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EFFECT OF NANOPARTICLES OF NATURAL MINERALS, IRON AND MANGAN COMPOUNDS, ON THE GROWTH AND SUPEROXIDE DISMUTASE ACTIVITY OF *BACILLUS SUBTILIS* IMV B-7023

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ABSTRACT

The cultivation of *Bacillus subtilis* IMV B-7023 in Spizizen medium, containing 1 g/L of bentonite or saponite stimulated the growth of these bacteria. The addition of 0.1–1 mM of Mn^{2+} ions to this medium resulted in increasing the number of bacteria. The cultivation of bacilli in the medium with similar concentrations of ferrum ions without NP`s had insignificant impact on their growth. However, the addition of 1 g/L of saponite to this medium led to considerable biomass increase, whereas bentonite had almost no impact on this value. The cultivation of *B. subtilis* bacteria in the medium, containing Mn^{2+} ions, resulted in nigligible increase in superoxide dismutase (SOD) activity. The addition of saponite and especially bentonite led to the considerable stimulation of SOD activity. The introduction of ferrum ions to the medium stimulated SOD activity of this strain, which maximum values amounted at the concentration of 1.5 mM Fe^{2+}.

Keywords: *Bacillus subtilis*, growth, superoxide dismutase activity, nanoparticles, ions

INTRODUCTION

The application of microbial preparations in agriculture is an important direction in correcting biotic processes in agroecosystems (**Kurdish *et al.* 2007; Kurdish 2010**). It is especially promising to use complex bacterial preparations, based on several strains of microorganisms, capable to cause diverse stimulating impact on the growth, development and performance of plants, protecting them from phytopathogens and phytophages (**Kurdish 2010; Roi *et al.* 2005; Roy *et al.* 2014; Soytong 2004; Volkogon 2006; Iutynska *et al.* 2010**).
The interaction of the selected strains of phosphate-mobilizing microorganisms *Bacillus subtilis* IMV B-7023 (**Patent of Ukraine No. 54923A**) and nitrogen-fixing bacteria *Azotobacter vinelandii* IMV B-7076 (**Patent of Ukraine No. 72856**) with particles of mineral clay bentonite was used to develop a highly efficient complex bacterial preparation Azogran for agriculture. It is components are characterized by the capability of synthesizing a wide range of biologically active substances (**Ocheretyanko *et al.* 2016; Chobotarov *et al.* 2017**), inhibiting the diffusion of phytopathogens and phytophages in the phytosphere (**Roi *et al.* 2005; Roy *et al.* 2014**), and causing antioxidant impact on the plant seeds, exposed to hydrogen peroxide (**Skorochod *et al.* 2011; Skorochod *et al.* 2013**).
The metabolic processes in living cells including microorganisms are accompanied by the formation of reactive oxygen intermediates (ROI), excessive amounts of which may lead to oxidative stress and damage to biomolecules. Among ROI a considerable role belongs to superoxide anion radical (O_2^-) – the product of incomplete reduction of molecular oxygen (**Fridovich 1995**). Superoxide dismutase (SOD) is one of the most relevant enzymes for the antioxidant protection of cells (**McCord *et al.* 1971**). The composition of the active center of SOD bacteria *B. subtilis* IMV B-7023 may contain ions of mangan and iron (**Halliwell *et al.* 1999**), the concentration of which in the medium may have impact both on the growth of bacteria and on the activity of this enzyme.
During Azogran application in agriculture, the bacteria in its composition may interact with nanoparticles of natural minerals (bentonite, saponite, etc.). The impact of mineral particles on the growth and SOD activity of this bacilli strain is not investigated yet.
Taking intoaccount the above, the aim of this work was to determine the impact of natural mineral nanoparticles and mangan and iron ions on the growth and superoxide dismutase activity of the strain *B. subtilis* IMV B-7023 – a component of complex bacterial preparation Azogran.

MATERIAL AND METHODS

Microorganisms, nutrient media and cultivation conditions

The cultures of *Bacillus subtilis* IMV B-7023 were cultivated in the nutrient medium of Spizizen (**Spizizen 1958**). The ions of Mn^{2+} and Fe^{2+} were added to the medium in the concentrations from 0.1 mM to 1.5 mM in the form of sulphates prior to its sterilization.
The bacteria were cultivated on the rotary shakers at 240 rpm at 28^{0}C for 24 h in the 750 mL conic Erlenmeyer flasks, containing 100 ml of the nutrient medium. The initial concentration of bacilli in the medium was $1 \cdot 10^6$ cells/mL. The number of viable bacteria in the suspension was determined by serial dilutions method on potato agar medium. The results were presented in the number of colony-forming units per 1 mL of suspension (CFU/mL) (**Zviagintsev 1980**).

Nanominerals used in the study

To obtain the nanoparticles of bentonite and saponite, 10 g of dry powdered mineral were added to 100 mL of Spizizen medium and dispersed for 5 min on the ultrasonic disintegrator UD-20 automatic, Poland. This method of minerals disintegration allowed to obtain nanoparticles with size up to 100 nm. After that the obtained nanocomposite was introduced into 0.9 L of nutrient medium, which was sterilized and used in the experiments.

Determination of superoxide dismutase activity

To obtain the supernatant, the cells of bacilli were centrifugated for 15 min at 5,000 g in the centrifuge OPn-8, Russia. The bacterial precipitate was washed twice with the phosphate buffer (0.05 M, pH 7.0), resuspended in 10 mL of this buffer, subjected to the freezing/thawing cycle and cells were disrupted on the ice bath, using the ultrasonic disintegrator UD-20 automatic (Poland) with the frequency of 22±1.65 kHz for 4 min.
The residues of cells were removed by centrifugation of the obtained suspension on the ultracentrifuge UTsP-50 at 20,000 g, for 30 min at 4˚C. The supernatant was used to study the superoxide dismutase activity. The impact of different factors on SOD activity was determined in the reaction mixture of the following composition: 0.05 M K-phosphate buffer, pH 7.0 – 1 ml; riboflavin (300 mg/L) – 6.5 ml; triphenyl tetrazolium chloride (TTC) (3 mg/mL) – 1 ml;

tetramethylethylenediamine (TEMED) – 48 μL; supernatant – 1.5 mL (**Bernas *et al*. 2000**).
The reaction mixture was exposed to ultraviolet for 15 min. The reaction mixture without TEMED was used as the negative control. The positive control was the reaction mixture, where the cell lysate was substituted for the similar volume of 0.05 M of the phosphate buffer (pH 7.65). The optical density was determined using the photoelectrocolorimeter KFK-2-UKhL 4.2 (Russia) in the cuvettes with d=1 cm at the wavelength of 540 nm (**Bernas *et al.* 2000**).
One unit of SOD activity (A) is defined as the difference in the amount of reduced formazan without the participation of SOD and the amount of formazan, reduced while inhibiting the SOD reaction by 50% for 15 min in 1 mL of the solution, per 1 mg of protein in the sample.

$$A = ((D_k – D_d)/D_k) \cdot 2k)/C \cdot l$$

where: **A** – a unit of enzyme activity, un/mg of protein; $\mathbf{D_k}$– the change in optical density of the solution of reducing TTC to formazan without SOD; $\mathbf{D_d}$– the change in optical density of the solution of reducing TTC to formazan in the presence of SOD of the investigated sample; **2** – a coefficient, corresponding to the inhibition of reaction with the participation of SOD by 50 %; **k** – a coefficient of diluting the investigated sample in the reaction, corresponding to 6.667; **C** – the concentration of protein, mg/mL; **l** – the length of the optical distance of the cuvette (**Bernas et al. 2000**).
Protein concentration in the samples was determined by it is binding to coomassie bright blue G-250 (**Bradford, 1976**), using bovine serum albumin as a standard.

Statistical analysis

The results of the studies were statistically processed (**Lakin, 1990**) in Microsoft Excel (Microsoft corporation, USA) by the data analysis of average mean of three replicates (±SE) obtained from three independent experiments.

RESULTS AND DISCUSSION

It was established that eightfold exposure of the *B. subtilis* suspension to ultrasound for 30 s each ensured the lysis of over 90 % viable cells. This allowed to obtain the lysates containing more than 1 mg/mL of protein. Taking into consideration the fact that natural nanomaterials are able to adsorb protein (**Herasimenko *et al.* 2015**), it was relevant to determine the permissible content of these nanoparticles in the reaction mixture, which would allow determining the impact of investigated minerals on SOD activity of these bacteria.
It was demonstrated that the introduction of 1 g/L of bentonite nanoparticles into the solution, containing 1 mg/mL albumin, resulted in low sorption on the particles of this mineral and did not exceed 5% of the initial content (Tab 1). However, this value increased rapidly with the increase of bentonite concentrations in the solution up to 5 g/L. At the same time, the sorption of albumin on saponite did not exceed 5% for all the investigated concentrations of the natural mineral particles (Tab 1).

Table 1 The dependence of protein sorption on the nanoparticles of natural minerals on their content in the solution

Type of disperse material	Content of nanomaterial, g/L	Sorption of albumin, %
Bentonite	0.5	3.0 ± 0.2
	1.0	4.0 ± 0.5
	5.0	21.0 ± 2.0
Saponite	0.5	1.5 ± 0.1
	1.0	3.2 ± 0.2
	5.0	4.6 ± 0.5

Note: The initial content of albumin in the solution was 1 mg/mL.

Taking into consideration the obtained results, the mineral nanoparticles were added into the medium at the concentration of 1 g/L.
It was established that the cultivation of *B. subtilis* IMV B-7023 for 24 h in Spizizen medium containing of 1 g/L of saponite or bentonite particles was accompanied with the increase in their growth (Fig. 1). During the bacteria cultivation in the medium with Mn^{2+} ions at concentration of 0.1 mM, the number of viable cells increased up to $2{\cdot}10^9$ cells/mL, both in the suspension without nanoparticles and with saponite at concentration of 1 g/L. With increase in the cation concentration in the medium without nanoparticles up to 1 mM, increase in the bacilli growth obtained, but at the concentration of 1.5 mM of Mn^{2+} this value decreased down to $2{\cdot}10^9$ cells/mL (Fig. 1).
While cultivating *B. subtilis* IMV B-7023 in the medium with bentonite, low concentrations of Mn^{2+} ions (0.1 mM) did not impact the growth of these bacteria. However, at the concentration of 0.5 mM of these cations therein, the growth was over $2.2 \cdot 10^9$ cells/mL in the medium. The growth of bacteria decreased with the increase of these ions concentration in the medium (Fig. 1).

With addition of Fe^{2+} ions into the *B. subtilis* cultivation medium, which did not contain mineral nanoparticles, resulted in low stimulation effect on the bacterial growth. During the bacteria cultivation in the medium containing 1 g/L of bentonite, increase in the number of viable cells was observed only at the concentration of 1 mM Fe^{2+} (Fig. 2).

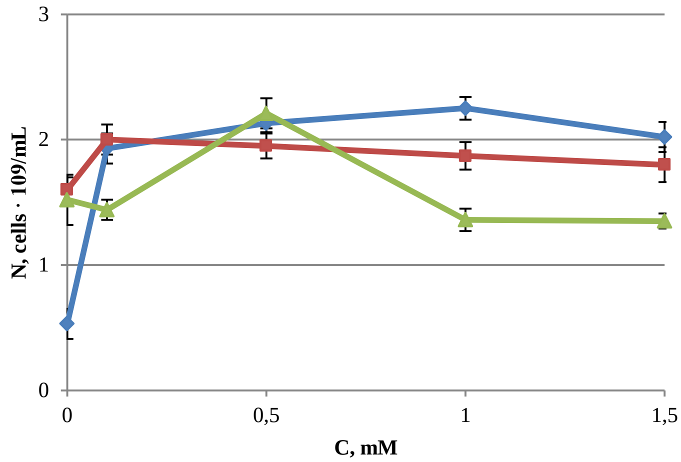

Figure 1 The number of viable cells (N) of *Bacillus subtilis* IMV B-7023 depending on the concentration (C) of mangan cations (II) in the medium without nanomaterials (1) and with 1.0 g/L of saponite (2) or bentonite (3)

A considerable impact on the growth of *B. subtilis* in the medium containing 1.0 g/L of saponite was caused by various concentrations of Fe^{2+} cations (Fig. 2). For instance, even at the concentration of 0.1 mM of Fe^{2+} ions in the medium the number of viable bacteria increased up to $2.53 \cdot 10^9$ cells/mL. With the increase in the concentration of Fe^{2+} in the medium with saponite up to 1.0 mM, there was an increase in the number of *B. subtilis* cells. The most significant increase in the growth of these bacteria was observed in saponite-containing medium at concentration of 1.5 mM of Fe^{2+} cations and amounted to $6.66 \cdot 10^9$ cells/mL (Fig. 2).
The cultivation of *B. subtilis* IMV B-7023 in the medium with nanoparticles had a considerable impact on SOD activity of these bacteria. In the medium without the nanoparticles, the SOD activity of the strain amounted 7 un/mg of protein (Fig. 3). With the introduction of 1 g of saponite or bentonite into the medium, the enzyme activity increased up to 8.3 and 9.3 un/mg of protein, respectively.

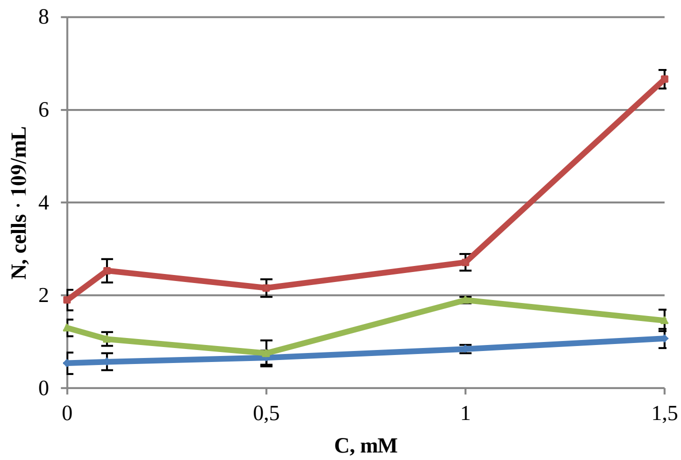

Figure 2 The number of viable cells (N) of *Bacillus subtilis* IMV B-7023 depending on the concentration (C) of Fe^{2+} cations in the medium without nanomaterials (1) and with 1.0 g/L of bentonite (2) or saponite (3)

It was established that the cultivation of *B. subtilis* IMV B-7023 in the medium with mangan ions was accompanied by the increase in superoxide dismutase activity of the bacteria (Fig. 3). For instance, addition of 0.1 mM of Mn^{2+} ions resulted in the SOD activity increase in 1.2 times compared to the control (without mangan ions). The increase in Mn^{2+} ions concentration led to decrease in the bacteria enzyme activity, however its obtained values were higher compared to the control (Fig. 3).
While cultivating the bacteria in the medium with mangan ions and 1 g/L of saponite, the SOD activity of bacilli increased considerably (Fig. 3), reaching the maximum values with the addition of 1.5 mM of mangan into the medium. In

these conditions, the SOD activity of *B. subtilis* was 27.2% higher compared to the corresponding medium without the nanomaterial (Fig. 3).

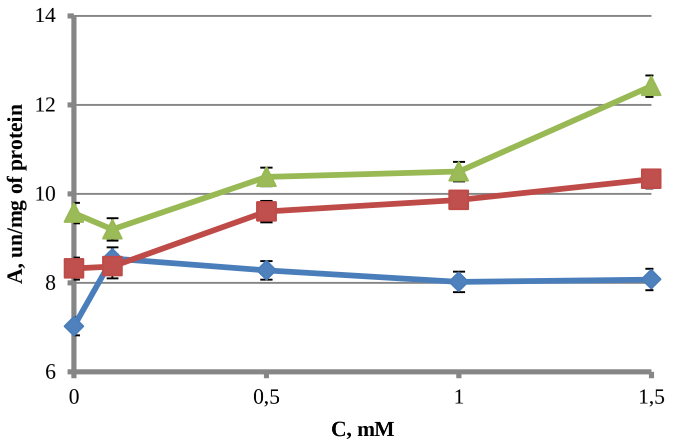

Figure 3 The dependence of superoxide dismutase activity (A) of *Bacillus subtilis* IMV B-7023 on the concentration (C) of mangan cations (II) in the medium without nanomaterials (1) and with the introduction of 1.0 g/L of saponite (2) or 1.0 g/L of bentonite (3)

It was established that the introduction of mangan ions into the medium of cultivating *B. subtilis*, containing 1 g/L of bentonite also had a considerable impact on the SOD activity of *B. subtilis* IMV B-7023 (Fig. 3). At the content of 0.1 mM of mangan ions in the medium, the enzymatic activity decreased compared to the control. However, when the concentration of these ions in the medium increased, the SOD activity increased considerably as well. Its maximum values were observed with the introduction of 1.5 mM of Mn^{2+} cations into the cultivation medium. In these conditions the SOD activity of bacteria amounted to 12.3 un/mg of protein and was 29.5% higher compared to the variant without these cations, and 53.7% higher compared to the corresponding variant without nanomaterials (Fig. 3).
The stimulation of the SOD activity of *B. subtilis* was caused by their cultivation in the medium with Fe^{2+} cations (Fig. 4). With the addition of 0.1 mM of these cations into the medium, not containing mineral nanoparticles, the investigated enzymatic activity increased up to 8.9 un/mg of protein (by 31% compared to the control) and at the concentration of 1.5 mM – 1.5 times.
Cultivation of *B. subtilis* in the medium, containing 1 g/L of saponite and 0.1 mM of Fe^{2+} ions resulted in low decrease of the bacteria superoxide dismutase activity. However, with higher concentrations of this cation, the enzyme activity increased, reaching its maximum values with the content of 1.5 mM Fe^{2+} in the medium (Fig. 4).

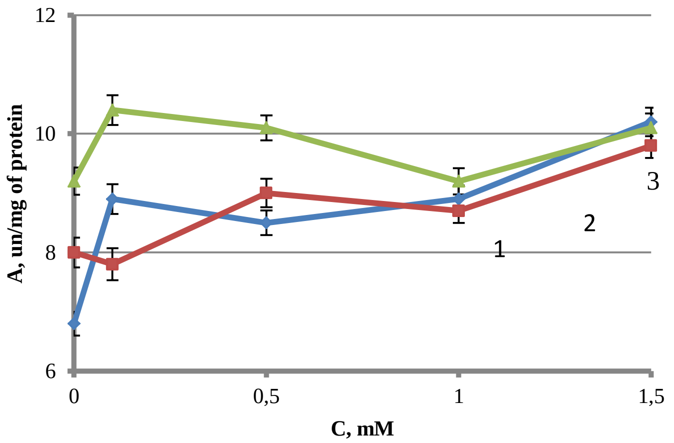

Figure 4 The superoxide dismutase activity (A) of *Bacillus subtilis* IMV B-7023 depending on the concentration (C) of Fe^{2+} cations in the medium without nanomaterials (1) and with the introduction of 1.0 g/L of saponite (2) or 1.0 g/L of bentonite (3)

The impact of Fe^{2+} ions on the superoxide dismutase activity of *B. subtilis* IMV B-7023 was investigated during the cultivation in the medium with bentonite nanoparticles. It was demonstrated (Fig. 4) that the addition of 0.1 mM of Fe^{2+} into the medium resulted in the increase in the SOD activity of bacteria up to 10.4 un. (by 13%). While cultivating in the medium containing 1 g/L of bentonite and 0.5–1.0 mM of Fe^{2+} ions, the level of SOD activity of bacteria was somewhat lower, while in the medium containing 1.5 mM Fe^{2+} this activity increased by 10% compared to the control.

CONCLUSION

It was established that the addition of 1 g/L of saponite or bentonite nanoparticles into the cultivation medium resulted in increase of the growth and superoxide dismutase activity of *B. subtilis* IMV B-7023 (Fig. 1–4). The introduction of 0.1 mM of Mn^{2+} ions to the medium without any nanoparticles and to the medium containing saponite therein was accompanied with the increase in the number of *B. subtilis* IMV B-7023 up to 2 · 10^9 cells/ml. When cultivated in the presence of bentonite, the bacteria growth decreased somewhat. However, at the concentration of 0.5 mM of mangan ions its value increased considerably. With the increase of mangan ions concentration up to 1.0 and 1.5 mM in the medium, bacteria growth was much lower compared to the values, obtained both in the media without nanoparticles and with the addition of saponite. The ions of Fe^{2+} at the concentration of 0.1–1.0 mM had nigligable impact on the growth of *B. subtilis* IMV B-7023. However, while cultivating in the medium, containing 1.5 mM of these ions and 1 g/L of saponite there was a considerable increase in the growth of the bacteria. These differences may be caused both by the impact of some concentrations of cations and the changes in the composition of the medium which may take place during its interaction with the particles of the investigated minerals. The possibility of such processes was demonstrated in previous investigations (**Chobotariov *et al*. 2010**).
While cultivating *B. subtilis* IMV B-7023 in the medium without nanomaterials, but containing 0.1 mM Mn^{2+}, the SOD activity of bacteria increased up to 8.5 un/mg of protein. However, the increase in the content of these cations in the medium resulted in some decrease in the value of enzyme activity. At the same time, cultivation of the bacteria with the particles of saponite and especially bentonite resulted in the SOD activity increase.
The cultivation of *B. subtilis* IMV B-7023 in the medium, containing Fe^{2+} ions also caused significant impact on the SOD activity of bacteria, especially at Fe^{2+} concentration of 1.5 mM. A similar dependence of the SOD activity on the content of these ions was obtained for bacteria *Desulfuromonas acetoxidans* IMV B-7384 (**Maslovska *et al.* 2015**). The results of our studies confirm the relevant role of Mn^{2+} and Fe^{2+} cations in the composition of the active center of SOD bacteria (**Halliwell *et al.* 1999**) for the functioning of this enzyme of *B. subtilis* IMV B-7023 – a component of Azogran preparation in conditions of the interaction of these bacteria with nanoparticles of the investigated natural minerals of different types of soils.

Conflict of Interest: The authors declare that they have no conflict of interest.

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