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EVALUATION OF ANIONIC COMPONENTS OF LEAD ON BIOTOXICITY AND BIOACCUMULATION ABILITY IN RESPECT OF PROBIOTIC STAMPS

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ABSTRACT: Lead is one of the most dangerous xenobiotic elements. It is actively accumulated not only in the environment, the distribution of lead in the water-soil-plant-animal-man chain directly depends on the initial content of elements in the geochemical province and is directly related to the health of the population. [1]. This paper presents the results of a study of the biotoxicity of lead cations in the structure of salts with different anionic components in relation to bacteria of the genus *Bacillus*, which are part of the probiotic preparations. These microorganisms are representatives of the soil microflora and are transitory to the organism of animals and humans. When conducting research, methods such as the agar wells were used, which not only visually but also qualitatively evaluate the effect of metal cations on the growth of the microorganisms studied, the colorimetric method was used to assess the effect of the element under study on the growth of the microorganism population, and the atomic absorption method made it possible to study accumulating characteristics of the studied bacteria. As a result of research, it has been established that lead acetate has a more pronounced toxic effect on the microorganisms under study. It should be noted that lead, which is present in the medium in high concentrations, does not have an inhibitory effect on bacterial strains; we associate this with the detoxification mechanisms of bacteria. The accumulating ability of microorganisms of the *Bacillus* genus of lead cations from nutrient substrates 24 hours after their cultivation in the presence of this element has high sorption characteristics with a percentage of its accumulation of more than 50%, the most active lead accumulates B. subtilis 534 with 66.3%, and the minimum values were recorded in *B. amyloliquefaciens* 10642 and amounted to 53.2%.

Keywords: Bioaccumulation; Biotoxicity; Lead, Probiotic strains

1. INTRODUCTION

A high degree of contamination is typical for the Mednogorsk industrial region, where the accumulation of heavy metals in all components of the surrounding medium occurs due to the exploitation of copper-zinc-pyrite deposits containing copper, zinc, sulfur, gold, silver, cadmium, germanium, lead, tin, manganese, arsenic natural environment. The pollution of the Buzuluk is also characterized by the dominance of lead. In 2009, the maximum excess was 12.1 MPC. In Kuvandyk, contamination is caused by an increased content of lead, nickel and cadmium, with the maximum excess being established for mobile lead forms of 2.7 MPC. Pollution by heavy metals in Orsk is due to elevated concentrations of nickel, cadmium, lead, zinc and copper.

Today, only cadmium, lead, mercury and antimony are toxic. The activity of a significant part of heavy metals in biological systems is different. It is true that there are no harmful substances, there are harmful concentrations [2].

The role of lead ions as a biogenic element is not great, it is known that lead participates in metabolic processes of bone tissue [3].

It is known that the ability to concentrate metals, including heavy metals, is very widespread in nature among various organisms. The true “record-breakers” for extracting heavy metals from the environment are microorganisms. The great interest is the study of this ability among microorganisms that make up probiotic preparations.

In this case, the greatest interest is the study of this ability among the microorganisms that make up probiotic drugs, in particular in bacteria of the genus Bacillus. It is important that the microorganisms of the genus Bacillus, which are part of the probiotic preparations, are self- selective antagonists and may have an antitoxic effect, manifested in the active excretion of toxic substances from the body, in particular heavy metals [4].

Based on the above data, the goal was set to study the effect of anionic components of lead on biotoxicity and bioaccumulating ability with respect to probiotic strains.

2. MATERIALS AND METHODS

For the implementation of the experiment, the

following probiotic preparations were chosen: “Sporobacterin” (the basis of *B. subtilis* 534, manufactured by “Bakoren”, Orenburg, Russia), “Bactisubtil” (the basis of *B. cereus* 5832, manufactured by “Marion Merrel”), “Vetom 1.1” (the basis of *B. subtilis* strain B-10641, the manufacturer “Research Center” (Russia), “Vetom 3” (the basis of the preparation *B. amyloliquefaciens* strain B-10642 (DSM 24614), manufacturer - “Research Center” (Russia), “Vetom 4” (the basis of *B. amyloliquefaciens* Amm B-10643 (DSM 24615), manufacturer - “Research Center” (Russia), as well as strain *Bacillus licheniformis 7038*.

The pure microorganisms` cultures obtained from the preparations were used for determining of the minimum suppressing concentrations of zinc salts having a subinhibitory and inhibitory effect, which was necessary for further work, namely, for determining of the anionic components` effect under study on the growth phases of the tested microorganisms and the subsequent determination of their ability to bioaccumulate anionic components. As toxicants, lead salts were used: acetate, nitrate. To carry out this stage of work, serial dilution methods and the method of agar wells were used. However, in the process of work, the priority was given to the method of agar wells, because it allows not only visually but also to qualitatively evaluate the effect of zinc on the growth of the microorganisms under study.

2.1 Method of Agar Wells

Method of agar wells: the probiotic strain was sown with a solid "lawn" on the surface of the agar plate in a Petri dish. After this, agar blocks in the amount of 7 pieces on one Petri dish, into which the investigated concentrations of substances were introduced to evaluate their inhibitory effect, were cut by a mirror drill (diameter 8 mm). The plates were placed in a thermostat for 24 hours at 37 ° C, followed by taking into account the growth and visual assessment of the effect of the test compound on the growth and morphology of the body test. The average value of the series of measurements was taken as the result of the analysis for each compound of salts.

2.2 Serial Dilution Methods

An evaluation of the influence of lead on the growth of microorganisms was carried out by measuring the optical density of a suspension of microorganisms in a liquid nutrient medium at intervals of 3 hours to obtain 3 close values that indicated the onset of a stationary growth phase.

To determine the optical density of the bacterial suspension in order to further construct the growth curve in the periodic culture, we used a colorimeter. To carry out this stage of work, previously prepared: sterile vials of 20 ml volume, sterile liquid nutrient medium (MPB), diurnal cultures of microorganisms, sterile metal solutions.

The experiments were carried out in triplicate. Bacteria were grown in the MPB using a periodic method of cultivation. 36 bottles were added: nutrient broth, suspension of microorganisms and working concentration of heavy metals (at which microorganisms are viable); 6 bubbles served as a control, which was the medium with microorganisms without the addition of metal solutions.

For the analysis, the colorimeter was adjusted and 20 minutes after switching on the device photometrically first standard, then test solutions. The average value of the series of measurements was taken as the result of the analysis for each element.

The measurement of the optical density of the bacterial suspension was carried out at intervals in three hours, starting at zero hour, and continued until three approximately equal values were obtained, which served as an indicator of the onset of the stationary phase. At intervals between measurements, the bubbles were placed in a thermostat at 37 ° C on shakers with constant stirring. The measurement was carried out 5 times and the final value was calculated as the arithmetic mean. In order to control the purity of the cultures of microorganisms present in the vesicles, a smear was periodically made, and then Gram staining was carried out according to a standard procedure.

Based on the results of the obtained optical densities, the Microsoft Excel program built up growth curves in periodic culture, including in the presence of toxicants. Each point of the growth curve is the average of three independent experiments [5].

The next and main stage in his work was the determination of the amount of metal accumulated by the bacteria under study from the nutrient medium. The quantitative determination of the metal was carried out using an atomic absorption method and both biomass and supernatant were analyzed. For this purpose, the analyzed metal was introduced into the culture medium at a working concentration, and cultivation was carried out before the onset of the stationary growth phase. At the end of the culture, the samples were processed and analyzed on an atomic absorption spectrophotometer (AASF) [6].

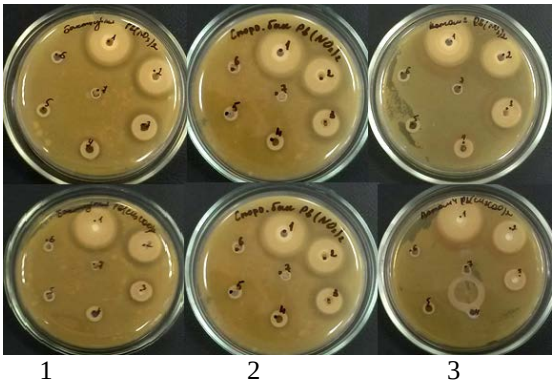
Preliminary work at this stage was to determine the amount of metal in the nutrient substrate before applying the working concentrations in order to exclude the possible impact on the accumulation indicators.

The obtained data were subjected to statistical

processing using Student's t-test [7].

3. RESULTS AND DISCUSSION

Lead compounds are known for their high toxicity. The individual susceptibility to lead poisoning varies widely, and the same doses of lead may have a greater or lesser effect for different people. Characteristic symptoms of poisoning are the pallor of the face, loss of attention, poor sleep, a tendency to frequent mood changes, increased irritability, aggressiveness, fatigue, and metallic taste in the mouth. Characterized by indigestion, loss of appetite, acute pain in the abdomen with spasms of abdominal muscles ("lead colic"). Conventional is the change in blood composition - from reticulocytosis, anisocytosis and microcytosis to lead anemia. At later stages, headache, dizziness, loss of orientation and vision problems are observed. Specific blackening ("lead line") may appear at the base of the gums. Possible paralysis ("lead cramps"), usually affecting primarily the fingers and hands. Children may be injured by the brain, which can lead to blindness or deafness or even death. Damage to the cerebral cortex is possible in adults after receiving large doses of lead. Lead enters the body from contaminated air, soil, dust in residential and outdoor areas, food, and inadequate personal hygiene. The risk of lead poisoning is usually assessed based on the determination of the concentration of lead in the blood. Concentration of lead in blood below 10 μg / dl in Russia and in the world is considered safe for the health of the child, although even the intake of small doses of lead in the body, depending on the duration and intensity, may affect health. The foregoing is a criterion for studying the influence of lead on the growth of microorganisms (Figure 1).



1 2 3

Figure 1 - Influence of $Pb(NO_3)$ 2 and $Pb(CH_3COO)_2$ on the growth of the investigated microorganisms (1 – *B. cereus* IP 5832, 2 – *B. subtilis* 534, 3 – *B. amyloliquefaciens* 10643)

Large concentrations of lead do not have a bactericidal effect on probiotic strains, but the main difference is that this element unlike cadmium has practically no effect on growth, but there is a change not only in the macromorphological characteristics of the cell culture near the well, but also morphological characteristics studied microorganisms.

The obtained data testify to the toxic effect of all the investigated lead salts, regardless of the anion component with respect to the studied probiotic strains, which actually confirms that lead is naturally a toxic metal. Lead toxin has the most pronounced toxic effect on the microorganisms under study. However, the values of the growth inhibition zones, which slightly exceeded the values of lead nitrate for the studied probiotic strains.

It was further found that, in the case of lead nitrate, the strains of *B. amyloliquefaciens* 10643 were the least resistant, and that of *B. amyloliquefaciens* 10642, respectively, in lead acetate. It should be noted that lead present in the medium in high concentrations does not have an inhibitory effect on bacterial strains, which in turn can be a criterion for increased bioaccumulation ability (Table 1). In all bacteria, the process of accumulation of metals is noted in the stationary phase of growth. The accumulation process is due to the fact that in this phase, substrate depletion and the accumulation of toxic products are observed, which forces bacteria to search for other sources of energy and detoxify the habitat. In connection with this, the next stage of our work was to determine the growth phases, in order to identify the optimal growth time in a periodic culture, and also the effect of anionic components on the growth dynamics of the microorganisms under study.

Determination of the optimal growth time on a periodic culture was carried out by cultivating the strains under study in a periodic culture on a liquid nutrient medium and measuring the optical density every 3 hours, starting at zero hour. The measurements were carried out until at least three approximately equal values of the optical density were obtained, which indicated the onset of a stationary growth phase. Summarizing the data obtained, it can be concluded that lead cations have the most pronounced effect on growth in all microorganisms under study (Figure 2).

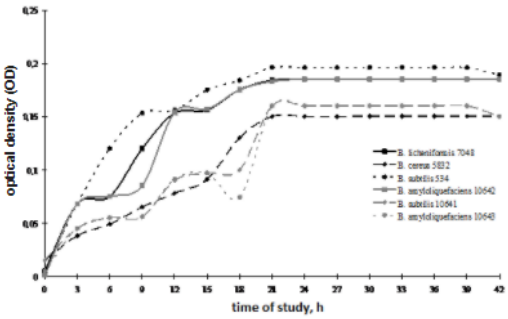


Figure 2 – Influence of $Pb(NO_3)_2$ on the growth of the investigated microorganisms

Table 1 - Assessment of the influence of lead salts on the growth of bacteria of the genus *Bacillus*

	B. licheniformis 7048				
	1 Моль	0,5 Моль	0,25 Моль	0,125 Моль	0,063 Моль
$Pb(NO_3)_2$	31,0±1,00	27,3±0,33	20,3±0,67	15,7±0,33	7,0±1,45
$Pb(CH_3COO)_2$	32,0±0,00	27,0±1,00	20,7±1,67	12,3±0,33	9,1±1,88
	B. cereus 5832				
$Pb(NO_3)_2$	30,3±0,33	25,3±0,67	19,3±0,33	10,7±1,20	7,3±0,33
$Pb(CH_3COO)_2$	32,3±0,33	22,3±1,45	14,0±1,53	9,7±0,33	7,7±0,33
	B. subtilis 534				
$Pb(NO_3)_2$	30,0±0,00	26,0±0,58	17,0±0,58	9,7±0,33	7,0±0,58
$Pb(CH_3COO)_2$	32,0±0,58	25,3±0,33	13,3±0,88	10,3±0,33	6,7±0,33
	B. amyloliquefaciens 10642				
$Pb(NO_3)_2$	36,0±0,58	27,7±1,20	24,7±2,19	10,7±0,33	7,3±0,33
$Pb(CH_3COO)_2$	38,7±0,33	30,3±0,88	23,3±0,88	10,3±0,33	7,0±0,58
	B. subtilis 10641				
$Pb(NO_3)_2$	23,7±0,33	18,7±0,33	14,7±0,33	9,7±0,33	6,7±0,33
$Pb(CH_3COO)_2$	23,3±0,33	19,3±0,33	13,0±0,58	9,7±0,33	6,7±0,33
	B. amyloliquefaciens 10643				
$Pb(NO_3)_2$	37,3±1,45	30,3±0,88	20,0±2,89	10,7±0,33	6,7±0,33
$Pb(CH_3COO)_2$	31,7±1,67	22,3±0,33	13,3±0,33	8,7±0,88	5,7±0,67

The duration of the lag phase is 3 hours, the exponential phase is 15 hours, the stationary phase begins after 21 hours of cultivation. In the case of *B. licheniformis*, there is a pronounced stimulating effect of lead ions on the growth dynamics of this microorganism.

Table 2 - Quantitative content of metal in a nutrient medium before the introduction of working concentrations of its salts

Metal	Concentrations introduced into the nutrient medium (M / l)	The minimum concentrations determined in AASP, (M / l)	The concentrations of metal in the nutrient medium, (M / l)
Pb	0,005 – 0,01	0,00003	–

Analysis of the growth dynamics of the probiotic strains of *B. amyloliquefaciens* included in the preparations Vetom 3 and Vetom 4, indicates the stimulating effect of lead on the growth of these microorganisms, with the most pronounced effect observed with *B. amyloliquefaciens* 10642.

Maximum values of population growth are noted in *B. subtilis* 534, and minimal in *B. cereus* IP 5832, however, it should be noted that the time of onset of the stationary phase in practically all microorganisms is 21 hours of growth in the periodic culture.

The next and main stage in our work was the determination of the amount of metal accumulated by the bacteria under study from the nutrient medium. The quantitative determination of the metal was carried out using an atomic absorption method and both biomass and supernatant were analyzed.

From the data obtained in Table 2, it follows that the metal concentration contained in the nutrient substrate is below the detection threshold in the AASF.

It should be noted that the presence of an anionic component does not significantly affect the sorption properties of the microorganisms under investigation.

Estimating the accumulating capacity of lead cations from microorganisms of the genus *Bacillus* from nutrient substrates (Fig. 3, 4, 5), it can be stated that this element has the highest sorption characteristics, since the percentage of its accumulation is more than 50%, with the lead most actively accumulating *B. subtilis* 534 which accounts for 66.3%, while the minimum values were recorded for strain *B. amyloliquefaciens* 10642 - 53.2%, respectively.

This study in our opinion confirms not only the data obtained during the assessment of the effect of lead cations on the growth of microorganisms by the method of agar wells, but also during the study of the effect of this element on the growth phase, where for all the strains studied this element acted as a "growth stimulator".

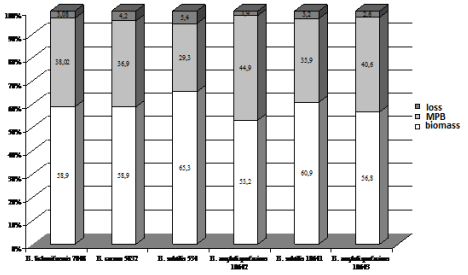


Figure 3 – Assessment of the bioaccumulation ability of *Bacillus* genus cations of lead cations from a substrate supplemented with $Pb(NO_3)_2$

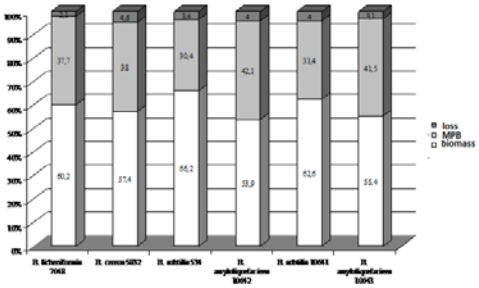


Figure 4 – Evaluation of the bioaccumulation ability of bacteria of the genus *Bacillus* cations of lead from the substrate with the addition of $Pb(CH3COO)_2$

Comparing the lead accumulation data (Fig. 5), depending on the anionic component, it can be noted that a direct relationship between the accumulation values in the presence of different lead salts was not detected, and the discrepancies were within a marginal error.

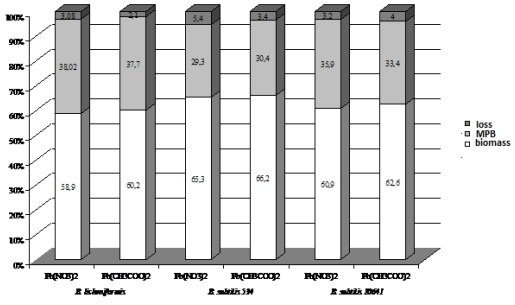


Figure 5 – Assessment of the bioaccumulation ability of *Bacillus* genus cations of lead cations from the substrate supplemented with $Pb(NO_3)_2$ и $Pb(CH_3COO)_2$

During the assessment of the influence of lead cations in complex with various anionic components, it was found that the most revealing method for this study is the method of agar wells, as it, unlike the conventional serial dilution method, allows not only to determine the minimal suppressing metal concentrations but also to determine them ability to accumulate cations.

The relationship between the toxicity of the compound and its accumulation on dense nutrient media was established, as for the presence of growth of the investigated microorganisms of the genus *Bacillus* near the hole with the maximum concentration of these metals, this research parameter can be used to develop strains of microorganisms that reduce the load level of these elements, as in environment, and in the human body and animals.

During the studying of the influence of heavy metal on the growth phases of the investigated microorganisms, it was found that the presence of lead in the nutrient substrate in the composition of salts with different anionic component has a stimulating effect on the growth of bacteria of the genus *Bacillus*, which in turn can be used in the development of selective and differential diagnostic media, for the isolation and identification of bacteria of this genus.

When assessing the bioaccumulation ability of bacteria of the genus *Bacillus*, it was found that all the microorganisms studied actively accumulate lead cations from the nutrient media (more than 60%), while it should be noted that the most active sorbent of these elements is *B. subtilis* 534 strain included in the preparation “Sporobacterin” developed and produced in the territory of the Orenburg region [8].

A review of the literature indicates an interest in studying the ability of microorganisms of the genus *Bacillus* to accumulate heavy metal ions. Thus, studies have been carried out to study the accumulation of lead bacteria by three species of bacteria (*Bacillus*, *Staphilococcus* and *Pseudomonas*). These bacteria were used as heavy metal sorbents in river water for the purpose of purification. Results were obtained showing that the share of accumulation of heavy metals by the microorganisms *B. subtilis*, *S. albus* and *P. aeruginosa* after 24 hours of exposure was: up to 94.5%, 85.7% and 90.8%, respectively. Thus, the best sorbent was the genus *Bacillus* [9].

The ability to accumulate lead ions was also found in *B. sphaericus* and *B. cereus*. Thus, *B. sphaericus* accumulates lead ions at concentrations up to 0.76 mol/g dry biomass respectively, and *B. cereus* up to 1.1 mol/g, respectively [10, 11]. *B. coagulans* actively sorbs lead ions and is an alternative to traditional physics in the removal of toxic metals from wastewater and underground sources. This strain can reduce the content of ions

of this metal by 65% with an initial total concentration of 500 mg/l [12]. Also, the ability to remove lead ions from an aqueous solution for *B. circulans* is known. The maximum accumulation of metals occurs at 72 and 96 hours of incubation of the microorganism and is 65% [13].

Based on the published literature, it follows that our studies do not contradict the general picture of ongoing scientific research.

4. CONCLUSION

As a result of the conducted studies it was found that the toxic effect of lead salts with different anionic component is mainly due only to the toxicity of the element itself and the level of dissociation of the molecule of its salt, as it has been experimentally established that nitrates, lead acetate have very close values both in assessing the effect on growth and growth phases of microorganisms, as well as in the evaluation of bioaccumulation ability, while oxides of these elements involved in the preliminary stage of the study did not influence the studied indicators.

Research data can be used to create probiotic preparations based on transient bacterial strains of the genus Bacillus with high bioaccumulative characteristics of cations of toxic elements, including lead, for the treatment of acute enterictions. In the future, these drugs will not only reduce the level of toxic effect on the body due to the sorption and detoxification characteristics of the microorganisms used, but also to remove excess concentrations of this element from the body.

5. ACKNOWLEDGEMENTS

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