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THE INFLUENCE OF MICRONUTRIENTS ON THE CAPACITY OF ACCUMULATING CELL BIOMASS IN *BACILLUS MEGATERIUM* STRAIN FROM SOIL

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ABSTRACT - *Bacillus megaterium* is a microorganism from soil, located mainly in rhizosphere, with capacities of biosolubilization of insoluble micronutrients, which are unavailable for plant nutrition. The biosolubilization of iron, manganese, zinc and cooper compounds, which are insoluble forms for plant nutrition, depended on the amount of microorganisms in soil. At the end of the experiment, the accumulation capacity of cell biomass was tested for *Bacillus megaterium* strain, which was isolated from forest soil, for decreasing the high degree of soil pollution. The quantity of accumulated biomass by *Bacillus megaterium* also depended on the level of microorganism adaptation to the presence of these metals in environment. Thus, it was demonstrated that an isolated strain from the metal polluted soil, in comparison with *Bacillus megaterium* isolated from a forest soil, would accumulate a significant superior biomass quantity.

Key words: *Bacillus megaterium,* phosphogypsum, accumulation of biomass

REZUMAT – Influenţa micronutrienţilor asupra capacităţii de acumulare a biomasei de către *Bacillus megaterium* din sol. *Bacillus megaterium* este un microorganism din sol, localizat în rizosferă, având capacităţi de biosolubilizare a micronutrienţilor insolubili, care nu sunt disponibili pentru nutriţia plantelor. Biosolubilizarea compuşilor fierului, manganului, zincului şi cuprului, care sunt forme insolubile pentru nutriţia plantelor, depinde de cantitatea de microorganisme din sol. La sfârşitul experimentului, s-a testat capacitatea de acumulare a biomasei de către *Bacillus megaterium,* care a fost izolat din solul forestier, pentru a diminua gradul mare de poluare a solului. Cantitatea de biomasă acumulată de tulpina *Bacillus megaterium* a depins şi de nivelul de adaptare a microorganismului la prezenţa acestor metale în mediu. Astfel, s-a demonstrat că o tulpină, izolată din solul poluat cu metale, în comparaţie cu *Bacillus megaterium,* izolat dintr-un sol de pădure, acumulează o cantitate de biomasă mult mai mare.

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Cuvinte cheie: *Bacillus megaterium,* fosfogips, acumulare de biomasă

INTRDUCTION

Bacillus megaterium is a soil microorganism, mainly located in rhizosphere, with capacities of biosolubilization of insoluble micronutrients unavailable for plant nutrition. Its ability to biosolubilize compounds of iron, manganese, zinc and cooper, which are insoluble forms for plant nutrition, depends on the amount of microorganisms in soil. We have tested the accumulation capacity of cell biomass for two strains of *Bacillus megaterium*. The area from which *Bacillus megaterium* strain was isolated is an area polluted by phosphogypsum. Phosphogypsum is a waste produced in the process of phosphoric acid production. The basic materials for the production of phosphoric acid are phosphoric rocks. The chemical composition of phosphogypsum varies according to the origin of phosphate rock. The main component of phosphogypsum is $CaSO_4 \cdot 2H_2O$ and it is used as an additive to calcareous soil (Dominguez *et al.,* 2001), as a source of calcium and sulphate, as a special amendment agent for soybean, wheat, peas, maize and vegetables (Vanlauwe *et al.,* 2000; Nogueira and Melo, 2003; Garrido *et al.,* 2005). On the other hand, phosphogypsum has macro- and microelements like potassium (2.31-3.1 g/kg), sodium (0.34-0.43g/kg), iron (0.464-0.545 g/kg), magnesium (0.026-0.035g/kg), zinc (13.5-15.2 mg/kg), copper (10.8-83.1 mg/kg), chromium (9.55mg/kg), molybdenum (0.024mg/kg) and manganese (49-57 mg/kg) (Gorecki *et al.,* 2005).

All these microelements are useful and necessary for living organisms; they play a role as enzymatic cofactor, growth factor or are indispensable for the metabolic cycle. For *Bacillus* genus, a great part of these metals plays the role of activators of the various enzyme systems necessary for sporulation.

Many scientists show that various biological roles have been associated with copper in animal, plant, and bacterial systems and there is a need of copper in various enzymatic reactions as a cofactor or a metal ion activator. Although manganese was stimulatory, its total effect was not as distinct as in case of copper. Iron was shown to be necessary at the early growth stage. Beskid and Lundgren (1961) and Lundgren and Cooney (1962) demonstrated that iron was used by *B. cereus* during the early stage of growth and returned to environment by the end of sporulation.

The goal of this scientific paper was to establish, on the one hand, how the concurring action of three metals, Mn, Zn and Fe, influenced the biomass accumulation, and on the other hand, the differences collected by two separate strains of *Bacillus megaterium*: a blank strain and an adapted one to the presence of metals in environment.

MATERIALS AND METHODS

Isolation and identification of bacterial strains. Two strains of *Bacillus megaterium* were isolated from soil samples. One sample was taken from a forest soil (BM_R) and another sample from phosphogypsum waste dump. Samples were heated at 80ºC for 15 min to kill the non-sporforming species. Individual bacterial strains were obtained by colony reisolation on Topping media (yeast extract – 2.5g, peptone – 2.5g, agar – 20g from 1000ml) plates at 32ºC, during 24-48h, and identified as *Bacillus megaterium* strains on the basis of their cell morphology and confirmation tests – production of catalase, anaerobic growth, growth in sodium chloride, hydrolysis of starch, reduction of nitrite to nitrate, Hydrolysis of casein, growth at 50-65˚C.

Fermentation. After the two strains are isolated and confirmed, the "inoculum" is prepared. For this, a mineral liquid media is used (sucrose - 10g, K_2HPO_4 – 2.5g, KH_2PO_4 – 2.5g, $(NH_4)_2HPO_4$ - 1g, $MgSO_4$ – 0.2g, $FeSO_4$ – 0.01g, $MnSO_4$ – 0.007g, distillate water from 1000ml). The average of pH is adjusted to 7.0, by adding 1N NaOH and later, is sterilized at 121ºC, 1.2 atm. for 15 minutes.

BM_R and BM_{30} strains are cultivated in a mineral liquid media and incubated on **Shaking Orbital Incubator GFL 3033** at 30°C and 190 rpm for a 24 h period, which will later serve as inoculum. The optical density of inoculum is OD_{600}=2.2848.

In a 500 ml Erlenmeyer glass, we have poured 100 ml of liquid mineral media, 1 ml of inoculum. For variant 1, we have used 10 ppm of Mn / 85 ppm of Zn / 9 ppm of Fe, for variant 2, we have used 30 ppm of Mn / 255 ppm of Zn / 27 ppm of Fe and for variant 3, we have used 50 ppm of Mn / 425 ppm of Zinc / 45 ppm of Fe. We have prepared eight Erlenmeyer glasses, one for every test, so the biomass accumulation could be observed at periods of 6h, 18 h, 22h, 26h, 30h, 42h and 54 h.

Analyses. At each of the above periods, the Erlenmeyer glass is removed from the orbital shaker, a sample is taken in a 10 ml inoculum and put in a centrifugal blender for about 15 minutes, at 6000 rpm, using a Rotofix 32 A equipment. The supernatant is removed, weighing the quantity of biomass formed at the preset period in a 100 ml of mineral liquid medium. The weighing process was made with a 0.001g precision scale.

RESULTS AND DISCUSSION

Isolation and identification of bacterial strains. Recently sampled soil was immediately bolted through a 4 mm bolter, and 10 g sample was put in 100 ml distilled water and then to a mechanical stirring machine, for 40 minutes.

The resulted soil suspension was later taken through an ultrasound machine for about 30 minutes (28 kHz/400W), leaving absorbed mineral or organic particles to detach themselves.

Samples were heated at 80ºC for 15 min to kill the non-sporforming species. From this soil suspension, a dilution process was made (10^{-1}, 10^{-2}, 10^{-3}, $10^{-4}$and 10^{-5}). The Topping environment was then poured through Petri plates and left to solidify for a while. The inoculation of plates was made through flooding with 1 ml of each soil sample. For a better result, it

is recommended to repeat the process three times.

Based on the specific features and with reference to Buchanan and Gibbon (1974), Bergey's manual of determinative bacteriology, it was concluded that strain belonged to *B. megaterium* (*Figure 1*).

Confirmation test of *Bacillus megaterium* strain. For an exact confirmation of a well-made *Bacillus megaterium* strain isolation, in addition to the microscopic examination, tests of selectivity were made, according to *Endospore-forming Gram-Positive Rods and Cocci*, Bergey's Manual of Systematic Bacteriology, with the results shown in *Table 1*.

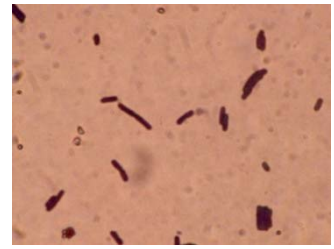

Figure 1 - Micrograph of Bacilli (stained with violet crystal), 100x objective (oil)

Table 1 - Confirmation test of *Bacillus megaterium* strain

Test	Repeatability	Results
Production of catalase	10	positive
Hydrolysis of starch	10	positive
Hydrolysis of casein	10	positive
Anaerobic growth	10	negative
Growth in sodium chloride	10	positive
Growth at 50-65˚C	10	negative
Reduction of nitrate to nitrite	10	positive

Obtaining positive results of above 90 % of soil samples on which were done the production of catalase, hydrolysis of starch, hydrolysis of casein, growth in sodium chloride and reduction of nitrate to nitrite proves indubitably that the isolated strain was *Bacillus megaterium.* The negative results, meaning the development at 50-65 ˚C and growing in an anaerobe medium, back up the results, confirmed by the literature, that *Bacillus megaterium* grows at 30-37˚C and it is an aerobe microorganism.

Fermentation. Testing both strains was made to establish the degree of biomass accumulation in the presence of metals (Mn, Zn, Fe), as well as the quantity differences between the blank strain and the strain adapted to these metals from the medium where it was isolated.

The variants taken under study were Variant 1: 10 ppm Mn / 85 ppm Zn / 9ppm Fe, Variant 2: 30 ppm Mn/ 255 ppm Zn/ 27 ppm Fe; Variant 3: 50 ppm Mn/ 425 ppm Zn/ 45 ppm Fe; the supplement of metals was increased for each variant with 30 %. For each strain, a blank sample was

made from 100 ml mineral liquid medium and 1 ml inoculum of *Bacillus megaterium.* In Table 2, there are shown the quantities of biomass accumulated for BM_R strain at different ages (*Table 2*).

In variant 2 and 3, an obvious flatting of the microorganism growth curve was reported, which explains an inhibition of the microorganism development at an increased supplement of metals in the growing medium. Between the medium of blank – without metal supplements – and the variant 1 there was found a 61% decreasing biomass quantity (*Figure 2*).

Table 2 - Dynamics of the biomass accumulation in BM_R strain

Age (h)	[g] biomass / 100 ml mineral liquid medium			
	Blank	Variant 1	Variant 2	Variant 3
6	2.672	0.8735	0.6726	0.5765
18	4.154	1.5349	1.0703	0.9835
22	3.8806	1.3268	0.9312	0.9175
30	3.4233	1.5748	0.882	0.8382
36	2.5973	0.9765	0.8068	0.7641
42	1.9124	0.7705	0.7054	0.6824
54	1.5792	0.7281	0.6834	0.5473
Average	1.8884	1.1121	0.8217	0.7585

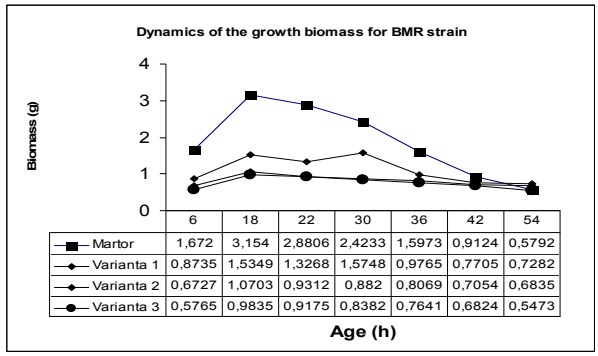

Figure 2 - Dynamics of the biomass accumulation in BM_R strain

In *Figure 3* and *Table 3*, an increased accumulated biomass reported to blank sample was observed. In variant 2, the best biomass accumulation was about 20%. Furthermore, three phases could be revealed from the increasing curves: exponential phase (log), stationary phase and death phase. In variant 3 – the one with the greatest metal supplement – a decreased biomass accumulation reported to the blank sample could be observed, the decrease being only of 15 %.

Table 3 – Dynamics of the biomass accumulation in BM30 strain

Age (h)	[g] biomass / 100 ml mineral liquid medium			
	Blank	Variant 1	Variant 2	Variant 3
6	0.87482	2.32125	2.90331	2.31482
18	3.57125	3.33296	3.84954	2.92115
22	3.50548	3.41036	3.90416	2.82073
30	3.38086	3.98794	3.75386	2.33226
36	3.38534	3.51887	3.77348	2.22748
42	2.29063	3.49893	2.94645	1.94733
54	3.04505	3.47374	2.74749	1.18774
Average	2.8647	3.3777	3.4111	2.2502

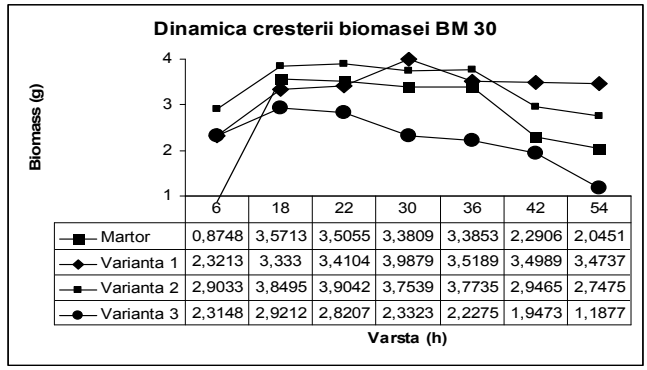

Figure 3 - **Dynamics of the biomass accumulation in BM_{30} strain**

Comparing the biomass quantities accumulated in the same metal supplement variants and at the same ages for BM_R strain – isolated in a non-polluted soil and BM_{30} – strain - adapted to the presence of metal growing environment, a biomass accumulation capacity could be detected that was significantly increased (Figure 3). If in the case of blank samples, the quantities of accumulated biomass were insignificantly equal, in the variant 1 and 2, they were notably different. Therefore, for variant 1, BM_{30} strain accumulated a quantity of biomass three times higher than BM_R strain, and for the variant 2, BM_R strain accumulated a quantity of biomass 4.2 times higher than BM_R strain. In variant 3, although the quantity of BM_{30} accumulated biomass was three times higher than BM_R, the effective quantity reported to the blank was 20 % lower.

CONCLUSIONS

This study shows that the presence of metals like Fe, Zn and Mn in the environment of *Bacillus megaterium* microorganism determines an increased biomass quantity.

The quantity of accumulated biomass in *Bacillus megaterium* also depends of the level of microorganism adaptation to the presence of these metals in its natural environment. Thus, it has been demonstrated that an isolated strain from the metal polluted soil – therefore adapted to these metals – in comparison with *Bacillus megaterium* isolated from a forest soil, would accumulate a significant superior biomass quantity. Based on these conclusions and considering the literature, these metals play an important role in the metabolic growth cycle of the microorganism.

From an enzymatic point of view, the presence in a higher quantity of these metals determines an increased enzymatic equipment activity. This results in increasing the accumulation of biomass quantity.

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