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EFFECT OF NANOPARTICLES OF DIFFERENT NATURE ON THE ADENOSINE TRIPHOSPHATASE ACTIVITY OF *AZOTOBACTER VINELANDII* IMV B-7076 AND *BACILLUS SUBTILIS* IMV B-7023

Under soil conditions, bacteria interact with nanoparticles of natural nanomaterials and ions. The study of such interaction and its effect on ATPase activity of bacteria is an important issue contributing to the understanding of the mechanisms underlying the functioning of living cells in their interaction with nanomaterials. ***Objective.*** *To investigate ATPase activity of nitrogen-fixing and phosphate-mobilizing bacteria exposed to silica and bentonite nanoparticles and some ions.* ***Methods.*** *ATPase activity of the culture was determined by the concentration of phosphate in the reaction mixture. Silica and bentonite were used as effectors for ATPase activity determination.* ***Results.*** *The level of ATPase activity of Azotobacter vinelandii IMV B-7076 was shown to increase by 241 % after 48 hours of culturing and by 97 % after 72 hours of culturing compared to 24-hr culture. Magnesium and calcium cations were found to significantly increase ATPase activity of A. vinelandii and B. subtilis, whereas sodium and potassium ions had little effect on this process.* ***Conclusions.*** *The ATPase activity of Azotobacter vinelandii IMV B-7076 and Bacillus subtilis IMV B-7023 was found to be the highest in the presence of magnesium and calcium ions. The interaction of these strains with bentonite nanoparticles significantly stimulated the ATPase activity of the bacteria, while silica nanoparticles negatively affected the ATPase activity of A. vinelandii and positively affected that of B. subtilis.*

Keywords*: Azotobacter vinelandii, Bacillus subtilis, ATPase, silicon dioxide, bentonite, nanoparticles.*

The intensive use of chemical fertilizers and pesticides in crop production is accompanied by environmental pollution, a decrease in the quality of agricultural products, and a negative impact on people's health (Patyka et al., 2005). Biologization of agriculture through the use of microbial preparations can reduce the use of chemical fertilizers and pesticides and improve the quality

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of agricultural products. Rhizosphere microorganisms included in preparations allow the correction of soil microbiota (Kurdish, 2010).

It is known that microorganisms play a key role in the cycle of nutrients and are an integral organic part of it (Dalal, 1998; Sparling, 1997; Volkogon et al., 2006; Iutynska et al., 2019). About 99% of soil microorganisms function in contact with particulate matter (Costerton, 1985; Nannipieri et al., 2003). The interaction of microorganisms with nanoparticles is accompanied by changes in physiological (Zvyagintsev, 1973; Kurdish, 2019; Srivastava et al., 2013; Chobotarov et al., 2017) and biochemical processes and is associated with the cellular energy metabolism (Pradet & Raymond, 1983).

Adenosine 5'-triphosphatase (ATPase), (EC 3.6.1.3), is one of the key enzymes of energy metabolism of bacterial cells, due to which the difference in electrochemical potentials at the membrane is generated; therefore, it can be an effective indicator of the general physiological state of microorganisms (Gruzina, 1997). It is known that the ATPase activity is significantly influenced by its interaction with cations, including Na^+, K^+, Mg^{2+}, and Ca^{2+} (Skulachev, 1989).

Based on the interaction of nitrogen-fixing bacteria *Azotobacter vinelandii* IMV B-7076 and phosphate-mobilizing bacteria *Bacillus subtilis* IMV B-7023 with bentonite particles, a highly active complex bacterial preparation Azogran for plants was developed at the Institute of Microbiology and Virology, NAS of Ukraine.

Thus, the study of nanomaterials' effect on ATPase activity of nitrogen-fixing and phosphate-mobilizing bacteria can be useful for the development of advanced biotechnology of high-performance microbial preparations for crop production and prediction of bacterial activity in agroecosystems.

Materials and Methods. Nitrogen-fixing bacteria *Azotobacter vinelandii* IMV B-7076 (State Enterprise «Ukrainian Intellectual Property Institute», 2006) and phosphate-mobilizing bacteria *Bacillus subtilis* IMV B-7023 (State Enterprise «Ukrainian Intellectual Property Institute», 2003), isolated in the Department of microbiological processes on solid surfaces of D.K. Zabolotny Institute of Microbiology and Virology, NAS of Ukraine, were the objects of research.

Nitrogen-fixing bacteria were grown in Burke's medium of the following composition, (g/L): 0.8 $K_2HPO_4 \times 3H_2O$; 0.6 KH_2PO_4; 0.5 $Na_3C_6H_5O_7$; 0.1 $CaCl_2$; 0.2 $MgSO_4 \times 7H_2O$; 0.015 $FeSO_4 \times 7H_2O$; 0.015 $Fe_2(SO_4)_3$; 0.005 Na_2MoO_4; 20.0 sucrose; pH 7.0—7.5. Phosphate-mobilizing bacteria were grown in glucose-mineral medium of the following composition, (g/L): 0.5 $(NH_4)_2SO_4$; 0.3 $MgSO_4 \times 7H_2O$; 0.3 NaCl; 0.3 KCl;5.0 $CaCO_2$; 0.001 $MnSO_4 \times 7H_2O$; 0.001 $FeSO_4 \times 7H_2O$; 10.0 glucose; 0.5 $K_2HPO_4 \times 3H_2O$; 0.5 KH_2PO_4; pH 7.0—7.4. Silicon dioxide or bentonite (0.05; 0.1; 0.5 g/L) was added into media for the study of nanomaterials influence on the ATPase activity of *A. vinelandii* IMV B-7076 and *B. subtilis* IMV B-7023. Cultivation temperature was 28 °C. Bacteria were cultured for 24—72 hours. Suspension of a one-day bacterial culture was used as inoculum. The initial viable bacterial content was 1×10^6 CFU/mL.

To determine ATPase activity of microorganisms, cells were freed from the culture medium by centrifugation on an OPn-8 centrifuge (JSC TNK DASTAN, Kyrgyzstan) at 6600 g for 15 min. The cells were then washed twice with 50 mM Tris-HCl buffer, pH 7.4, and resuspended in the same buffer. After that, they were disintegrated on an ultrasonic disintegrator (UD-20, Poland) with the operating frequency of 22±1.65 kHz for 4 min in an ice bath (Roy et al., 1982). Cell residues were freed from the sample by centrifugation of the resulting suspension on a refrigeration centrifuge at 10000 g and 4 °C for 30 min. The amount of protein in cell-free supernatants was determined by the Bradford assay (Bradford, 1976).

ATPase activity was determined in the reaction medium of 2.5 mL of 16 mM ATP in 50

mM Tris-HCl, pH 7.4, 2.5 mL of 16 mM $MgCl_2$ in 50 mM Tris-HCl, and 5 mL of sample in 50 mM Tris-HCl (for experimental variant) or 5 mL of 50 mM Tris-HCl (for control variant) at 28 ± 1°C. The salts NaCl, KCl, $CaCl_2$, and $MgCl_2$ were added to the reaction medium to reveal the influence of the ions on the ATPase activity. After 40 min, the reaction was stopped with 2 mL of 10 % trichloroacetic acid. The resulting precipitate was precipitated for 5 min at 6600 g. The amount of inorganic phosphate was determined by the Fiske-Subbarow method with ammonium molybdate at wavelength 590 nm, and the ATPase activity of the culture was calculated. ATPase activity (A) was expressed as the amount of inorganic phosphate (Pi) detached from the substrate (ATP) in 1 min per 1 mg of protein.

Statistical analysis for the data obtained were performed using Minitab statistical software (Minitab Inc.). All experiments were performed in at least three independent replicates.

Results. It was found that the highest level of ATPase activity of *A. vinelandii* IMV B-7076 was determined after 48 hours of cultivation. Under these conditions, the culture activity was 3.42 times higher than during the first day of cultivation. Upon further culturing (72 hours), the ATPase activity decreased but was still higher than after 24 hours of cultivation (Table 1).

ATPase activity of *B. subtilis* IMV B-7023 decreased with increasing time of culture in glucose-mineral medium (Table 1). Thus, after two and three days of cultivation, the ATPase activity of the culture decreased sharply to 1.60 μmol × 10^{-2}/min × mg protein and 1.40 μmol × 10^{-2}/min × mg protein (by 75.87 % and 78.88 % respectively) compared to the culture which was grown for 24 hours (Table 1).

Table 1. **The influence of cultivation time on the ATPase activity of *A. vinelandii* IMV B-7076 and *B. subtilis* IMV B-7023**

Time, h	ATPase activity, μmol × 10^{-2}/min × mg protein	
	A. vinelandii IMV B-7076	*B. subtilis* IMV B-7023
24	2.58 ± 0.25	6.63 ± 0.30
48	8.81 ± 0.31	1.60 ± 0.35
72	5.10 ± 0.39	1.40 ± 0.47

The level of ATPase activity of *A. vinelandii* IMV B-7076 was found to increase with increasing ATP concentration in incubation medium up to 4 mM. With further increase in substrate content, ATPase activity decreased (Fig. 1). In view of this, in further studies to determine the level of ATPase activity of bacteria, the ATP concentration of 4 mM (optimum activity) was used, and the culture was grown for 48 hours.

The dynamics of ATPase activity of *B. subtilis* depending on ATP concentration is shown in Fig. 1. Thus, the level of ATPase activity of *B. subtilis* increased with increasing ATP concentration in incubation medium (up to 4 mM) and was 6.63 μmol × 10^{-2}/min × mg protein. With further increase in substrate — ATPase activity decreased slightly but was higher than in the other variants.

Considering the results for the dependence of ATPase activity level of culture on time of cultivation and substrate concentration in incubation medium, in further experiments on determining the ATPase activity level of *B. subtilis*, we used an ATP concentration of 4 mM (optimum activity) and culture of 24 hours.

When studying the level of ATPase activity of *A. vinelandii* depending on the content of the ions added to the reaction medium, it was found that the highest activity was observed in the medium with Mg^{2+} ions (Fig. 2). Under these conditions, the activity of the ATPase of the culture was at 8.81 μmol × 10^{-2}/min × mg protein. When Ca^{2+} ions were added to the incubation medium, the ATPase activity of *A. vinelandii* was decreased by 2.72 % as compared to the variant with Mg^{2+} ions. However, the lowest ATPase activity of bacteria was observed when Na^+ and K^+ ions were added to the reaction medium. Thus, the level of ATPase activity in the presence of Na^+ ions was at 7.94 × 10^{-2}/min × mg protein,

and in the presence of K^+ ions it was 8.1 μmol × × 10^{-2}/min × mg protein.

Based on the obtained results, further experiments were conducted using a 48-hour culture of *A. vinelandii*, a substrate concentration of 4 mM, and the addition of Mg^{2+} ions in the reaction medium.

When studying the level of ATPase activity of phosphate-mobilizing bacteria depending on the ions introduced into the reaction mixture, it was found that the highest activity was recorded in the medium with divalent ions Mg^{2+} and Ca^{2+} (Fig. 3). Thus, it was shown that the introduction of both Mg^{2+} and Ca^{2+} ions into the incubation medium resulted in an abrupt activation of the ATPase. Under these conditions, the ATPase activity of *B. subtilis* was 6.63 μmol × 10^{-2}/min × mg protein for Mg^{2+} ions and 6.55 μmol × 10^{-2}/min × × mg protein for Ca^{2+} ions. When single-valent ions (Na^+ and K^+) were added to the incubation mixture, a low level of ATPase activity of the culture was recorded. Similar results of ion impact on ATPase activity were observed for nitrogen-fixing bacteria (Fig. 3).

It was found that when *A. vinelandii* was in Burke's medium, to which bentonite nanoparticles were added, the ATPase activity of azotobacter increased (Fig. 4).

Thus, when 0.05 g/L bentonite was added, the level of ATPase activity increased significantly (by 19.18%) compared to the control (without bentonite).

The ATPase activity of the culture increased with increasing concentration of the mineral nanoparticles (Fig. 4). Maximum ATPase activity of bacteria was fixed at 0.5 g/L of bentonite in Burke's medium. Under these conditions, the level of ATPase activity was 28.26 % higher than in the control.

At the same time, when silicon dioxide nanoparticles were added to the medium, ATPase activity of azotobacter decreased compared to the control (Fig. 5). Thus, when 0.05 g/L silicon dioxide was added to the medium, the ac-

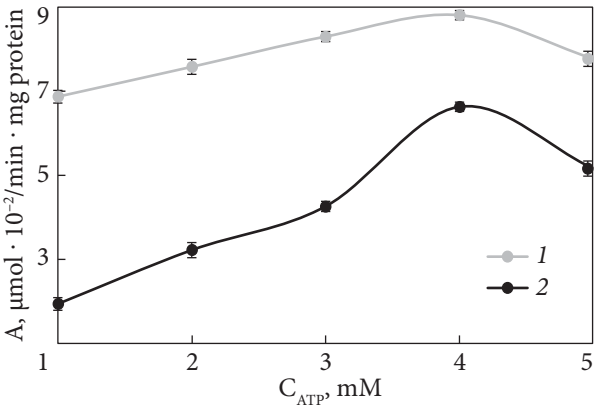

Fig. 1. The influence of ATP concentration in the reaction medium on ATPase activity (A) of *A. vinelandii* IMV B-7076 (1) and *B. subtilis* IMV B-7023 (2)

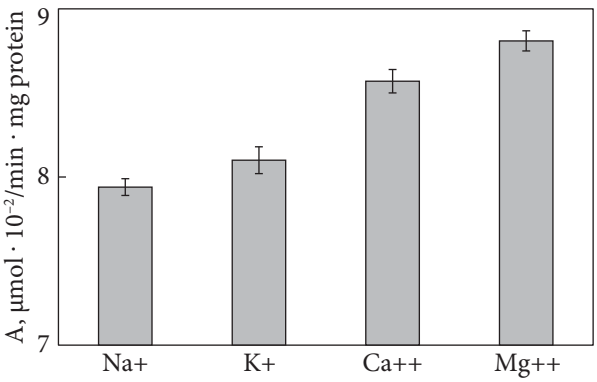

Fig. 2. The influence of various ions on ATPase activity (A) of *A. vinelandii* IMV B-7076 in the reaction medium

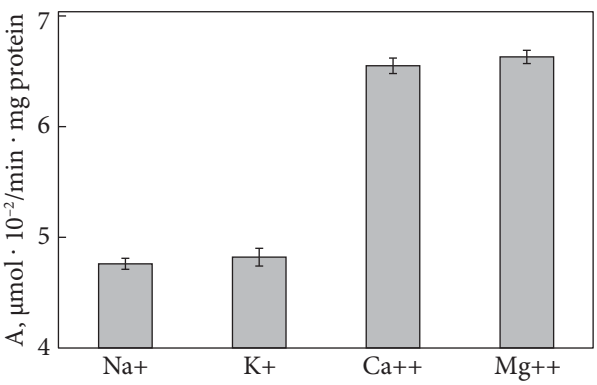

Fig. 3. The influence of various ions on ATPase activity (A) of *B. subtilis* IMB B-7023 in the reaction medium

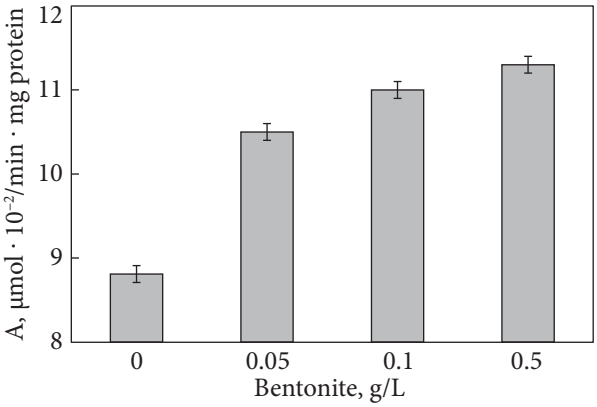

Fig. 4. The influence of bentonite nanoparticles on ATPase activity (A) of *A. vinelandii* IMV B-7076

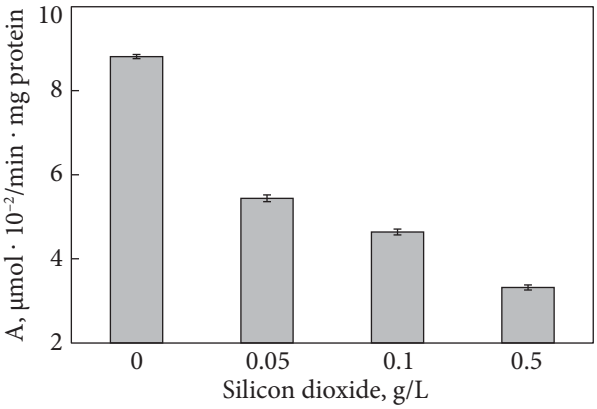

Fig. 5. The influence of silicon dioxide nanoparticles on ATPase activity (A) of *A. vinelandii* IMV B-7076

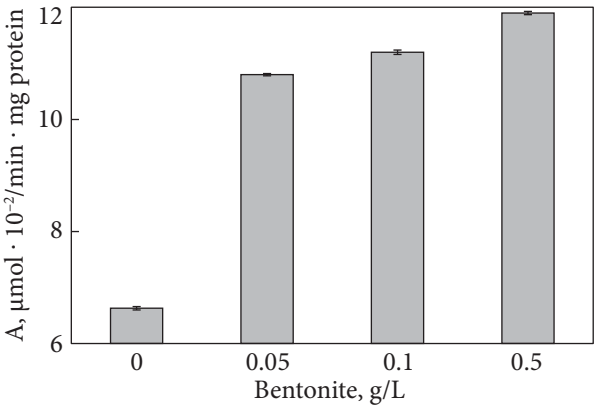

Fig. 6. The influence of bentonite nanoparticles on ATPase activity (A) of *B. subtilis* IMV B-7023

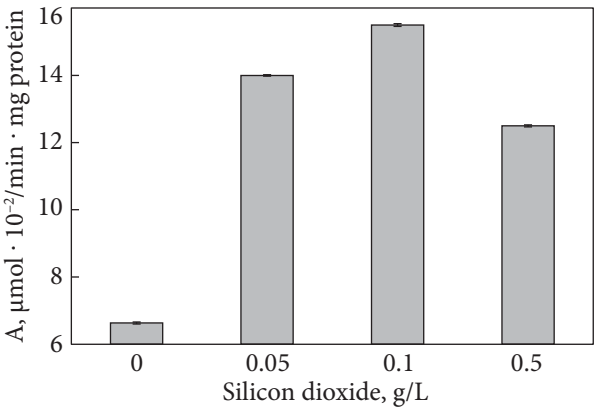

Fig. 7. The influence of silicon dioxide nanoparticles on ATPase activity (A) of *B. subtilis* IMV B-7023

tivity decreased by 38.25 % (down to 5.44 μmol 10^{-2}/min × mg protein).

When the content of silicon dioxide nanoparticles was increased (up to 0.5 g/L) in Burke's medium, the ATPase activity of the culture decreased. Under such conditions, the ATPase activity level of *A. vinelandii* IMV B-7076 was 3.32 μmol × 10^{-2}/min × mg protein (Fig. 5).

When investigating the effect of dispersed materials on ATPase activity of bacilli, slightly different results were observed than those obtained for the *A. vinelandii* culture. When bentonite was added to the glucose-mineral medium, ATPase activity of bacilli increased sharply (Fig. 6). Thus, when 0.05 g/L bentonite nanoparticles were added, ATPase activity was 62.90% higher compared to the control. An increase in ATPase activity of *B. subtilis* was observed with the increase in the content of nanoparticles of natural material in the bacterial culture medium (Fig. 6). The highest ATPase activity of the culture was recorded when 0.5 g/L bentonite was added to the glucose-mineral medium. Under these conditions, the ATPase activity of *B. subtilis* was 11.9 μmol × 10^{-2}/min × mg protein.

When bacilli were cultured in the medium with silicon dioxide nanoparticles, ATPase activity increased, which is not typical for *A. vine-*

landii, compared to the control variant (without dispersed material) (Fig. 7). Thus, at the content of silicon dioxide particles in the medium of 0.05 g/L, a sharp increase in ATPase activity of the culture was observed. Under such conditions, the level of ATPase activity was 14.0 μmol × × 10^{-2}/min × mg protein. When 0.1 g/L of dispersed material was applied, the highest level of ATPase activity of the culture was recorded (15.5 μmol 10^{-2}/min × mg protein) (Fig. 7). When 0.5 g/L of silicon dioxide nanoparticles was added, a slight decrease in ATPase activity of the culture was recorded.

Discussion. The functioning of plants, living organisms, and microorganisms in the environment is closely related to the generation of energy in the form of ATP and its consumption during the hydrolysis of this compound by ATPase (Skulachev, 1989). The ATPase activity of microorganisms is significantly influenced by external environmental factors (temperature, pH, the composition of their functioning medium, and the content of cations in the reaction medium) (Varbanets, 1974; Kushkevich et al., 2007). Cells use the energy of ATP hydrolysis to support biochemical processes (synthesis of biomolecules, active transport, etc.) (Subramanian, 2022).

We have shown that ATPase activity of bacteria *A. vinelandii* IMV B-7076 and *B. subtilis* IMV B-7023, components of high-active complex bacterial preparation for crop production Azogran, reaches the highest values when cultured in a medium for 48 hours. When introducing bacterial preparations into agroecosystems, the cells will interact with nanoparticles of natural minerals, which also has a significant effect on the physiological and biochemical activity of microorganisms (Chobotarov, 2020). Upon interaction with nanoparticles of natural minerals, these strains are capable of enhancing the synthesis of phytohormones (Chobotarov et al., 2017). The application of the Azogran preparation, developed on the basis of these bacteria, protected barley plants from oxidative stress (Skorochod et al., 2020) and potato plants from damage by the Colorado beetle (Kurdish et al., 2021). We have shown that the ATPase activity of both *A. vinelandii* and *B. subtilis* significantly increases when bentonite nanoparticles are introduced into the medium. At the same time, when these bacteria were cultured in a medium with silica nanoparticles, the indices of this activity decreased for azotobacter and increased for bacilli. The reasons for this effect need further investigation.

The level of ATPase activity of the enzyme complex of microorganisms is largely determined by the content of univalent and divalent cations introduced into the incubation medium (Varbanets, 1974; Kushkevich et al., 2007). Magnesium is believed to be one of the most important regulators of cellular energy exchange, which affects the energy supply of membrane ion channels (Swaminathan, 2003), including the Na^+/K^+ pump (Grycova et al., 2009).

The highest values of this activity for *A. vinelandii* and *B. subtilis* were determined in the medium containing 4 mM ATP and Mg^{2+} ions. The addition of Ca^{2+} ions to the reaction medium less markedly stimulated ATPase activity compared to Mg^{2+} ions, while the addition of potassium and sodium ions to this medium insignificantly stimulated ATPase activity of these bacteria. Similar results of the influence of these cations were obtained earlier in the study of ATPase activity of *A. chroococcum* (Varbanets, 1974). Thus, our results testify to a significant influence of a number of factors on ATPase activity of bacteria *A. vinelandii* IMV B-7076 and *B. subtilis* IMV B-7023, which are components of complex bacterial preparation Azogran.

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ВПЛИВ НАНОЧАСТОК РІЗНОЇ ПРИРОДИ НА АДЕНОЗИНТРИФОСФАТАЗНУ АКТИВНІСТЬ *AZOTOBACTER VINELANDII* IMB B-7076 ТА *BACILLUS SUBTILIS* IMB B-7023

У грунтових умовах бактерії взаємодіють з наночастками природних наноматеріалів та іонами. Дослідження такої взаємодії, її вплив на АТФ-азну активність бактерій є важливим питанням, яке сприятиме розумінню механізмів, що лежать в основі функціонування живих клітин за їх взаємодії з наноматеріалами. **Мета.** Дослідити АТФ-азну активність азотфіксуючих та фосфатмобілізуючих бактерій за впливу наночасток діоксиду кремнію і бентоніту та деяких іонів. **Методи.** АТФ-азну активність культури визначали за концентрацією фосфату в реакційній суміші. Як ефектор для визначення зміни АТФ-азної активності використовували діоксид кремнію та бентоніт. **Результати.** Показано, що рівень АТФ-азної активності *Azotobacter vinelandii* IMB B-7076 зростав на 241 % після 48 годин та на 97 % після 72 годин культивування у порівнянні з 24-х годинною культурою. Встановлено, що катіони магнію та кальцію суттєво підвищують АТФ-азну активність азотобактера та бацили, тоді як іони натрію та калію незначно впливають на цей процес. **Висновки.** Встановлено, що АТФ-азна активність як *Azotobacter vinelandii* IMB B-7076, так і *Bacillus subtilis* IMB B-7023 досягає найвищих значень за наявності в середовищі іонів магнію та кальцію. Взаємодія даних штамів з наночастками бентоніту значно стимулює АТФ-азну активність бактерій, тоді як наночастки діоксиду кремнію негативно впливають на АТФ-азну активність *A. vinelandii* та позитивно на *B. subtilis*.

Ключові слова*: Azotobacter vinelandii*, *Bacillus subtilis*, АТФ-аза, *SiO_2, бентоніт, наночастки.*