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ABIOTIC FACTORS INFLUENCE ON *BACILLUS SUBTILIS* IMV B-7023 PHYTASE ACTIVITY

Bacteria of the Bacillus genus can synthesize specific phytase enzymes. This property is especially important for soil bacteria. It helps to mineralize phytin and phytates and to provide these bacteria and plants (in the root zone of which they live) with the available phosphorus. Our previous studies have demonstrated that the Bacillus subtilis IMV B-7023 strain exhibits a phytase activity and can use phytate as a nutrition source. It is a component of the «Azogran» complex bacterial preparation for crop production. As known, abiotic environmental factors can influence the phytase activity of bacteria. In particular, the phytase activity changes significantly under different pH and temperatures. Solid soil particles, including nanosized minerals, can also influence bacteria's enzymatic activity. The influence of abiotic factors on Bacillus subtilis IMV B-7023 phytase activity has not previously been studied, so this was the ***aim*** *of our research.* ***Methods.*** *The phytase activity of bacteria was studied by measuring the amount of phosphate released from sodium phytate during the enzymatic reaction, and the nanomaterials' influence on growth — by cultivation methods.* ***Results.*** *The highest B. subtilis IMV B-7023 phytase activity was observed at 28 °C. Also, there was no B. subtilis IMV B-7023 phytase activity at pH 4—6. However, this activity increased at pH 7 and did not change significantly with increasing the buffer system pH to 12. Silicon dioxide influence on the B. subtilis IMV B-7023 growth activity during cultivation in a media with phytate as a phosphorus source depended on the nanomaterial concentration. Thus, at 0.05 and 0.5 g/L of silicon dioxide in the medium, this strain growth activity increased by 8—18%, and at 5.0 g/L of these nanoparticles, bacteria growth inhibition by 19% was observed. At the same time, clay mineral bentonite did not affect the B. subtilis IMV B-7023 growth under the studied cultivation conditions. In addition, silicon dioxide and bentonite stimulated B. subtilis IMV B-7023 phytase activity at all studied concentrations. So, phytase activity increased by 1.82—3.34 times upon adding silicon dioxide and by 2.54—5.83 times upon adding bentonite into the medium. Since the optimal values for phytase activity of most genus Bacillus bacteria are within neutral pH values and temperatures within 50—55 °C, a property of B. subtilis IMV B-7023 to show maximum phytase activity at alkaline pH and lower temperatures (28 °C) and also stimulation of this activity by soil minerals increases competitiveness of this strain as a component of a bacterial preparation for crop production.* ***Conclusions.*** *Abiotic environmental factors influence the B. subtilis IMV B-7023*

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growth and phytase activity. Optimal physicochemical factors for the phytase activity of these bacteria are temperature 28 °C and pH 7—12. The concentrations 0.05, 0.5, and 5.0 g/L of silicon dioxide and bentonite increase B. subtilis IMV B-7023 phytase activity. The effect of these nanoscale minerals on the B. subtilis IMV B-7023 growth depends on their type and concentration during cultivation in the medium with phytate as a phosphorus source. The obtained results indicate the potential ability of the B. subtilis IMV B-7023 strain to effectively assimilate phytates in neutral and alkaline soils, especially due to the interaction of these bacteria with bentonite and silicon dioxide nanoparticles. This expands the possibility of using B. subtilis IMV B-7023 in agricultural technologies.

Keywords: *Bacillus subtilis IMV B-7023, abiotic factors, phytase activity, growth activity, bentonite, silicon dioxide, nanoparticles.*

Bacteria of the *Bacillus* genus can synthesize phytase enzymes [1—4]. These specific enzymes hydrolyze mio-inositol 1,2,3,4,5,6-hexakisphosphate (phytate) to form less phosphorylated inositol derivatives. This property is especially important for soil bacteria because it helps to mineralize phytin and phytates and to provide the bacteria and plants (in the root zone of which they live) with the available phosphorus. Our previous studies have demonstrated that the strain *Bacillus subtilis* IMV B-7023 has phytase activity and can actively use phytate as a nutrition source [5]. It is a component of the complex bacterial preparation «Azogran» used for crop production.

It is known that abiotic environmental factors can influence the phytase activity of bacteria. In particular, it changes significantly under different pH and temperature [2, 3]. Solid soil particles, including nanosized minerals, can also influence bacteria's enzymatic activity [6]. In addition, many preparations for crop production contain various minerals, for example, bentonite, vermiculite, and silica [6—8]. The influence of abiotic factors on *B. subtilis* IMV B-7023 phytase activity has not previously been studied, so this was the aim of this research.

Materials and methods. Strain *B. subtilis* IMV B-7023 was the study object [9]. The bacteria were isolated from the soil at the Department of microbiological processes on solid surfaces of the Zabolotny Institute of Microbiology and Virology, NAS of Ukraine [10]. Strain *B. subtilis* IMV B-7023 was plated onto a phytase screening medium of the following composition: 2% sucrose, 0.4% sodium phytate, 0.2% $CaCl_2$, 0.5% $(NH_4)_2SO_4$, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 5H_2O$, 1.5% agar (pH 7.0) [11, 12]. Bacteria were cultivated in a liquid medium (in Tris-HCl buffer, 50 mM, pH 7.2) of the following composition (g/L): 1.0 sodium phytate, 0.5 $(NH_4)_2SO_4$, 0.3 $MgSO_4 \cdot 7H_2O$, 0.3 NaCl, 0.3 KCl, 0.001 $MnSO_4 \cdot 5H_2O$, 0.001 $FeSO_4 \cdot 7H_2O$, 2.0 $CaCO_3$, and 10 glucose. Bentonite or silicon dioxide (0.05, 0.5, 5.0 g/L) was added to the media to study the influence of nanomaterials on *B. subtilis* IMV B-7023's growth and phytase activity. The bacteria were cultured for 2 days at 28 °C, since the previous studies have demonstrated their maximum growth under the same conditions [5].

The natural mineral bentonite and synthetic silicon dioxide were used in the research. Bentonite was selected from the Dashukovskyi deposit of bentonite clays (Cherkasy region, Ukraine). It was ground in a porcelain mortar and then was homogenized by an ultrasonic disintegrator to obtain a fraction with a not more than 100 nm particle size. Silicon dioxide is provided by the Chuiko Institute of Surface Chemistry, NAS of Ukraine. Its particle size was 5—20 nm.

To determine the *B. subtilis* IMV B-7023 extracellular phytase activity, bacteria were precipitated by centrifugation at 10000 g, 4 °C to get cell-free supernatants. Phytase activity assays were performed by measuring the amount of inorganic phosphate released from sodium phytate during the enzymatic reaction [4, 13]. The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.2) or another buffer (see below), 2 mM sodium phytate, and cell-free superna-

tants. For investigation of the temperature effect on the phytase activity, the mixture was incubated at 20 °C, 28 °C, 37 °C, 50 °C, and 60 °C for 40 min. All other assays were performed at 28 °C. The pH effect on *B. subtilis* IMV B-7023 phytase activity was studied in the following buffer systems: pH 4 — sodium citrate/HCl, pH 5, 6 — sodium citrate/NaOH, pH 7, 8 — Tris-HCl, and pH 9—12 — glycine/NaOH. The reaction was stopped with 10% trichloroacetic acid. The amount of inorganic phosphate was determined by the Fiske-Subbarow method with ammonium molybdate at a wavelength of 590 nm [14]. The amount of protein in cell-free supernatants was determined by the Bradford assay [15]. One unit of phytase activity was the amount of crude enzyme that catalyzes the sodium phytate hydrolysis with the formation of 1 μmol of inorganic phosphate for 1 min under the assay conditions. The results were presented as the specific activity, that is, the number of activity units per milliliter of cell-free supernatant (U/mL).

Statistical analysis for the data obtained was performed using the Minitab statistical software (Minitab Inc.). All experiments were made in triplicate.

Results. It was shown that *B. subtilis* IMV B-7023 grew on a phytase screening medium with a halo zone (4—10 mm) around the colonies (Fig. 1). This clearly indicates phytate-degrading properties by these bacteria.

It was found that *B. subtilis* IMV B-7023 phytase activity depends on physicochemical parameters of the reaction medium. Thus, the highest phytase activity in the Tris-HCl buffer system and glycine buffer was observed at 28 °C (Fig. 2). At further temperature increase, *B. subtilis* IMV B-7023 extracellular phytase activity decreased expressively. It should be noted that at 20 °C, 28 °C and 37 °C, the phytase activity in a glycine buffer was lower than in the Tris-HCl system. In addition, the level of *B. subtilis* IMV B-7023 phytase activity was almost the same in the above two buffer systems at 50 °C and 60 °C

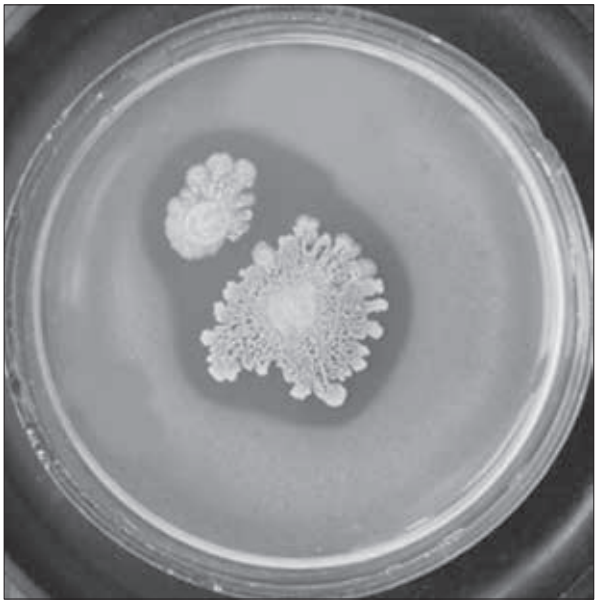

Fig. 1. Phytate hydrolysis assay for *B. subtilis* IMV B-7023

(Fig. 2). Since the phytase activity of bacteria was higher in Tris-HCl buffer at most studied variants, this buffer system was used in the following assays.

While studying different pH effects on the *B. subtilis* IMV B-7023 phytase activity, it was found that at pH 4—6, this activity was not observed (Fig. 3). However, the phytase activity increased to 5.67 U/mL at pH 7. The further increase in the buffer system pH to 12 units did not lead to substantive changes in the bacterial phytase activity (Fig. 3).

It was shown that silicon dioxide influences the *B. subtilis* IMV B-7023 growth activity during cultivation in media with phytate as the phosphorus source depends on the nanomaterial concentration (Table 1). Thus, at 0.05 and 0.5 g/L of silicon dioxide in the medium, this strain growth activity increased by 8—18%, and at 5.0 g/L of the nanoparticles, bacteria growth inhibition by 19% was observed (Table 1). At the same time, the clay mineral bentonite did not affect the *B. subtilis* IMV B-7023 growth under the studied cultivation conditions. Thus, when ben-

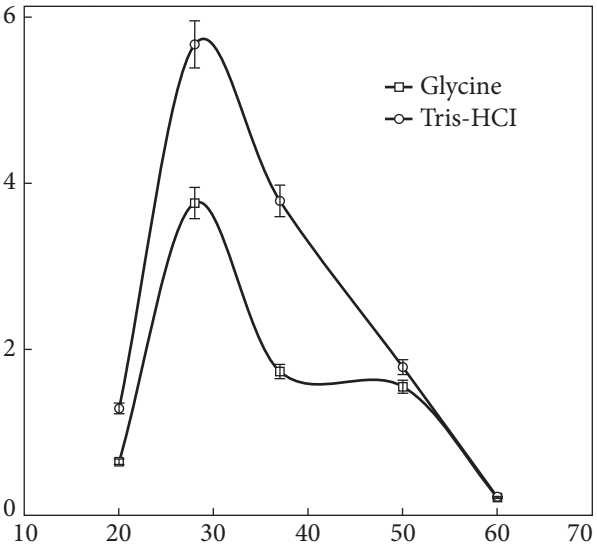

Fig 2. The temperature influence on *B. subtilis* IMV B-7023 phytase activity in Tris-HCl buffer (pH 7.2) and glycine buffer (pH 7.0)

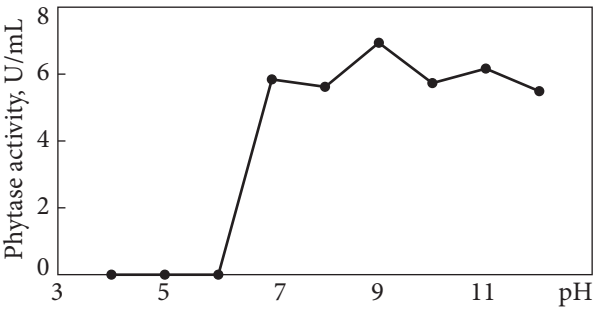

Fig 3. The pH influence on *B. subtilis* IMV B-7023 phytase activity at 28 °C

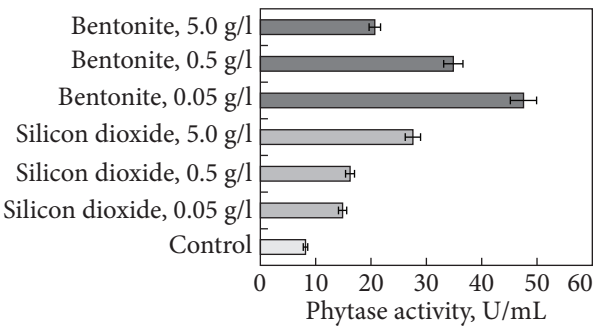

Fig. 4. Nanomaterials' influence on the *B. subtilis* IMV B-7023 phytase activity cultivation with phytate as a phosphorus source

tonite was added to the medium with sodium phytate with the same concentrations, *B. subtilis* IMV B-7023 number remained at the same level as the control variant (Table 1).

In addition, silicon dioxide and bentonite stimulated *B. subtilis* IMV B-7023 phytase activity at all studied concentrations. So, upon adding silicon dioxide to the medium, the phytase activity increased by 1.82—3.34 times, whereas upon adding bentonite — by 2.54—5.83 times (Fig. 4).

Discussion. Since *B. subtilis* IMV B-7023 phytase activity was noted at pH 7—12 and it did not change within these limits, it was reasonable to conclude that the optimal pH values for phytases of these bacteria were neutral and alkaline pH. The optimal temperature for the *B. subtilis* IMV B-7023 phytase activity was 28 °C. Regarding the other members of the genus, it is known that, for example, the strain *B. subtilis* VTTE-68013 phytase is active in the pH range from 2.5 to 9 and temperatures from 25 °C to 80 °C. However, the optimal values of physicochemical factors for the phytase activity of these bacteria were pH 7.5 and temperature 55 °C [16]. The *B. nealsonii* ZJ0702 phytase activity increased with temperature increasing from 20 °C to 50 °C and reached a maximum value

Table 1. **Nanomaterials' influence on *B. subtilis* IMV B-7023 growth activity during cultivation in media with phytate as a phosphorus source**

Medium		The number of bacteria (CFU/mL)*	% to control
with phytate (control)		$(6.03 \pm 0.24) \cdot 10^8$	100
with phytate and			
silicon dioxide, g/L	0.05	$(6.55 \pm 0.18) \cdot 10^8$	108
	0.5	$(7.12 \pm 0.25) \cdot 10^8$	118
	5.0	$(4.86 \pm 0.27) \cdot 10^8$	81
bentonite, g/L	0.05	$(6.07 \pm 0.22) \cdot 10^8$	100
	0.5	$(5.77 \pm 0.25) \cdot 10^8$	96
	5.0	$(6.17 \pm 0.24) \cdot 10^8$	102

Note: * the start number of cells was $(7.8 \pm 0.1) \cdot 10^6$/mL.

at 55 °C. At higher temperatures the enzyme activity decreased rapidly. It varied depending on pH and was the highest at pH 7.5 [3]. The *B. subtilis* DR6 phytase activity was determined at pH 3.5—8.5 and temperature 20—70 °C, and pH 5.5 and temperature 50 °C were revealed to be optimal [13]. The highest *B. subtilis* B.S.46 phytase activity was observed at temperature 56.5 °C and pH 7.3 [4].

Accordingly, the optimal values for the phytase activity of most genus *Bacillus* bacteria are in the range of neutral pH and 50—55 °C temperatures. It should be noted that the strain *B. subtilis* IMV B-7023 does not lose phytase activity even at alkaline pH and has a lower temperature optimum (28 °C). Based on the fact that *B. subtilis* IMV B-7023 is a component of the «Azogran» complex bacterial preparation for crop production, such its properties will expand the possibilities of using it for soils in agricultural technologies.

Studied nanomaterials differently influenced the growth and phytase activities of *B. subtilis* IMV B-7023 during cultivation in a medium with phytate as a phosphorus source. In particular, bentonite had no impact on the growth whereas silicon dioxide stimulated the growth at 0.05 and 0.5 g/L concentrations and inhibited it at 5 g/L. Previously, it has been shown that *B. subtilis* IMV B-7023 growth activity increases during cultivation in a medium containing 0.11 g/L of PO_4^{3-} and 0.1—1.0 g/L of silicon dioxide. But it has been observed that the number of viable cells at 5.0—20.0 g/L of this nanomaterial decreases. Moreover, silicon dioxide stimulated *B. subtilis* IMV B-7023 growth at 0.1—0.5 g/L and 10.0 g/L concentrations and inhibited it at 1.0 and 20.0 g/L concentrations during bacteria cultivation with 0.56 g/L PO_4^{3-} in the medium [17]. Given that the media with sodium phytate at 0.5, 1.0, and 2.0 g/L concentrations contain 0.072, 0.144, and 0.288 g/L of PO_4^{3-}, it is possible to note a similarity of the obtained in this work dependence of *B. subtilis* IMV B-7023 growth in the medium with silicon dioxide. That is, the same but differently created conditions for *B. subtilis* IMV B-7023 cultivation in a medium with low phosphate concentration (0.05—1.0 g/L) were accompanied by an increase in bacilli growth activity. However, under the same conditions and at a content of 5.0 g/L of silicon dioxide, the growth decreased. It can be assumed that this decrease is due to an increase in the availability of nutrients for bacteria that can be sorbed on nanomaterialparticles, or due to changes in the permeability of bacterial membranes [18].

The phytase activity of *B. subtilis* IMV B-7023 increased under the influence of nanosized silicon dioxide and bentonite. It is known that the catalase activity of another strain of *B. subtilis* increases in the presence of titanium dioxide and copper nanoparticles [19]. The stimulation of the phytase activity of *B. subtilis* IMV B-7023 by bentonite can also be explained by the effect of cations contained in this mineral. According to the database webmineral.com, bentonite may contain ions of sodium, aluminum, iron, zinc, magnesium, sodium, potassium, and calcium. As known, metal ions can stimulate the activity of phytases [4, 20]. However, the mechanisms of most of the obtained dependences are not yet known and need further study. Basing on the above data, we can conclude that to improve the mineralization of phytates by strain *B. subtilis* IMV B-7023, it is advisable to use these bacteria in crop production together with mineral preparations of bentonite and silicon dioxide or with these materials as a basis for bacterial preparations.

The obtained results expand the understanding of the influence of abiotic environmental factors, such as temperature, pH, and soil minerals, on the *B. subtilis* IMV B-7023 growth and phytase activity. They indicate the strain *B. subtilis* IMV B-7023's potential ability to effectively assimilate phytates in neutral and alkaline soils, in particular due to the interaction of these bacteria with nanosized bentonite and silicon dioxide.

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ВПЛИВ АБІОТИЧНИХ ФАКТОРІВ НА ФІТАЗНУ АКТИВНІСТЬ *BACILLUS SUBTILIS* IMB B-7023

Бактерії роду *Bacillus* можуть синтезувати специфічні ферменти-фітази. Така властивість особливо важлива для ґрунтових бактерій, оскільки дозволяє їм мінералізувати фітин та фітати, таким чином забезпечуючи доступним фосфором як самих себе, так і рослини, в кореневій зоні яких вони мешкають. Нашими попередніми дослідженнями було показано, що бактерії *Bacillus subtilis* IMB B-7023, які є компонентом комплексного бактеріального препарату для рослинництва Азогран, мають фітазну активність та можуть використовувати фітат в якості джерела живлення. Відомо, що на активність бактеріальних фітаз можуть впливати абіотичні фактори навколишнього середовища. Зокрема, за різних pH та температури фітазна активність значно змінюється. Тверді частки ґрунту, серед яких є нанорозмірні мінерали, також можуть впливати на ферментативну активність бактерій. Вплив абіотичних факторів на фітазну активність *B. subtilis* IMB B-7023 був раніше не досліджений, тому це стало **метою** даної роботи. **Методи.** Фітазну активність бактерій визначали за кількістю фосфату, що вивільнявся з фітату натрію в ході ферментативної реакції, а вплив наноматеріалів на ріст — культуральними методами. **Результати.** Встановлено, що фітазна активність *B. subtilis* IMB B-7023 залежала від фізико-хімічних факторів середовища, в якому проводили ферментативну реакцію. Так, найвищу фітазну активність спостерігали за температури 28 °C. З подальшим збільшенням температури фіксували різке зниження активності позаклітинних фітаз *B. subtilis* IMB B-7023. Дослідження впливу різних значень pH на фітазну активність *B. subtilis* IMB B-7023 показало, що за pH 4—6 вона не проявлялася. Однак, за pH 7 фітазна активність бацил різко зростала і вже не змінювалася з підвищенням pH буферної системи до 12 одиниць. Показано, що вплив діоксиду кремнію на ростову активність *B. subtilis* IMB B-7023 за умов культивування в середовищі з фітатом як джерелом фосфорного живлення залежав від концентрації наноматеріалу. Так, за вмісту в середовищі 0,05 та 0,5 г/л нанорозмірного діоксиду кремнію ростова активність бацил зростала на 8—18 %, а за вмісту 5,0 г/л спостерігали пригнічення росту бактерій на 19 %. Водночас, глинистий мінерал бентоніт за досліджених умов культивування не вливав на ріст *B. subtilis* IMB B-7023. Поряд з цим, діоксид кремнію і бентоніт в усіх досліджених концентраціях стимулювали фітазну активність *B. subtilis* IMB B-7023. Так, за додавання діокиду кремнію в середовище культивування фітазна активність бацил зростала в 1,82—3,34 рази, а бентоніту — в 2,54—5,83 рази. Оскільки оптимальні значення фітазної активності для більшості бактерій роду *Bacillus* знаходяться в межах нейтральних pH та температури 50—55 °C, властивість *B. subtilis* IMB B-7023 проявляти максимальну фітазну активність за лужних значень pH та нижчої температури (28 °C), а також стимулювання цієї активності ґрунтовими мінералами підвищує конкурентоспроможність цього штаму як компонента бактеріального препарату для рослинництва. **Висновки.** Абіотичні фактори навколишнього середовища впливають на фітазну активність і ріст *B. subtilis* IMB B-7023. Оптимальними значеннями фізико-хімічних факторів для фітазної активності цих бактерій є температура 28 °C та pH 7—12. 0,05; 0,5 і 5,0 г/л діоксиду кремнію і бентоніту підвищують фітазну активність *B. subtilis* IMB B-7023. Вплив цих нанорозмірних мінералів на ріст *B. subtilis* IMB B-7023 за культивування в середовищі з фітатом як єдиним джерелом фосфорного живлення залежить від їх типу та концентрації. Отримані результати свідчать про потенційну здатність штаму *B. subtilis* IMB B-7023 ефективно асимілювати фітати в нейтральних і лужних ґрунтах, особливо за взаємодії цих бактерій з наночастинками бентоніту та діоксиду кремнію, що розширює можливості його використання в агротехнологіях.

Ключові слова: *абіотичні фактори*, *Bacillus subtilis IMB* B-7023, *фітазна активність*, *ростова активність*, *бентоніт*, *діоксид кремнію*, *наночастинки*.