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THE TECHNOLOGY OF CHEMICAL COMPOUND BIOTOXICITY ASSESSMENT BY THE METHOD OF AGAR BASINS

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ABSTRACT

The assessment of various compound biotoxicity with respect to microorganisms is an essential stage of research in the field of medicine, biology, pharmacology, synthesis of new chemical and chemotherapeutic compounds. In our work, we presented a technology of biotoxicity assessment for various chemical compounds to the representatives of pathogenic and opportunistic microflora applying the method of agar basins with diffusion into the substrate. The proposed technology allows to significantly reduce the time of biotoxicity studies, because, by its nature, it combines the agar diffusion method and the method of serial dilution, which lets us assess the degree of the studied compound influence on the growth of test organisms in the same conditions of cultivation. One of the main advantages of the research technology is the capacity to assess biotoxicity of the

various concentrations of compounds not only visually but also we can describe the quantitative characteristics of the compound influence on macrocultural growth characteristics of microorganisms.

The only defect of this technology is the inability to assess biotoxicity of the compounds with low dissociation in aqueous solutions or with the complete lack of solubility. It should be noted that compounds with high levels of dissociation the high bactericidal and bacteriostatic characteristics, so, in this work, we give biotoxicity estimates of heavy metal salts, which led us to suggest that creation of the peak concentrations of the essential elements such as zinc and copper have the pronounced toxic effects on microorganism population growth.

The effects of the toxic elements such as lead and cadmium activate the detoxification mechanisms in bacteria under the excessive concentrations of these elements in the substrate. This technology also allows evaluating the perspective sorption characteristics of microorganisms in interaction with various xenobiotic agents.

Keywords: Technology of biotoxicity assessment, Essential and toxic elements, Test organisms and Assessment of biological effects of synthesized chemical compounds.

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1. INTRODUCTION

1.1. Timeliness

Currently, intensification of the opportunistic microorganisms is observed in all countries of the world. The bacteria of this group are characterized by a pronounced heterogeneity, being, on the one hand, representatives of human's transient normal microflora and, on the other hand, agents of infections. In this regard, the problem of infections, especially caused by the representatives of the family *Enterobacteriaceae*, is paid much attention [4].

Moreover, intoxication with heavy metals is a question of present interest. The extent of toxic effects depends on the biological characteristics and the individual sensitivity of the organism. The main way of bringing heavy metals into the body is the gastrointestinal tract, which is most vulnerable to the effects of man-made ecotoxicants [5]. Heavy metals and their compounds can come in a living organism through the lungs, mucous membranes, skin, and the gastrointestinal tract. Mechanisms and speed of their penetration through different biological barriers and mediums depend on physicochemical properties of the substances, chemical composition and the conditions of internal body environment [6].

Formation of microorganism sustainability to chemical compounds (in our case, to heavy metals) remains one of the most actual problems of modern microbiology [7]. The acquired resistance is associated with the ability of individual strains of micro-organisms to remain viable under high concentrations of chemical compounds. One of the promising ways to overcome the microorganism persistence is the search and implementation of new technologies that will allow assessing the toxicity of chemical compounds.

In our work, we present a technology of biotoxicity assessment for various chemical compounds to the representatives of pathogenic and opportunistic microflora applying the method of agar basins with diffusion into the substrate [8]. The proposed technology allows to significantly reduce the time of biotoxicity studies, because, by its nature, it combines the agar

diffusion method and the method of serial dilution, which lets us assess the degree of the studied compound influence on the growth of test organisms in the same conditions of cultivation.

2. MATERIALS AND METHODS

We used bacterial strains of the genus Bacillus as test organisms on the approbation of the effectiveness of the proposed technological approach, also we applied salts of heavy metals and the organic compounds synthesized on their basis as a xenobiotic influencing factor.

We developed the microbiological punch for cutting basins out of agarized feed medium for research optimization. This device significantly reduces the time of research but also allows to present experimental research with a high percentage of authenticity.

"The facility for cutting basins in the agar gel" is the closest to the proposed device [1, 2, 3]. The device for cutting basins in the agar gel contains a stamp with punches and the plate for agar. The punches are located at radii relative to the longitudinal axis of symmetry to ensure directional diffusion of the investigated material while conducting reactions of immunodiffusion. The plate is recessed with symmetrical notches which sides are parallel to the symmetry axes, the last are the radii of the punch location.

The distinctive feature of the device we construct is that it possesses seven fixed punches that allow doing seven equally spaced basins with one click. Cut out blocks are removed from the punch by blowing tubes using a rubber bulb. The punch is provided with detent mechanisms, a return spring, and a return plate which allow not only to fix the device on the Petri dish surface, but also to deepen the tubes of the punch in precise, given distance to avoid leaks of the liquid from a basin into the crack between the agar and the bottom of the dish.

On the basis of our drawings, we managed to represent the model in 3D for better visibility, elimination of all shortcomings, and miscalculation of the nuances (Figure 1).

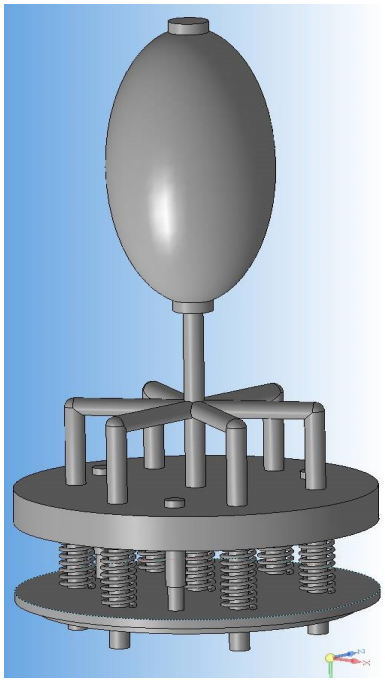


Figure 1 3D-model of the constructed device

We needed to pick up conforming materials for manufacturing the experimental model of the punch. The plates should be durable, able to withstand high temperatures under sterilization, as transparent as possible. The tube-punches should be of metal, durable, able to avoid dulling with multiple uses.

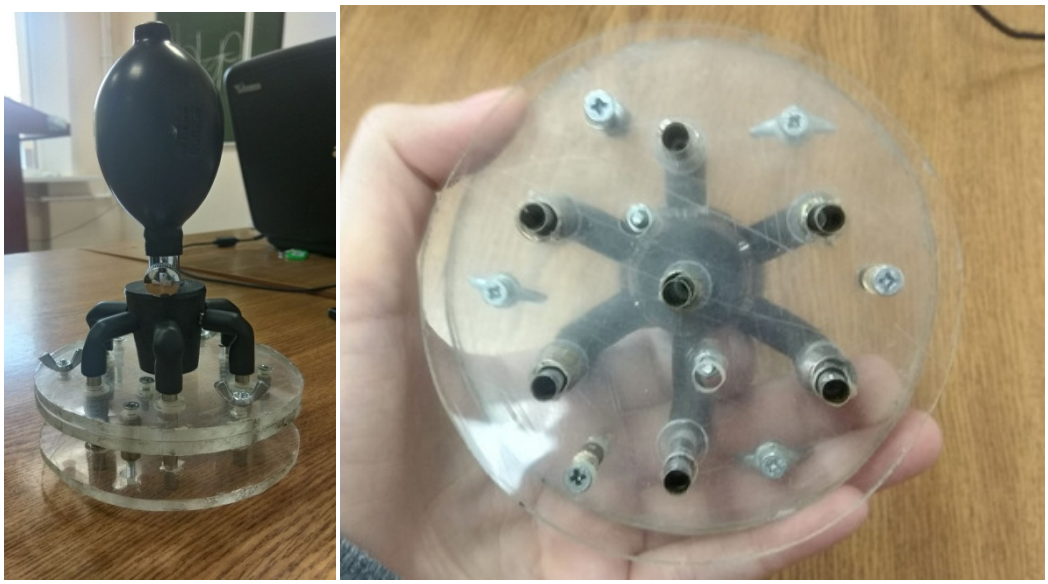
As a result, our choices were the plexiglass for the plates and the medical steel for the tube-punches, the detent mechanisms.

The plexiglass consists entirely of a thermoplastic resin. The chemical composition of the standard plexiglass of all manufacturers is the same. The main advantages of the plexiglass are: small heat conductivity; high transparency, which does not change over time; shock resistance, which is 5 times higher than that of glass; lightness; resistance to moisture, bacteria, and microorganisms; eco-friendly, it emits no toxic gases when it burns; the ability to shape a variety of forms using thermoforming, which goes without violation of the optical properties, but with decent detailing; easiness for machining; sustainability to the external factors, frost-resistance; resistance in chemical mediums; electrical insulation properties; recyclable.

The disadvantages of the plexiglass are: outputting the harmful monomer – methyl methacrylate – in pyrolysis; tendency to superficial damage; technological difficulties in thermal- and vacuum moulding (the emergence of internal stresses in bending points, which leads to the subsequent appearance of microcracks); flammable material.

The medical steel is used for manufacturing of medical instruments, it is associated with the exact property of the material, and this is primarily due to its density. The fact of the matter is that stainless steel 18/10 is optimal for its density and hardness for use in hospitals, scratches where dirt would accumulate, providing a fertile breeding ground for microbes, occur rarer on this steel. The medical steel is resistant to acids and alkalies even at high temperatures. That is why medicine uses the stainless steel 18/10.

After drawing and 3D-modelling we faced the task to make an experimental model of the microbiological punch (figure 2).



(1) **(2)**

Figure 2 The experimental model of the microbiological punch (1 – side view, 2 – bottom view)

The technical results of the proposed device are a simplified design; reduction of the facility cost and maintenance costs. The design has the following changes: we added the return platform which allows placing the device on the surface of the Petri dish; immersion depth of breakout drills was limited by studs of the detent mechanisms, and the presence of the return springs allows the device to uniformly lift the breakout drills up, which significantly reduces the chance of damaging the agarized plate (the nutrient medium). The technical result is achieved by the fact

that the proposed facility combines the punch and the return platform (playing the role of as a stencil).

We chose the diffusion method of agar basins to implement the efficiency of applying the technology of chemical compound biotoxicity assessment by means of the method of agar basins. This method combines the two methods: the method of making basins in the agar layer and the serial dilutions. Zinc salts with a high level of dissociation in aqueous solutions such as sulfates, chlorides, nitrates, acetates and the organic compounds synthesized on their basis were used as sources of excessive cation concentrations of the essential elements in this work. The advantage of this method is a visual estimate of the chemical compound toxicity in different concentrations under the same conditions, in addition, this technique is not only about quality but also about quantitative biotoxicity assessment of the studied chemical compounds [7].

The constructed facility was applied in the way described below.

The nutritious medium (taking into account the physiological needs of the studied microorganisms) fills a Petri dish. The medium contains 2% agar-agar in quantities of 20 ml (prerequisite is placing cups on the surface of balanced level because the thickness of the agarized plate should have the identical values all around the diameter of the dish). After full hardening, we sowed the studied microorganisms of the surface of the medium by the lawn technique (50 µl of the suspension at a concentration of 10^9 CFU/ml are distributed with a sterile microbiological spatula on the surface of the nutrient substrate).

On the surface of the Petri dish there gets placed the microbiological punch by installing the return platform. It is notable that the diameter of the plate corresponds to the outer diameter of the Petri dishes. The tubes of the punch are located in that way so the distance from the inside edge of the dishes is 1.5 cm and 3.0 cm between the tubes. After placing the punch, we click on the plate which fixes the breakout drill for dipping the drill (d=5 mm) in agarized medium aiming to cut out equidistant identical holes. The device is equipped with the return springs and the detent mechanisms limiting the depth of the punch tube penetration into a Petri dish (length calculation the detent mechanism length was made in such a way that the tubes reached the bottom of the Petri dishes, but made no excessive pressure on the bottom of the dish with the agarized medium, which could lead to damage of the punch tubes and the Petri dishes). The device is provided with a rubber bulb to remove cut out blocks. The bulb allows clearing the cavity of breakout drills of the punch with one touch.

We preliminarily prepared a series of serial dilutions of the studied compounds (salts of metals, and synthesized chemical compounds) with a high level of dissociation in aqueous solutions. After removing the agar blocks from the agarized medium we added 30 µl of the investigated solution into the basins. Next, we incubated the studied microorganisms in the thermostat, taking into account the physiological characteristics of the test organism. We made response accounting by means of measuring the zones of growth suppression of the studied microorganisms.

3. RESULTS

The reactions of the microorganism to the various concentrations of the studied chemical compounds as the size of the growth suppression zones are listed below, in Table 1.

The values from Table 1 reveal a high bactericidal activity of the studied essential substances in salts with a various anionic component and in the composition of synthesized organic compounds, which, in our view, is associated with the lack of protective detoxification mechanisms of this organism to excessive concentrations of the studied compounds, as these elements are involved in various metabolic processes and become deposited by a bacterial cell as a result. It is notable that the synthesized organic compounds based on copper ($C_{24}H_{20}O_{12}N_2Cu$)

maintains a high level of bactericidal activity in all series of dilutions that may be used in the development of disinfectants.

Table 1 Studying the bioactivity of the investigated compounds on *B. subtilis* 10641

	The concentration of the compound				
	1 mol/l	0,5 mol/l	0,25 mol/l	0,125 mol/l	0,063 mol/l
$C_{24}H_{20}O_8Br_2Zn$	18,00±1,15*	18,00±1,53	14,67±1,20	12,67±1,45**	8,33±0,33
$ZnSO_4$	34,0±1,69	33,3±0,56	20,3±1,98	12,3±1,18	1,5±1,33
$Zn(CH_3COO)_2$	34,8±0,69	31,3±1,33	26,0±1,00	24,3±1,96	19,7±1,33
$ZnCl_2$	25,7±1,85	19,7±2,60	12,0±0,66	10,7±1,33	7,4±2,88
$Zn(NO_3)_2$	35,0±1,59	32,9±0,20	30,6±2,06	22,3±1,17	19,0±2,66
$C_{24}H_{20}O_8Br_2Mg$	20,00±0,02	15,00±0,06	10,00±1,00	5,33±1,33	–
$C_{24}H_{20}O_{12}N_2Cu$	21,33±1,20	21,33±0,67	19,33±0,33	18,00±1,53	16,67±0,33
$CuSO_4$	32,3±0,88	23,7±1,76	18,7±1,76	16,7±0,33	6,67±2,06
$Cu(CH_3COO)_2$	20,3±1,67	13,3±4,09	8,1±2,66	7,7±2,00	–
$CuCl_2$	32,0±0,00	26,7±2,73	20,3±2,60	15,7±1,33	10,0±3,33
$C_{24}H_{20}O_{12}N_2Co$	21,33±0,33	19,33±0,33	13,00±0,58	9,67±0,33	6,7±0,33
*P≤ 0,05, **P≤ 0,01, ***P≤ 0,001					

4. CONCLUSION

The technical result of the proposed methodology is an increase in productivity at biotoxicity studying for various chemical compounds and determination of the minimum suppressive concentrations for the microorganisms. The task is solved by constructing a mechanical device for cutting basins in the agarized nutritional medium. The device carries out cutting out equidistant identical basins in the agarized nutrient substrate filling a Petri dish.

This technique allows assessing the resistance of the studied microorganisms to various chemical compounds, their level of diffusion process into the structure of the nutrient medium, as well as the ability of microorganisms to accumulate certain chemical elements. Application of the device significantly reduces run time of researches on biotoxicity of compounds and determination of the minimum suppressive concentrations to various microorganisms.

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