CHAPTER «BIOLOGICAL SCIENCES»

REPRODUCTION OF THE STRAIN OF BACTERIA *BACILLUS SUBTILIS* IMV B-7023 IN THE PRESENCE OF NANOMATERIALS WITH DIFFERENT CHEMICAL COMPOSITION

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Abstract. An aim of the study is to investigate an influence of nanomaterials (NMs) of different chemical composition on the growth of the strain *Bacillus subtilis* IMV B-7023. The following NMs have been used in the study: cerium (Ce) nanoparticles (NPs), titanium (Ti) NPs, nickel (Ni) and zinc (Zn) NPs, compositions of NPs of iodine and sulfur (I+S), selenium and iodine (Se+I) and "Avatar-2 protection" substance. All NMs were colloidal solutions. The strain *B. subtilis* IMV B-7023 has been cultivated in the liquid culture medium (peptone water) during 7 days. NMs have been added to the medium at the concentration of 100 $cm^3 \cdot L^{-1}$. Culture of the strain, which has been cultivated without addition of NMs, served as a control. CFU/cm^3 has been measured to study the influence of NMs on the growth of the strain. The measurements has been done on 1st, 2^{d} and 7th days by means of inoculation of agar plates with the liquid culture.

After the measurement of CFU/cm^3 on 1st, 2^{d} and 7th days, an average CFU/cm^3 for the whole term of cultivation has been calculated.

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Statistical analysis has been performed to assess the statistical significance of the results of the study.

Ce NPs, Ti NPs, Ni NPs and composition of Se+I NPs increase the CFU/cm^3 by 113,23; 34,34; 82,59 and 17,87 % respectively.

Composition of NPs I+S and "Avatar-2 protection" substance decrease CFU/cm^3 by 11,14 and 45,71 % respectively.

Zn NPs exhibit strong bactericidal activity against *B. subtilis* IMV B-7023; there was no viable cells in the presence of Zn NPs.

Changes in cultural-morphological characteristics under the presence of NMs have been also observed. Ce NPs, composition of NPs I+S, Ti NPs, composition of NPs Se+I and "Avatar-2 protection" substance decrease average diameter of colonies. For instance, Ce NPs, composition of NPs I+S and "Avatar-2 protection" substance decrease the average diameter by 16,95; 41,69 and 49,70 % respectively. Ni NPs have no significant effect on the cultural-morphological characteristics.

Statistical analysis has proven the significance of the results.

Thus, the influence of NMs on the reproduction of *B. subtilis* IMV B-7023 depends on the chemical composition of NMs strongly: different NMs exhibit opposite effects on the growth of the strain. Some of them increase CFU/cm^3 significantly, while others decrease it (to the extent of total absence of viable cells in the culture). Most of NMs decrease the average diameter of colonies.

NMs, which increase CFU/cm^3 can be useful in the production of microbiological substances (microbiological fertilizers, insecticides, fungicides, etc.), for the enhancement of biological activity of *B. subtilis* and other purposes.

Zn NPs are promising bactericidal agents.

Investigation of the influence of NMs on *B. subtilis* has to be continued. For instance, investigation of physiological-biochemical characteristics of *B. subtilis* under the presence of NMs is a promising topic for further research.

1. Introduction

Importance of the investigation of the influence of NMs on the reproduction of bacteria from Bacillus genus. Studying the influence of NMs on bacteria from the *Bacillus* genus is especially important because of the profound value of this genus.

Many species of *Bacillus* genus have valuable positive effects on growth and development of plants. Mechanisms of these effects can be divided into direct and indirect. Direct mechanisms include synthesis and excretion of plant hormones from classes of auxin and gibberrellin derivatives [1, pp. 173, 176–177], phosphorus mobilization and iron reduction in rhizosphere [2, pp. 63–64]. Indirect ones include synthesis of antibiotics and other compounds that suppress growth of pathogenic microorganisms [1, pp. 173, 176, 178-179; 2; pp. 63–64; 3, pp. 109, 114]. *Bacillus* species produce and excrete cytokinins in rhizosphere. 30 % gain of plants mass in presence of cytokinins derived exactly from *B. subtilis* shown [4, pp. 201, 205].

Besides positive influence on growth and development of plants *B. subtilis* bacteria have many other beneficial properties, which are valuable for agriculture.

Inoculants, which include probiotic strains of *B. subtilis* are widely used for silage fermentation [5, pp. 128–135; 6, pp. 151, 153–154]. Addition of *B. subtilis* to silage inoculants increases amount of lactic acid, nutritious compounds and beneficial microorganisms in silage, while amount of butyric acid and harmful microorganisms decreases. Feeding animals with such silage decreases incidence of gastrointestinal infections in youth, increases reproductive activity [6, pp. 153].

Species *B. subtilis* is a promising bioeffector for corn preservation [7, pp. 57–60; 8, pp. 49–52; 9, pp. 77–82; 10, pp. 62, 64–66]. Strong antifungal activity of *B. subtilis* against casual agents of preserved cord spoilage and its potentially huge efficiency for corn preservation were shown [10, pp. 62, 66].

This species is efficient destructor of organic residues. Efficient decomposition of straw by strains *B. subtilis* IMV B-7516 and *B. subtilis* IMV B-7515 was shown [11, pp. 52, 54–58].

Ability of *B. subtilis* to reduce phenol, 2.4-dichlorophenol and chlorophenoxyacetic acids [12, pp. 71–73; 13, pp. 52–55] makes it useful for bioremediation.

Considering mentioned beneficial properties, various strains of *B. subtilis* species are widely used as bioeffectors in microbiological agricultural inoculants.

For instance, this species is a component of highly efficient microbial inoculant “Azogran”. The inoculant includes bacterial strains *B. subtilis* IMV B-7023 and *Azotobacter vinelandii* IMV B-7076. Strain *B. subtilis*

IMV B-7023 has high ability to mobilize phosphorus from its organic and poorly soluble compounds, enhances phosphorus uptake by plants, has antagonistic activity against phytopathogenic microorganisms, produces compounds, which stimulate growth and development of plants [14, pp. 50]. "Azogran" inoculant enhances productivity of tomato, cucumber, potato, beat and other vegetables. Among horticultural crops, positive effects of "Azogran" on growth of roses, pines [15, pp. 101, 102] and lawn grass were shown. Studies show that exactly addition of strain *B. subtilis* IMV B-7023 is responsible for especially high biological activity of "Azogran", which is much higher as compared to monoculture of *A. vinelandii* IMV B-7076 [14, pp. 51]. "Azogran" elevates productivity of plants by 16 – 37 % [16, pp. 29, 30]. Productivity of winter wheat increases by 0,57 – 0,62 t ha^{-1} owing "Azogran" use [17, pp. 67, 71].

Thus, studying effects of NMs on beneficial *Bacillus* species and especially *B. subtilis* has a profound scientific and practical importance for number of reasons. Besides using microbial inoculants and traditional approaches, scientists look for new highly efficient methods for application in agriculture. Nanotechnology is a promising solution in this search and NMs can revolutionize agriculture soon [18, pp. 44–51]. However, at certain concentrations NMs can be harmful for humans, animals, plants, beneficial soil microorganisms. Authors of this study have already investigated the influence of some NMs on eukaryotic cell lines (pigs' embryonic kidney cell line) and established threshold limit values [19, pp. 91–94; 20, pp. 107, 109–110]. However, influence of NMs on beneficial soil microorganisms is not studied sufficiently yet and need further extensive investigation.

Another important area in NMs research is studying their antibacterial activity. Besides the majority of *Bacillus* species are beneficial, some of them are dangerous human pathogens. *B. anthracis* is a causal agent of anthrax. *B. cereus* produces enterotoxins, causes foodborne illnesses, including nausea, diarrhea and vomiting. It can also cause skin infections and keratitis.

Antibacterial activity of NMs. NMs are promising antibacterial agents. For instance, cerium dioxide NPs show high antibacterial activity. Cerium dioxide (CeO_2) is semiconductor with a band gap 2,96 – 3,19 eV and high exciton binding energy [21, pp. 527–529]. CeO_2 NPs show antibacterial activity against *Streptococcus pneumoniae*, *B. subtilis*, *Pseudomonas aeruginosa* and *Proteus vulgaris* [22, pp. 2348, 2351]. Mechanism of antibacterial

activity of the most of metal and metal oxide NPs is based on interaction of NPs with bacterial cell wall, which occurs because of attraction of positively charged NPs to negatively charged bacterial cell walls due to electrostatic forces. This interaction leads to growth inhibition, reactive oxygen species (ROS) generation and cell death. Antibacterial activity of CeO_2 NPs differs by their ability to pass through cell wall, bind to mesosomes, disrupt cell respiration and DNA replication, induce oxidative stress, which lead to cell death [22, pp. 2351]. Antibacterial activity of some CeO_2 NMs is pH-dependent with higher values at alkaline pH [23, pp. 1, 4, 6-8]. Cubic CeO_2 NPs exhibit antibacterial activity against *Streptococcus pneumoniae*, *B. subtilis* [24, pp. 295, 300], *Escherichia coli*, *Salmonella typhimurium* and *Enterococcus faecalis* [25, pp. 1, 4–5]. Disruption of *E. coli* cell wall integrity after treatment with cubic CeO_2 NPs has been shown [25, pp. 1, 4].

Other promising antibacterial NMs are Ni and NiO NPs. Studies show that Ni NPs obtained by chemical and "green" routes have different activity spectrums [26, pp. 751–752]. Ni NPs obtained by "green" route are more efficient against *Klebsiella pneumoniae* and *Proteus vulgaris*, while chemically synthesized NPs are more active against *Staphylococcus aureus* and *Vibrio cholerae*. Ni NPs obtained from $Ni(NO_3)\times 6H_2O$ have strong antibacterial activity against *Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella* sp. [27, pp. 990, 996–997].

Thereby, studying effects of NMs on *Bacillus* species and especially *B. subtilis* in order to find NMs that either enhance reproductive activity of these bacteria or exhibit antibacterial activity is a topical area of research, which has huge scientific and practical value.

2. Materials and methods

Strain of bacteria. The strain *B. subtilis* IMV B-7023 was kindly given to us by vice-president of the Danylo Zabolotny Institute of Microbiology and Virology of the NASU, doctor of biological sciences, professor, head of the department of microbiological processes on solid surfaces, Kurdish Ivan Kyrylovych.

Nanomaterials. NMs, which were used in the study were kindly given to us by the head of "Nanomaterials and nanotechnologies" LTD., Kaplunenko Volodymyr Heorhiiovych. "Avatar-2 protection" substance was kindly given to us by "Scientific-industrial Company "Avatar" LTD. "Avatar-2 pro-

tection" substance contains NPs of 20 chemical elements, which determine its biological activity. NPs, compositions of NPs and "Avatar-2 protection" substance were given to us in form of colloidal solutions (suspensions). 4 colloidal solutions contained NPs of one chemical element in each one (Ce, Ti, Ni and Zn). 2 other colloidal solutions were compositions of NPs of 2 chemical elements in each one (I+S and Se+I). "Avatar-2 protection" substance is a mixture of NPs of 20 chemical elements at different concentrations. Suspensions of NPs, compositions of NPs and "Avatar-2 protection" substance were decided to be called by general term nanomaterials (NMs) to make discussion easier. Concentrations of NMs, which were used to treat *B. subtilis* IMV B-7023 were measured in cm^3 per 1 L of culture medium.

Liquid medium variants preparation. Initial suspensions of NMs were diluted in half by distilled water addition. pH was adjusted to 7,0 via addition of 20-% NaOH solution by drops. Then suspensions were autoclaved at pressure 0,7 atm. during 20 min. Peptone water was autoclaved at pressure 0,7 atm. during 15 min. Sterile test tubes were filled with 1,8 cm^3 of sterile peptone water and 0,2 cm^3 of NMs suspensions. 0,2 cm^3 of sterile water was added to the control variant.

Table 1

Blueprint of the study

Variant of the medium	NMs	Concentration of NMs, cm^3 L-1	Strain of bacteria
1	No NMs (sterile H2O)	100	*B. subtilis* IMV B-7023
2	Ce NPs	100	*B. subtilis* IMV B-7023
3	I+S NPs	100	*B. subtilis* IMV B-7023
4	Ti NPs	100	*B. subtilis* IMV B-7023
5	Ni NPs	100	*B. subtilis* IMV B-7023
6	Se+I NPs	100	*B. subtilis* IMV B-7023
7	Zn NPs	100	*B. subtilis* IMV B-7023
8	"Avatar-2 protection"	100	*B. subtilis* IMV B-7023

Inoculation of liquid medium. Portions of *B. subtilis* IMV B-7023 colonies were picked up by sterile wire loop from fresh culture and transferred to liquid medium. Cultures were incubated in thermostat at temperature 37 °C for 7 days.

Viable cells measurement. Numbers of viable cells per cm^3 of cultures were measured via methods of serial dilutions and standard plate counts. 1:1000000 dilutions of each variant of culture were made. Dilutions were transferred to agar plates via spread plate method. 0,1 cm^3 of dilutions were placed on the center of cooled peptone agar and spread evenly over medium's surface with a sterile Drigalski spatula. Transfers were done on 1st, 2d and 7th days after the onset of incubation to study effects of NMs not only on general number of viable cells, but also on growth dynamics. 3 agar plates were used for each variant of culture. On the 2d and 7th days, number of plates for Se+I NPs variant was increased to 4. Plates were incubated in thermostat at temperature 37 °C for 5 days. Numbers of colony forming units (CFU)/cm^3 of cultures were calculated by the following formula:

$$A = \frac{X}{0,1} \times 10^n$$

Where A – number of CFU/cm^3 of the culture, X – number of colonies that were formed on the agar plate after the transfer, n – number of the dilution.

Diameter estimation. Colonies diameters were estimated in ImageJ software.

Calculations and statistical analysis. Numbers of CFU/cm^3 were calculated in Microsoft Office Excel. Statistical analysis was done in StatSoft STATISTICA software. Numbers of CFU/cm^3 were divided into samples by 2 factors: variant of culture (type of NMs added) and duration of incubation (1, 2, 7 days and general sample for the whole term). Normality of data distribution was measured by the Shapiro-Wilk test. Homoscedasticity was measured by the Brown-Forsythe test, Bartlett's test and Cochran's C test. Effects of NMs on numbers of CFU/cm^3 in dynamics were estimated by multivariate analysis of variance (MANOVA). Levels of significance were assessed by Duncan's new multiple range test (DMRT) and Fisher's least significant difference test (Fisher's LSD). Effects of NMs on average number of CFU/cm^3 during the whole term of incubation were estimated by analysis of variance (ANOVA). Levels of significance were estimated with Student's t-test, DMRT and Fisher's LSD. Differences between weighted means (WM) in percent were calculated and visualized in Microsoft Office Excel.

3. Results

Effect of NMs on colony numbers. Colony numbers in all cultures with NMs differed from that in control culture (Figure 1, Table 2). NPs of Ce, Ti, Ni and composition of Se+I NPs increased numbers of colonies, while composition of NPs I+S and "Avatar-2 protection" substance decreased them. There were no colonies on agar plates inoculated with culture containing Zn NPs (Figure 1, Table 2).

Morphology of colonies. Colonies of cultures incubated with Ce NPs, composition of I+S NPs and "Avatar-2 protection" substance were smaller than in control by 16,95; 41,95 and 49,70 % respectively. Ti NPs and composition of NPs Se+I also affected colonies morphology. Ni NPs did not show any significant or noticeable effects on colonies morphology.

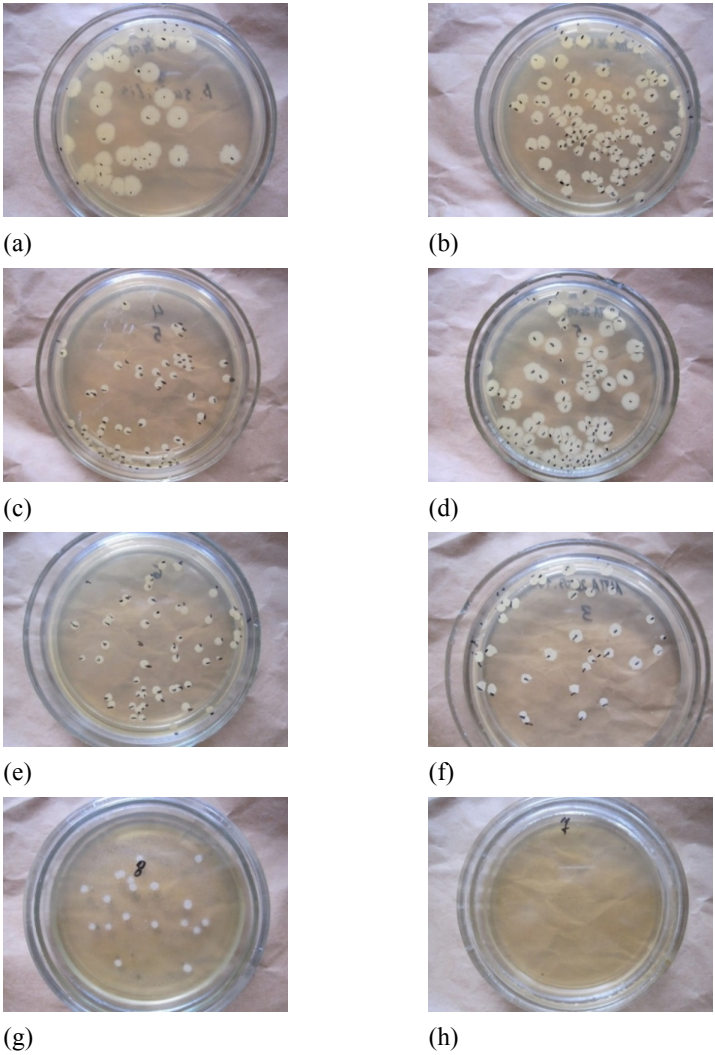
Numbers of CFU/cm^3 calculation. Numbers of colonies on agar plates were used to calculate CFU/cm^3 (table 3). Obtained numbers of CFU/cm^3 were used to form samples for statistical analysis.

Approaches to data analysis. Two approaches were used. Separate samples for each culture variant and each CFU/cm^3 measurement (on 1st, 2^{d} and 7th days) were formed to analyze effects of NMs on strain growth in dynamics. To analyze effects of NMs on average number of CFU/cm^3 during the whole term of cultivation, all measurements were integrated to one general sample for each variant of culture.

Table 2

Numbers of colonies on agar plates inoculated with different variants of culture

No	NMs	Average number of colonies (WM ± Std.Err)		
		1 day	2 day	7 day
1	Ce	82,67±4,18	96,67±5,55	96,33±5,33
2	I+S	32,67±1,20	40,33±0,33	42±1,00
3	Ti	55,67±2,03	58,67±3,71	59,33±4,91
4	Ni	71±5,03	81,67±3,71	83,33±3,38
5	Se+I	42,33±2,16	53,75±1,79	54,25±1,70
6	Zn	0	0	0
7	"Avatar-2 protection"	20,33±1,33	24,33±2,85	25,67±2,91
8	No NMs	39±4,16	44,67±3,84	45,67±3,71



(a) (b) (c) (d) (e) (f) (g) (h)

Figure 1. Colonies on agar plates inoculated with different variants of culture, 7th day

(a) no NMs, (b) Ce NPs, (c) Ti NPs, (d) Ni NPs, (e) composition of NPs Se+I, (f) composition of NPs I+S, (g), "Avatar-2 protection" substance, (h) Zn NPs

Table 3

Results of numbers of CFU/cm^3 calculation

No	NMs	Average number of CFU/cm^3, ×10^8 (WM ± Std.Err)		
		1st day	2^d day	7th day
1	Ce	8,27±0,42	9,67±0,56	9,63±0,53
2	I+S	3,27±0,12	4,03±0,03	4,2±0,10
3	Ti	5,57±0,20	5,87±0,37	5,93±0,49
4	Ni	7,10±0,50	8,17±0,37	8,33±0,34
5	Se+I	4,23±0,22	5,38±0,18	5,43±0,17
6	Zn	0	0	0
7	"Avatar-2 protection"	2,03±0,13	2,43±0,29	2,57±0,29
8	No NMs	3,90±0,42	4,47±0,38	4,57±0,37

Choosing methods of analysis. To choose proper methods of statistical analysis, data distribution and homogeneity of variances were analyzed in all samples. Results showed that data in all samples were distributed normally (table 4).

Table 4

Normality of data distribution in samples, according to the Shapiro-Wilk test

No.	Variant of culture	Level of significance, p			
		Variant of culture + measurement day			Variant of culture
		1st day	2^d day	7th day	
1	Ce NPs	>0,13	>0,71		>0,25
2	I+S NPs	>0,46			>0,08
3	Ti NPs	>0,84	>0,29	>0,33	>0,06
4	Ni NPs	>0,21	>0,29	>0,32	>0,21
5	Se+I NPs	>0,25	>0,58	>0,27	>0,14
6	Zn NPs	-	-	-	-
7	"Avatar-2 protection"	-	>0,19	>0,78	>0,10
8	No NMs	>0,53	>0,58	>0,29	>0,80

Tests for homoscedasticity shown all variances being homoscedastic (table 5).

Thus, the data in all samples were distributed normally and all variances were homoscedastic. It allowed us to use parametric methods for further analysis.

Table 5

Results of homoscedasticity measurements

No	Samples by factors	The Brown-Forsythe test		Cochran's C test and Bartlett' test		
		F	p	Cochran's C	Bartlett's chi	p
1	Variant of culture	>1,92	>0,08	>0,31	>1,68	>0,94
2	Variant of culture + measurement day	>0,55	>0,93	>0,12	>15,14	>0,76

Effect of Ce NPs in dynamics. Average number of CFU/cm^3 with Ce NPs was larger than in control by 112,05; 116,33 and 110,72 % on 1st, 2^{d} and 7th days respectively (table 6). The difference was highly significant (table 6).

Table 6

Effect of Ce NPs on CFU/cm^3 of *B. subtilis* in dynamics

No.	Day	Variant of culture	CFU/cm^3 (×10^8) (WM ± Std.Err)	Δ, %	Level of significance, p	
					DMRT	Fisher's LSD
1	1st	No NMs	3,90±0,42		<0,0001	<0,00002
2	1st	Ce	8,27±0,42	+112,05		
3	2^{d}	No NMs	4,47±0,38		<0,0001	<0,000004
4	2^{d}	Ce	9,67±0,56	+116,33		
5	7th	No NMs	4,57±0,37		<0,0001	<0,000005
6	7th	Ce	9,63±0,53	+110,72		

Thus, Ce NPs exhibit strong stimulatory activity towards strain *B. subtilis* IMV B-7023 at concentration 100 cm^3 L^{-1}.

Effect of composition of I+S NPs in dynamics. Average number of CFU/cm^3 with composition of I+S NPs was smaller than in control by 16,15; 9,84 and 8,09 % on 1st, 2^{d} and 7th days respectively (table 7). The difference has exhibited low significance, which, however, it does not mean that result is negligible (table 7).

Thereby, composition of NPs I+S exhibits slight bacteriostatic activity against strain *B. subtilis* IMV B-7023 at concentration 100 cm^3 · L^{-1}.

Effect of Ti NPs in dynamics. Average number of CFU/cm^3 with Ti NPs was larger than in control by 42,82; 31,32 and 29,76 % on 1st, 2^{d} and 7th days respectively (table 8). The difference was significant (table 8).

Thus, Ti NPs exhibit significant stimulatory activity towards strain *B. subtilis* IMV B-7023 at concentration 100 cm^3 · L^{-1}.

Table 7

Effect of composition of NPs I+S on CFU/cm³ of *B. subtilis* in dynamics

No.	Day	Variant of culture	CFU/cm³ (×10⁸) (WM ± Std.Err)	Δ, %	Level of significance, p	
					DMRT	Fisher's LSD
1	1st	No NMs	3,90±0,42		>0,14	>0,14
2	1st	I+S	3,27±0,12	-16,15		
3	2d	No NMs	4,47±0,38		>0,32	>0,30
4	2d	I+S	4,03±0,03	-9,84		
5	7th	No	4,57±0,37		>0,40	>0,37
6	7th	I+S NPs	4,20±0,10	-8,09		

Table 8

Effect of Ti NPs on CFU/cm³ of *B. subtilis* in dynamics

No.	Day	Variant of culture	CFU/cm³ (×10⁸) (WM ± Std.Err)	Δ, %	Level of significance, p	
					DMRT	Fisher's LSD
1	1st	No NMs	3,90±0,42		<0,02	<0,01
2	1st	Ti	5,57±0,20	+42,82		
3	2d	No NMs	4,47±0,38		<0,04	<0,03
4	2d	Ti	5,87±0,37	+31,32		
5	7th	No NMs	4,57±0,37		<0,04	<0,03
6	7th	Ti	5,93±0,49	+29,76		

Effect of Ni NPs in dynamics. Average number of CFU/cm³ with Ni NPs was larger than in control by 82,05; 82,77 and 82,28 % on 1st, 2d and 7th days respectively (table 9). The difference was highly significant (table 9).

Thus, Ni NPs exhibit strong stimulatory activity towards strain *B. subtilis* IMV B-7023 at concentration 100 $cm^3 \cdot L^{-1}$.

Effect of composition of Se+I NPs in dynamics. Average number of CFU/cm³ with composition of Se+I NPs was larger than in control by 8,46; 20,36 and 18,82 % on 1st, 2d and 7th days respectively (table 10). The difference was significant according to Fisher's LSD on 2d and 7th (table 10).

Thereby, composition of NPs Se+I exhibit noticeable stimulatory activity towards strain *B. subtilis* IMV B-7023 at concentration 100 $cm^3 \cdot L^{-1}$.

Effect of Zn NPs in dynamics. There were no colonies on agar plates inoculated with culture that was incubated with Zn NPs; hence, calculated numbers of CFU/cm³ were 0 for each measurement. The average numbers of CFU/cm³ were smaller than in control by 100 % in each measurement (table 11).

Table 9

Effect of Ni NPs on CFU/cm³ of *B. subtilis* in dynamics

No.	Day	Variant of culture	CFU/cm³ (×10⁸) (WM ± Std.Err)	Δ, %	Level of significance, p	
					DMRT	Fisher's LSD
1	1st	No NMs	3,90±0,42		<0,0003	<0,0002
2	1st	Ni	7,10±0,50	+82,05		
3	2d	No NMs	4,47±0,38		<0,0002	<0,00003
4	2d	Ni	8,17±0,37	+82,77		
5	7th	No NMs	4,57±0,37		<0,0002	<0,00003
6	7th	Ni	8,33±0,34	+82,28		

Since, numbers of CFU/cm³ in variant with Zn NPs were be 0, estimation of significance was done formally. Levels of significance were high (table 11).

Thus, "Avatar-2 protection" substance exhibits strong bacteriostatic activity against strain *B. subtilis* IMV B-7023 at concentration 100 $cm^3 \cdot L^{-1}$.

Thus, number of CFU/cm³ in the culture with Zn NPs was 0 during the whole term of incubation. It can be interpreted as the absence of viable cells in the culture. The first measurement was done on the 1st day after the onset of incubation; thereby the data indicates that there were no viable cells on the 1st day after the onset of incubation and further. Possibility of the presence of viable cell in the culture at earlier stages of incubation is not excluded, however, according to our data; there were no viable cells in the culture as soon as after 24 hours of incubation.

Thereby, Zn NPs exhibit extreme bactericidal activity against strain *B. subtilis* IMV B-7023, causing the loss of viability by cells of the bacteria.

Effect of "Avatar-2 protection" substance in dynamics. Average number of CFU/cm³ with "Avatar-2 protection" substance was smaller than in control by 47,95; 45,64 and 43,76 % on 1st, 2d and 7th days respectively (table 12). The difference was highly significant (table 12).

Significance of results and role of chemical composition of NMs. Effects of NPs of Zn and Ce on strain growth were the most significant ($p<0,0001$). Effect of Ni NPs also had high significance ($p<0,0003$). Effects of "Avatar-2 protection" substance, Ti NPs and composition of Se+I NPs were significant at levels $p<0,003$, $p<0,04$ and $p<0,05$ respectively. Effect of I+S NPs had low significance (table 13).

Table 10

Effect of composition of NPs Se+I on CFU/cm³ of *B. subtilis* in dynamics

No.	Day	Variant of culture	CFU/cm³ (×10⁸) (WM ± Std.Err)	Δ, %	Level of significance, p	
					DMRT	Fisher's LSD
1	1st	No NMs	3,90±0,42		>0,42	>0,44
2	1st	Se+I	4,23±0,22	+8,46		
3	2d	No NMs	4,47±0,38		>0,05	<0,04
4	2d	Se+I	5,38±0,18	+20,36		
5	7th	No NMs	4,57±0,37		>0,06	<0,05
6	7th	Se+I	5,43±0,17	+18,82		

Table 11

Effect of Zn NPs on CFU/cm³ of *B. subtilis* in dynamics

No.	Day	Variant of culture	CFU/cm³ (×10⁸) (WM ± Std.Err)	Δ, %	Level of significance, p	
					DMRT	Fisher's LSD
1	1st	No NMs	3,90±0,42		<0,0001	<0,000001
2	1st	Zn	0	-100		
3	2d	No NMs	4,47±0,38		<0,0001	<0,000001
4	2d	Zn	0	-100		
5	7th	No NMs	0		<0,0001	<0,000001
6	7th	Zn	8,33±0,34	-100		

Table 12

Effect of "Avatar-2 protection" substance on CFU/cm³ of *B. subtilis* in dynamics

No.	Day	Variant of culture	CFU/cm³ (×10⁸) (WM ± Std.Err)	Δ, %	Level of significance, p	
					DMRT	Fisher's LSD
1	1st	No NMs	3,90±0,42		<0,003	<0,002
2	1st	"Avatar-2 protection"	2,03±0,13	-47,95		
3	2d	No NMs	4,47±0,38		<0,002	<0,001
4	2d	"Avatar-2 protection"	2,43±0,29	-45,64		
5	7th	No NMs	4,57±0,37		<0,002	<0,001
6	7th	"Avatar-2 protection"	2,57±0,29	-43,76		

Thus, the way NMs affect *B. subtilis* growth strongly depends on NMs chemical composition. NPs of Ce, Ni, Ti and composition of Se+I NPs stimulated strain growth. NPs of Ce and Ni had especially strong stimulatory activity (table 13).

Table 13

Distribution of effects by significance and role of chemical composition of NMs

No.	NMs	Significance	Effects of NMs on *B. subtilis* reproduction
1	Zn	p<0,0001	Bactericidal. Cells loose viability within 1 day.
2	Ce	p<0,0001	Stimulatory. CFU/cm^3 doubles.
3	Ni	p<0,0003	Stimulatory. CFU/cm^3 increases by > 80 %.
4	"Avatar-2 protection"	p<0,003	Bacteriostatic. CFU/cm^3 decreases by > 43 %.
5	Ti	p<0,04	Stimulatory. CFU/cm^3 increases by > 29 %.
6	Se+I	p<0,05	Stimulatory. CFU/cm^3 increases by > 8 %.
7	I+S	p>0,14	Bacteriostatic. CFU/cm^3 decreases by > 8 %.

"Avatar-2 protection" substance and composition of I+S NPs suppressed growth of the strain, but did not cause cell death. Zn NPs exhibited extreme bactericidal effect, causing loss of viability by cells within 1 day (Table 13, Figure 2).

Effects of NMs on strain growth during the whole incubation. All measurements (1st, 2^{d}, 7th days) were united in one sample for each variant of culture. Significance testing showed that effects of NMs on strain growth of are highly significant (table 14).

4. Possible mechanisms of the influence of NMs on the growth of *B. subtilis*

Effects of NMs on *B. subtilis* depend on their chemical composition. Despite the majority of studies describe NMs as solely antibacterial mate-

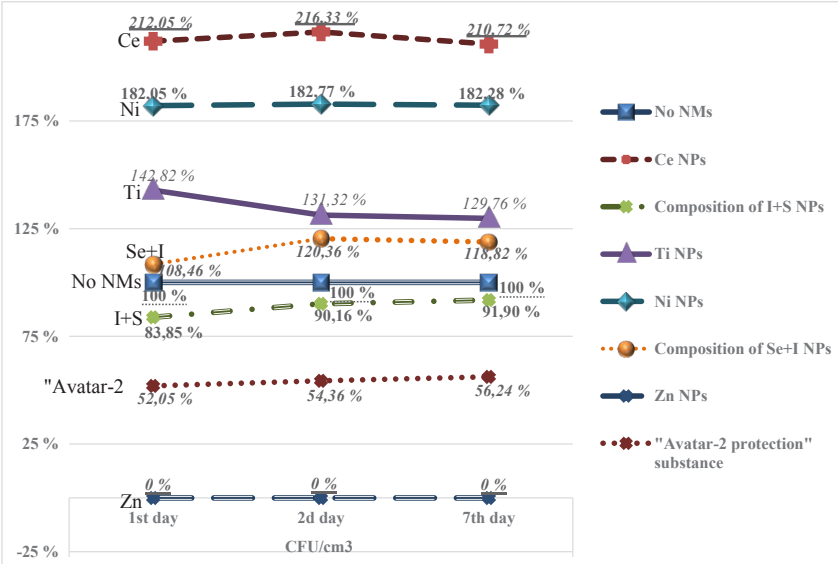


Figure 2. Effects of NMs with different chemical composition on growth of *B. subtilis*

rials, we have found that NPs of Ce, Ti, Ni and composition of Se+I NPs significantly stimulate growth of *B. subtilis*, which is quite opposite to dominating concept in science so far.

Stimulatory effect of Ce NPs can be explained by the fact that Ce is an essential cofactor of bacterial enzymes. Thus, Ce is a cofactor of methanol dehydrogenase of *Methylacidiphilum fumariolicum* and *Bradyrhizobium* sp. [28, pp. 1–3; 29, pp. 613–617]. *Pseudomonas putida* produces pyrroloquinoline quinone-dependent alcohol dehydrogenases (PQQ-ADHs). PedH, one of their homologs, reduces aliphatic and aromatic primary and secondary alcohols and aldehydes, but only in presence of La^{3+}, Ce^{3+}, Pr^{3+}, Sm^{3+}, or Nd^{3+} ions. The enzyme is inactive without these ions. Concentration of these ions, which activates enzyme, is as little as 1 – 10 nM [30, pp. 1, 4, 6–7, 9–10]. We can suppose that *B. subtilis* also has enzymes, activity of which is enhanced by Ce.

Stimulatory effect of Ni NPs can be explained by the role of Ni in bacterial metabolism. Ni is a component of bacterial metalloenzymes and

Table 14

Effects of NMs on average CFU/cm³ of *B. subtilis* during the whole incubation

NMs	CFU/cm³ (×10⁸) (WM ± Std.Err)	Δ, %	Level of significance, p		
			Student's t-test	DMRT	LSD test
No NMs	4,31±0,22		-	-	-
Ce	9,19±0,34	+113,26	<0,000001	<0,00004	<0,000001
I+S	3,83±0,15	-11,14	>0,09	>0,11	>0,12
Ti	5,79±0,19	+34,34	<0,0002	<0,0001	<0,00001
Ni	7,87±0,28	+82,59	<0,000001	<0,00006	<0,000001
Se+I	5,08±0,19	+17,87	<0,02	<0,02	<0,02
Zn	0,00×108	-100	<0,000001	<0,00006	<0,000001
"Avatar-2 protection"	2,34±0,15	-45,71	<0,000003	<0,00006	<0,000001

is a vital element for bacteria. One of Ni-containing enzymes is urease [31, pp. 4312]. All ureases contain Ni atoms, which determine their activity and functions. Accessory proteins are required to produce active urease [32, pp. 207–208, 212–219]. *B. subtilis* genome contains urease structural genes, but does not contain genes of accessory proteins, necessary for Ni^{2+} ions incorporation into enzyme's active sites. However, functional urease is present in *B. subtilis* cells [32, pp. 212–213, 217, 219]. It indicates that *B. subtilis* have to be highly dependent on external sources of Ni to maintain efficient functioning of at least some metabolic pathways. Despite Ni insolubility in water and, accordingly, high stability of Ni NPs in water-based colloids, Ni NPs form thin surface layers of NiO, which is a product of Ni oxidation and protects it from further oxidation. Studies show that native Ni NPs have thin NiO surface layers, which can be as thin as 0,6 nm [33, pp. 1079]. NiO solubility is often referred as "negligible", but it is actually 1,1 mg $\cdot L^{-1}$ [34]. Despite NiO low solubility and thinness of NiO layers, amount of Ni^{2+} ions released by Ni NPs can be sufficient to affect bacteria. As we know from biological role of Ce, nanomolar concentration can be sufficient. It can explain positive effect of Ni NPs on *B. subtilis*.

Another NM, which stimulated *B. subtilis* growth, was composition of NPs Se+I. Most of available studies emphasize only antibacterial activity of Se NPs. Only very few of them elicit positive effects of Se NPs on bacteria. We show that Se+I NPs can elevate number of CFU/cm³ of

B. subtilis by 20,36 % and this effect is significant ($p<0,04$). It is predictable, as Se is vital for living organisms, including bacteria. However, positive effects of Se NPs on bacteria are not studied sufficiently. It was shown that chitosan-modified Se NPs (CS-SeNPs) are much less toxic to *Lactobacillus bulgaricus* than sodium selenite (Na_2SeO_3). Both CS-SeNPs and Na_2SeO_3 did not affect bacteria at concentration 1 μg/mL (by Se). At concentration 10 μg/mL CS-SeNPs caused death of 20 % cells, while Na_2SeO_3 – 60 % at the same concentration. CS-SeNPs shown to pass through bacterial cell wall, but without damaging it. When CS-SeNPs was added to culture, *L. bulgaricus* transformed most of available Se to organoselenium compounds, while in presence of Na_2SeO_3 production of organoselenium compounds was much lower and most of Se that was identified in culture incubated with Na_2SeO_3 had oxidation state IV [35, pp. 1-2, 12-19]. Ukrainian scientists have shown that addition of Se NPs to a probiotic based on *Lactobacillus plantarum* significantly elevates its activity [36, pp. 15]. Evidences on positive effects of Se NPs on lactic acid bacteria (LAB) are especially promising, because as mentioned in introduction, *B. subtilis* is perspective for use in probiotics and its use along with LAB was proposed. We have shown *B. subtilis* growth promotion in presence of Se+I NPs, so we suppose that at some concentrations Se NPs can be beneficial for both *B. subtilis* and LAB, which makes Se NMs promising materials for probiotic production.

Besides discovery of positive effects of NMs, some NMs showed antibacterial activity. Zn NPs are the most active, causing total loss of viability by cells.

Thus, different NMs show either antibacterial or stimulatory effects on strain *B. subtilis* IMV B-7023. These findings open up new horizons for invention and production of new generation of both microbial inoculants and antibacterial substances, produced using NMs and having unique qualities.

NMs, which enhance growth of *B. subtilis* can be used in production of microbial inoculants. For instance, use of NMs can be an efficient solution for elevating titers of microbial inoculants and speeding up processes of fermentation. NMs can be used to boost biological activity of microbial inoculants based on *Bacillus* species. NMs can increase viability of bacteria, their ability to colonize substrates and compete with indigenous microorganisms, enhance their ability to grow and persist in soil, organic residues, on surfaces of leaves, etc.

NMs can elevate plant hormones production. As mentioned, Ce and Ni play a key role in activity of many bacterial enzymes. It is suggested that the last enzyme in indole-3-acetic acid (IAA) synthetic pathway of *B. amyloliquefaciens* is indole 3-acetaldehyde dehydrogenase [37, pp. 1, 3–4]. Considering role of Ce in activity of dehydrogenases of other bacteria, we suppose that NMs of Ce and other elements can elevate production of IAA and other plant hormones by *Bacillus* species.

Besides changes in growth, NMs also influenced colonies morphology. Diameters of colonies decreased in all variants, except Ni NPs. In some variants, shape and color of colonies have also changed.

In total, data obtained in our study (changes in reproductive activity and colonies morphology) along with theoretical data indicate that other characteristics of the bacteria could undergo dramatic changes. Among them there are: enzymatic activity, functioning of transport proteins, intracellular concentration of ions (for instance Ca^{2+}), plasma membrane permeability, cell wall integrity, cell morphology, biologically active compounds production and others.

It needs to be pointed out that besides huge scientific and practical value of our results, many of them are surprising and challenge the dominating scientific thought about NMs as almost only antibacterial materials.

Therefore, effects of NMs on a vast number of *Bacillus* species characteristics, including physiological and biochemical ones need further rigorous investigation. The value of discoveries, which can be made in this area, is difficult to overestimate.

5. Conclusions

1. Effects of NMs on the reproduction of the strain *B. subtilis* IMV B-7023 depend on the chemical composition of NMs strongly.

2. Ce NPs, Ti NPs, Ni NPs and composition of NPs Se+I have shown significant stimulatory effect on growth of the strain, increasing its average number of CFU/cm^3 by 113,23; 34,33; 82,59 and 17,87 % respectively.

3. Composition of NPs I+S and "Avatar-2 protection" substance showed bacteriostatic activity, decreasing CFU/cm^3 by 11,14 and 45,71 % respectively.

4. Zn NPs exhibited extreme bactericidal activity at concentration used in the study. They caused loss of viability by all cells available in culture within 1 day.

5. All studied NMs, except Ni NPs, caused significant decrease of average colony diameter. Some NPs also influenced shape and color of colonies.

6. Our study shows that certain NMs are promising to be applied in many areas of human life. NPs of Ce, Ti, Ni and Se+I can be used to facilitate production of microbial inoculants, as well as to dramatically enhance activity of bioeffectors. Zn NPs can be used to develop efficient bactericidal agents. In total, NMs open up horizon for creation of new generation of revolutionary products with unique qualities, which can be applied either in agriculture or in any other area of human life.

7. Besides the results of the study are promising, they also challenge dominating scientific thought about NMs as primarily antibacterial materials. Some of these challenging results can be explain with theoretical data. Stimulatory effect of Ni NPs can be explained by the release of nanomolar amounts of Ni^{2+} ions, which traditionally considered being negligible, but can be enough to change bacterial metabolism. At the same time, stimulatory effect of Ce NPs is harder to explain. Ions of Ce have profound biological role, but due to chemical properties of Ce, the possibility of their release from Ce NPs is unclear. All this data leads us to urgent necessity of studying interactions between NMs and bacteria, as origins of many phenomena are unclear.

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