and membrane-tropic agent that is safe for the experimenter. We opted for acoustic impact.

2. Materials and Methods

Experimental ultrasonic exposure varied within the following limits: time from 1 to 300 sec, SATA-intensity of 0.01–2.0 W/cm^2, generation frequency was 0.88 or 2.64 MHz, standing or traveling wave. The modulation frequency range was within 0.1–150 Hz. Devices: UST-1-01F; UST-5 and UST-1.02C.Modulating generators G3–112 and CP–110. US-treatment of model cells was carried out according to the authors' method [4, 5].As indicated above, the *in-vitro* effects of ultrasound (US) of therapeutic intensities in cells has been tested in several objects.

The MDBK cell culture was grown according to the traditional method in 50 ml-mattresses. The monolayer in the control was formed on the second or third day. The cultivation of the sonicated and control cells was carried out at a temperature of 37°C in Eagle's medium with the addition of 10% bovine serum, antibiotics lincomycin or kanamycin (100 μg/ml), 5 % glutamine and sodium bicarbonate until the pH was neutral. The initial concentration of cells was 8,010^4–1,010^{5}per 1 ml.

The composition of the growth medium for the cultivation of bacteria included (in g/l): peptone – 10, yeast extract – 1, NaCl – 30, Na_2HP0_4– 5.3; KH_2P0_4– 2.1; $(NH_4)_2S0_4$– 0.5, $MgS0_4$– 0.l;glycerol – 3 ml; pH of the medium was 7.4. Growth medium for extremely halophilic archaea included (in g/l): yeast extract – 10, casamino acids – 7.5;Nacitrate – 3, KCl – 2, NaCl – 250, $MgSO_4\times 7H_2O$ – 20, $FeCl_2\times 4H_2O$ – 0.036, $MnCl_2\times 4H_2O$ – 0.36 мг; pH 7.4. The bacteria were grown at room temperature in 100-ml conical flasks with 50 ml of medium on a shaker at 90 rpm. Archaeaweregrownin Erlenmeyer flasks with a volume of 500 ml with 100 ml of medium for five days at 37°C on a shaker at 180 rpm and lighting with monochrome light with a wavelength of □ = 560 nm.

Suspensions of synchronized cell cultures at different stages of the cell cycle were used for research; the initial number of cells with an intact membrane in the control and experiment was at least80 %. The integrity of the membrane of the studied cells

was determined by the absence of staining with trypan blue. Statistical processing of the results was carried out using a package of applied programs «Statistica 6.0». The significance of differences in mean values was determined using the paired Student's t-test. The differences were considered significant when$p < 0.05$.

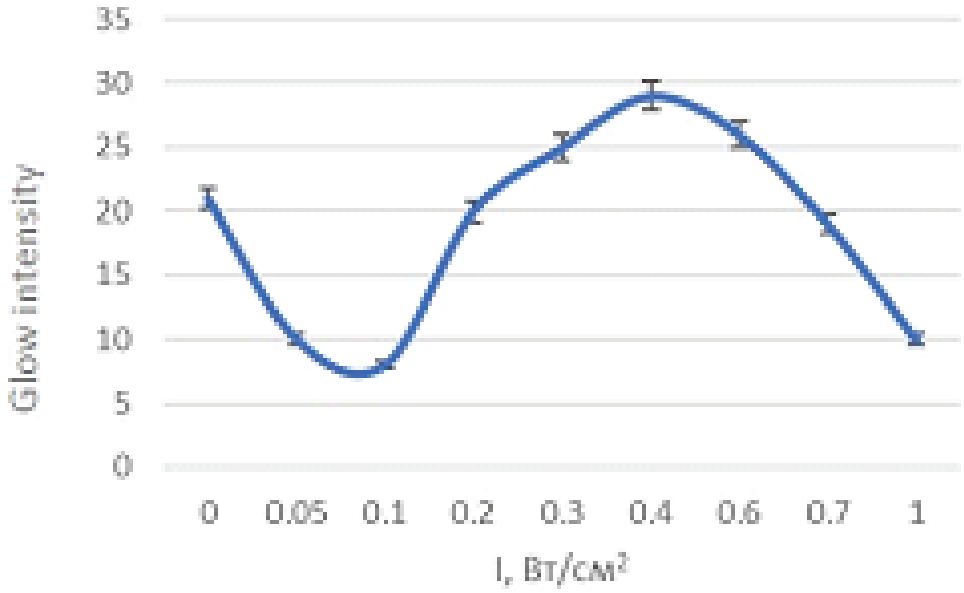
3. Results and Discussion

3.1. Stimulation of the growth of MDBK cells

Stimulation of MDBK cell growth was initiated by US-intensity of 0.03–0.05 W/cm^2 with an exposure of 5–30 sec. The increase in cell mass in comparison with the control was 65–130 %, depending on both the duration of exposure and the stage of the cell cycle. The maximum stimulating effect during the treatment of cells with continuous ultrasound was set at 0.05 W/cm^{2}with an exposure of 10 seconds after a single "pre-plant" irradiation.The growth of cells in the control did not exceed 3,110^5 /ml.As a result of US-stimulation, the proliferation index increased from 3.8 to 9.0($p < 0,05$). Also, in the "experimental" cultures, an increase in the proliferation rate was recorded — the monolayer was finally formed 10–12 h faster than in the control. In cases of US-treatment of cells at the stage of monolayer formation, no positive effect was observed. It was revealed that the maximum proliferation index (9.1-13.7) in all cases of stimulant application was noted in cultures with minimal initial proliferative activity in the control (2.7-3.0).The authors exclude the possibility of a direct effect on the mitotic apparatus of cells, since the work was carried out in the range of minimum therapeutic US-intensities. The increase in cell mass is the result of a change in the permeability of the cytoplasmic membrane after ГЫ-irradiation (data from the trypan blue test), therefore more nutrients can penetrate the cell.

3.2. Ultrasonic effect in marine luminescent bacteriaAliivibriofischeri

Marine luminescent bacteria are used as biosensors. Due to the presence of luciferase and various types of sensitive structures, photobacteria react objectively and quickly to the effects of factors of variousnature.We studied the effect of ultrasound on the growth and emission characteristics of *A. fischeri* culture, determined the parameters of suppression and stimulation of its growth and biochemiluminescence (0.88 MHz, 3 min, I_{SATA}of0.4 W/cm^2, continuous mode, standing wave).It has been proven experimentally



Authors' own work. The abscissa axis is the luminescence intensity, rel. units; Y-axis — US-intensity, W/cm^2

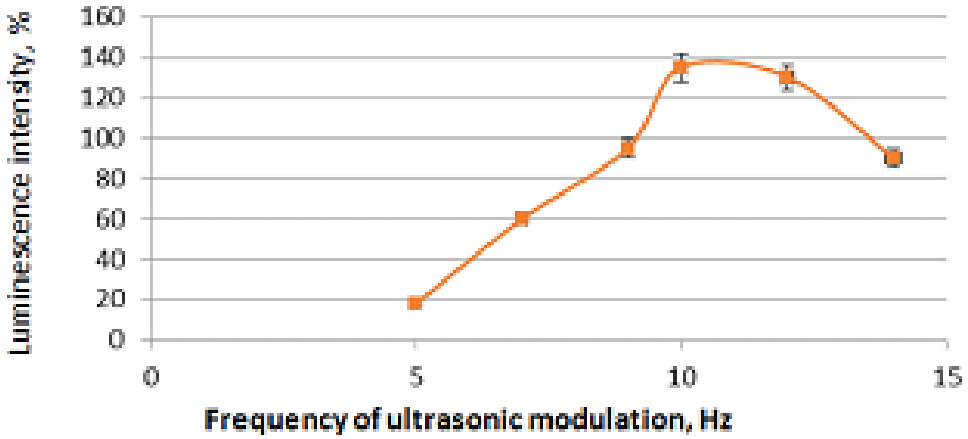
Figure 1: Changes in the luminescence of photobacteria after exposure to ultrasound of various intensities

[4] that ultrasound of low intensities 0.05–0.1 W/cm^2 with an exposure time of 1–3 min has practically no effect on the subsequent growth and development of the bacterial culture, but it compresses the rate of emission of insonated cells, which probably did not reach the *quorumsensing*-state. However, the intensity of luminescence quickly recovered after the cessation of irradiation. With an increase in the US-intensity from 0.1 to 0.2 W/cm^2, the cell luminescence sharply increased and gradually reached the control value. Exposure to ultrasound with an intensity of 0.4 W/cm^2 had a stimulating effect on bioluminescence (Figure 1) and, to a lesser extent, on an increase in the growth rateof*A. fischeri*. In the interval between 0.4 and 0.6 W/cm^2, no visible changes in the luminescence of bacteria were observed. Perhaps, under these intensities, the effects of suppression and stimulation of bioluminescence turned out to be equal.Irreversible suppression of bioluminescence was observed at intensities above 0.6 W/cm^2. The number of viable cells decreased progressively.TheUS-intensity at which the suppression of bioluminescence begins, coincides with the threshold of cavitation in the suspension. This was confirmed by experiments on the occurrence of ultrasonic cavitation luminescence in medium free of bacterial cells at intensities exceeding 0.6 W/cm^2.

After treatment with continuous ultrasound, a two-phase change in the luminescence was observed. For the first time, the luminescence intensity after exposure to ultrasound returned to the control level upon treatment with0.2 W/cm^2. A maximum was observed for the second time in the intensity range of 0.4–0.6 W/cm^2. Therefore, to study the

TABLE 1: Change in emission activity of *A. fischeri* depending on the modulation frequency of ultrasound at an intensity of 0.2 **W/cm²**

Ultrasonic frequency (v), Hz	control, intact cells	10–25	35–45	55–65	75–80	85–90	100	120
Photoemissio intensity, rel. units	20.8±0.3	20.9±0.3	19.8±0.2	20.4±0.3	20.8±0.2	25.2±0.4	29.4±0.2	0



The abscissa axis is the modulation frequency, Hz. Y-axis — luminescence intensity, percentage in relation to control.Authors' ownwork

Figure 2: Dependence of changes in the luminescence intensity of A. fischeri on the frequency of ultrasonic modulation at 0.4 W/cm²

possibility of directional modulated action on microorganisms, the same US-intensities of 0.2 W/cm²and 0.4 W/cm² were chosen, under the influence of which an increase in photoemission was noted.

It was found that the directional effect of ultrasound with an intensity of 0.2 W/cm²and a modulation frequency of less than 85 Hz had little significant effect on the degree of emission of cellcultures, while at an US-modulation frequency of 120 Hz, bioluminescence was completely suppressed (Table 1). At the specified intensity of 0.2 W/cm², the action of ultrasound significantly increases the degree of emission($p < 0.05$).

The ultrasonic intensity of 0.4 W/cm²with a modulation frequency of 9 and 14 Hz does not significantly affect the photoemission. But at lower modulation frequencies, 5–8 Hz, at first, a partial loss of emission was recorded, and then the luminescence intensity significantly decreased by 2–5 times. Stimulation of luminescence was observed only at modulation frequencies of 10–12 Hz.The growth and intensity of luminescence of *A. fischeri* bacterial cells (Figure 2) were significantly stimulated by insonation with a US-modulation frequency of 10 Hz; cell luminescence was not detected in the area of influence of 3 Hz and more than 20 Hz. The regulation of luminescence depends on the density of the cell population and occurs at the time of transcription.Proportionally

to the growth rate and density of bacteria, *A. fischeri* cells synthesize and release into the medium a small amount of a signaling compound — the autoinducer *N*(3-oxohexanoyl)–*L*–homoserine lactone.The latter, upon accumulation and reaching the threshold concentration — the *quorum-sensing*-effect — induces emission. The autoinducer easily penetrates the cell membrane and binds to the receptor — transcription regulator*Lux-R.Lux-R*is a member of the homoserine lactone-derived signaling molecule family, activates expressionof*luxGDABEGH*locusThe intensification of growth and an increase in luminescence authorsexplained, as in the MDBK culture, by an increase in membrane permeability after US-exposure (trypan blue test data) and substrate consumption, the release of an autoinducer and activation of cellular respiration (data not shown).

The photoemission catalyst is the cytoplasmic enzyme luciferase — monooxygenase, which carries out the conjugate oxidation of *NADPH* and long-chain aldehyde *(R–CHO)* with molecular oxygen. Reactive oxygen species that intensify of emission are formed in biological media during the passage of an acoustic wave[6, 7]. Also, the bacterial bioluminescence system includes fatty acid reductase. In our opinion, the ultrasonic action can stimulate the catalyst yield.Then, in the presence of reactive oxygen species, an exothermic reaction is initiated, accompanied by luminescence in the visible mid-wavelength part of the spectrum.Moreover, the released free energy is spent exclusively on emission, the intensity of which directly depends on the □□*P*and*NADPH* concentration

Natural and recombinant luminescent bacteria have now become a widespread tool for biotesting of surface water bodies, of industrial drains and soils, as well as for determining the degree of toxicity of newly synthesized chemical compounds and pharmaceuticals. An attractive feature of bioluminescence sensors is that they do not require an excitation source, since light is generated in a reaction that provides extremely high sensitivity. An equally important advantage of light emission is the ease of detection and the availability of modern equipment for photon's counting.Speed, accuracy and sensitivity to the integral action of pollutants are important features of luminescent test systems [8–10].

3.3. Effect of modulated ultrasound in culture Halobacteriumhalobium

The cells of the experimental culture of the archaea *H. halobium* were sonicated with different US-intensity and modulation frequency. It is found that at a higher ultrasonic

Table 2: Dependence of the optical density of archaea culture on the frequency of ultrasound modulation at an intensity of 0.4 W/cm^2($p < 0.05$)

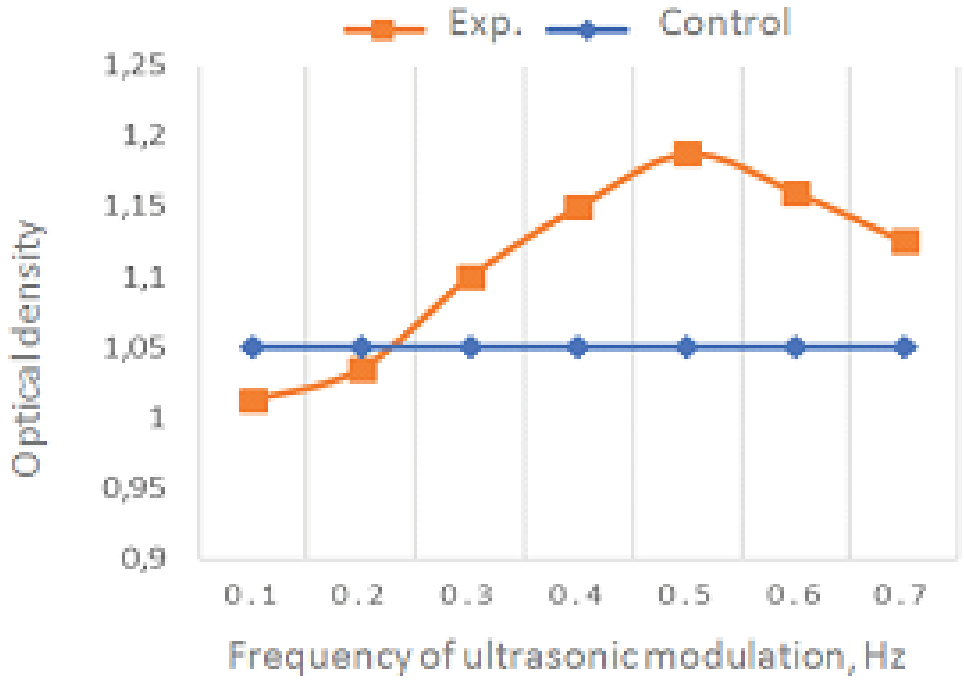
ν, Hz	control, intact cells	0.1	0.2	0.25	0.3	0.5	0.7
D, rel. units; ΔD = ± 0.0011	1.0500	1.0125	1.0350	1.0602	1.1005	1.1875	1.1250
ν, Hz		10–25	35–45	55–65	75–80	85–90	100
D, rel. units; ΔD = ± 0.0015		1.0475	1.0450	1.0452	1.0505	1.0311	1.0215

intensity, equal to 0.4 W/cm^2, only low modulation frequencies from 0.25 to 0.7 Hz can stimulate the growth of archaea.The rest of the experimental frequencies of US modulation had little effect on the growth of the culture.

Exposure to ultrasound with higher modulation frequencies from 10 to 100 Hz (table 2), mainly caused a decrease in the optical density of the cell suspension of archaea, reaching the level of control values only in a narrow range of 75–80 Hz.

The cells of the culture of archaea *H. halobium* (Figure 3) were more sensitive ($p < 0.05$)to the modulated ultrasound of the selected frequency range and intensity.Ultrasonic frequencies 0.1–0.2 Hz at an intensity of 0.4 W/cm^{2}and an exposure time of 3 min changed the growth pattern, slightly suppressing it in comparison with the growth of control cultures. The frequency range from 0.25 to 0.5 Hz led to an intensification of the growth of the strain, and an increase to 0.7 Hz resulted in a decrease in the growth rate and optical density in comparison with the most effective frequency of 0.5 Hz, nevertheless leaving the optical density higher than the density of the control population.10 Hz-modulation of US, in contrast to *A. fischeri* with sonication of the same intensity, did not significantlyaffect the growth of archaea culture, and the frequency range of 85–100 Hz resulted in a depressing effect.

The third model object — archaea *H. halobium* (*salinarum; N. pallidum*) — depending on the cultivation conditions, is a photo- or chemo-organo-heterotrophs.Due to the natural protein, archaeon bacteriorhodopsin can convert light energy into the electrochemical energy of generated protons H^+ and ATP, which is important for the industry of photoconverting nanomaterials and biomolecular electronics [11, 12].Thereby, the authors consider the direction they have found to intensify the technology of biomass obtaining to be promising.



.The abscissa axis is the modulation frequency, Hz. Y-axis — optical density of the cell suspension, rel. units. The blue line marks the control level.Authors' own work

Figure 3: Dependence of the optical density of the cell suspension of archaea *Halobakteriumhalobium* on the modulation frequency at US-intensity of 0.4 W/cm^2

4. Conclusion

Despite the various origin of cells and serious differences in the structure of the cell integument of the model objects, the possibility of controlling of the functional state of *MDBK*, photobacteria *Aliivibriofischeri*and *Halobacterium halobium* using the single membranotropic agent has been proven.That allows us to speak about the universality of US-influence on the physiological processes in biotechnological cultures. The optimal modes of exposure depend on the population density and the structure of cell covers, but the authors showed that the maximum energy of the ultrasonic field in all cases should not exceed 0.4 W/cm^2.US-stimulation of the growth and proliferation of cell cultures made it possible to increase the growth and shorten the time to obtain a unit volume of cell mass; significantly reduce the cost of its production, and, consequently, to significantly simplify, to speed up the production and to reduce the cost of control, therapeutic, prophylactic and diagnostic drugs in the production of which cells are used.

The practical significance of this stage of work determines the need to find environmentally friendly and relatively easy-to-implement methods and nanotechnologies when obtaining biological products [13, 14]. The authors expect that the study of the possibilities of acoustic continuous and modulated waves applying for these purposes will make it possible to develop a new biotechnological method.

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References

[1] Oesterhelt D,Stoeckenius W.Rhodopsin-like protein from the purple membrane of *H. halobium*. Nature. 1971;233(89):149–160.

[2] Mosin OV,Skladnev DA, Shvets VI. Incorporation of [2,3,4,5,6-$^{2}H_{5}$]phenylalanine, [3,5-$^{2}H_{2}$]tyrosine and [2,4,5,6,7-$^{2}H_{5}$]tryptophan into the *Halobacterium halobium* bacteriorhodopsin molecule. Applied Biochemistry and Microbiology. 1999;35(1):34–42.

[3] Ismailov ADLuminescence functions of marine photobacteria in the natural environment. Autotrophic microorganisms: 5th All-Russian symposium with International participation. Moscow: Lomonosov Moscow State University; 2015.

[4] Oleshkevich AA, Kaminskaya EV, Nosovskiy AM. (2014) Experimental and theoretical substantiation of methods for increasing the production of cells of various etiologies after treatment with acoustic (ultrasonic) waves. Part 1. Method of intensification of metabolic processes of bacterial cells in suspension. Biomedical Radioelectronics. 2014;2:53–57.

[5] Oleshkevich AA, Pashovkin TN. (2015) Biochemical and biophysical effects of continuous and modulated ultrasonic waves in *Alivibriofischeri* and *Natrinema pallidum*. Veterinary, Zootechnics and Biotechnology. 2015;12:50–56.

[6] Firestein, F & Rozenszajn, LA & Darvish, Leili & Elimelech, R & Radnay, J & Rosenschein, Uri. Introduction of apoptosis by ultrasound application in human malignant lymphoid cells: Roles of mitochondria-caspase pathway activation.Annals of the New York Academy Sciences. 2003;1010:163–166.

[7] Lagneaux, Laurence & Meulenaer, Eric & Delforge, Alain et al. Ultrasonic low-energy treatment: A novel approach to induce apoptosis in human leukemic cells. Experimental Hematology. 2002;30:1293–1301.

[8] KhrulnovaSA,Manukhov IV, Zarubina AP, Zavilgelsky GB. *Aliivibriologei* KCh1 (Kamchatka isolate): Biochemical and luminescent characteristics, cloning of the *lux*-operon. Microbiology. 2010;79(3):368–375.

[9] MazhulMM, Zarubina A.P., Yudina T.P., Danilov V.S., Zavil'gel'skii G.B. FMN-reductase from *Escherichia coli* and its effect on luciferase activity from marine bacteria *Vibrio*

fischeri. Microbiology. 1999;68(2):149–154.

[10] Yudina TP, Danilov VS. (2015) Investigation of the genotoxicity of substances by a luminescentbacterial test. Autotrophic microorganisms: Materials of the 5th All-Russian symposium with international participation.Moscow; Lomonosov Moscow State University; 2015.

[11] Akopyan VB.Baksin: Monograph. Moscow: International Center for Scientific and Technical Information; 2000.

[12] Mosin OV, Ignatov I. (2014) Synthesis of photochromic transmembrane protein bacteriorhodopsin by halobacterium*Halobacteriumhalobium*. Science of Science. 2014;2 10-17

[13] Kozlov NA. Electromyostimulationat the postoperative period for herniated intervertebral disc in dogs. International Veterinary Bulletin. 2012;4:31–34.

[14] Wu J, Nyborg WL, editors. Emerging therapeutic ultrasound.Burlington: Imperial College Press; 2006.