MICROBIOLOGICAL STUDY OF A *BACILLUS MEGATERIUM* STRAIN WITH SOIL PHOSPHORUS SOLUBILIZATION POTENTIAL

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The isolation of bacterial strains exhibiting high ability to solubilize soil phosphorus is a matter of great interest with practical applicability. This paper focused on the isolation, selection, characterization and identification of *Bacillus megaterium* strains from different natural environments to the purpose of solubilizing the phosphorus from the soil. The research investigations conducted resulted in the isolation of 15 bacterial strains as pure cultures from 5 samples of cultivated soil; upon the examination of the micromorphological characteristics, the strains were assigned to the *Bacillus* (13 strains) and *Staphylococcus* (2 strains) genera. The researches on their morphological, growth-related characters and the physiological and biochemical properties allowed the taxonomic classification of the R_4-UAIC strain into the *Bacillus* genus, *megaterium* species, in which the capacity to solubilize soil phosphorus was observed.

Key words: Bacillus megaterium, macromorphology, micromorphology, phosphorus.

INTRODUCTION

Phosphorus is an essential element in the life of plants. It has specific functions, is found in the composition of cell structures and acts as an energy conveyer. The total content of phosphorus in the soil cannot change very easily and its conversion from not easily accessible forms into readily accessible ones is tightly connected to the microbial activity in the soil, chemical reactions occurring in the soil, soil type, as well as soil heat and water concentration (Caramete, 1974).

Microorganisms have an important role in the phosphorus conversion processes in the soil. Phosphorus can be found in the chemical structure of microorganisms as phosphates. It plays a significant part in the energy metabolism (in the phosphorylation reactions of high energy compounds formation, etc.), the respiratory and fermentative processes. In addition, it has a plastic function, being involved in the synthesis of nucleoproteins, lipids and sugars (Madigan *et al.*, 2000).

Ever since 1956, based on their phosphorus nutrition, microorganisms have been classified as follows: bacteria assimilating to the same extent mineral and organic phosphorus; bacteria assimilating particularly mineral phosphorus; bacteria assimilating particularly organic phosphorus; bacteria solubilizing phosphorus in the form of glycerophosphate; bacteria solubilizing tricalcium phosphorus. This bacteria grouping is characteristic for every type of soil (Zarnea, 1983).

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Phosphorus is used differently by the various microorganisms due to their participation in the processes of solubilization, mineralization and fixation of phosphates in the soil. There is evidence that the chemical nature of most of the organic phosphorus in the soil is of microbial origin (Seshardi, 2000).

Most microorganisms possess an enzymatic system which enables them to mineralize phosphorus-containing organic compounds. For instance, the phosphatase activity of the fungus *Aspergillus niger* has been known for a long time now. Up to 50 % of the microorganisms isolated from the soil and rhizosphere exhibit phytase activity (Greaves *et al.*, 1963, cit. Eliade, 1975). Atlas (1998) considers that bacteria have both phytase and nuclease and, as a result, are able to mineralize not only glycerophosphate but also lecithin. In 1964, Mazkin and Kuznețova (cit. Eliade, 1975) divide soil microorganisms according to their enzymatic mechanism as follows: microorganisms with low phosphatase activity (*e.g.* mycobacteria, micrococci, *Bacillus mycoides*); microorganisms with high phosphatase activity (*e.g. Pseudomonadaceae* family representatives); microorganisms with high phosphatase and ribonuclease activities (*e.g. Bacillus megaterium*, which possesses a glucose-induced adaptive ribonuclease); microorganisms with high phosphatase, ribonuclease and desoxyribonuclease activities (*e.g. Bacillus subtilis, Bacillus cereus*). Nucleotides are readily metabolized in the soil by microorganisms which at the same time use them as carbon, nitrogen, and phosphorus source. The DNA is more resistant to the microbial attack than the RNA, while phytin is more difficult to dephosphorylate than lecithin. In 1956, Picci (cit. Eliade, 1975) detected the ability to solubilize phosphates in fungi belonging to the *Aspergillus* and *Penicillium* genera. Phosphate reduction to phosphites, hypophosphites, phosphides, a process occurring in the soil in exceptional cases of anaerobiosis, is caused by microorganisms as well.

Microorganisms also solubilize sparingly soluble phosphates by decreasing the pH of the surrounding environment or acting on the calcium, iron, aluminum, and magnesium salts. In rice plantations, for example, a large amount of organic acids is generated, this increasing phosphorus availability to rice (Nimițan, 1997).

Bacillus megaterium var. *phosphaticum* was used to create a bio-preparation called *Phosphobacterin* with the purpose of enhancing mineral phosphorus solubilization. If phosphorus is present in the complex structures of the soil and, at the same time, readily decomposable carbon sources, such as manure, are incorporated in the soil, phosphorus solubilization can be increased due to biological activity stimulation. This organic carbon increase may aid to complexing the soil aluminum in acids, thus reducing the aluminum phosphate (Sylvia *et al.*, 1999).

Taking into account that the isolation of bacterial strains exhibiting high potential of soil phosphorus solubilization has been little studied in Romania, we believe it would be useful to approach this subject of great interest and practical applicability.

The objectives of this scientific paper were the isolation of bacterial strains from different soil samples, micromorphological investigation of the bacterial strains isolated, examination of the macro- and micromorphological characters, identification of the physiological and biochemical properties, taxonomic classification of the R_4-UAIC strain, and detection of soil phosphorus solubilization by the *Bacillus megaterium* R_4-UAIC strain.

MATERIAL AND METHOD

Sample collection. The successful performance of the experiments depends to a great extent on the technique used for collecting samples (Angle, 1994). The various strains of the *Bacillus* genus were isolated from many samples of soil planted with corn, sun flower and wheat. The plots belong to the Agricultural Research & Development Station from Podul Iloaiei and the Didactic Station of the "Ion Ionescu de la Brad" University of Agronomy and Veterinary Medicine Iaşi – the Ezăreni Farm, Iaşi County. Upon collection, the sampling area was delimited and soil samples of equal amount were collected at random, from different points of the area. The samples were collected from a 5 to 10 cm depth, after the superficial soil layer consisting mainly of vegetable residues had been removed. The samples were transferred to sterile paper bags labeled with the sampling date and place, type of soil, and an identification number (Norrell, 1997).

Sample preparation. The 5 soil samples (designated as I to V) were subjected to a thermal pre-treatment (stored for 10 minutes at 80 °C) to isolate the strains belonging to the *Bacillus* genus, since the spores of the bacteria included in this genus can survive a temperatures exceeding 70 °C, while the vegetative forms of most bacteria known are destroyed at such temperature level (Vinter, 1987). The pre-treatment was carried out in a drying oven.

Soil dilution suspensions preparation. Serial 10-fold dilutions (*i.e.* 10^{-1}, 10^{-2}, and so on) of the samples collected were made with sterile water under routine aseptic conditions. Using this technique, a series of dilutions in which the number of bacterial cells decreased in geometric progression was obtained. To prepare these dilutions, the sterile water was distributed in sterile 300 ml iodine flasks, 45 ml per flask. 5 g of sample were weighed in sterile conditions and transferred to the first dilution flask. Following mechanical vigorous stirring for 20 minutes, the first suspension was obtained (the 1:10 dilution). 5 ml of this suspension were transferred using a sterile pipette to a second flask. Following a 15 minute stirring, a new suspension was obtained (the 1:100 dilution). The other dilutions were obtained in a similar manner.

Inoculation on a solid medium. A *special growth medium* containing molasses and corn extract was used. The medium was distributed in Petri dishes and was inoculated using the last three dilutions (*i.e.* 10^{-4}, 10^{-5}, and 10^{-6}). Two

plates were used for each dilution. The inoculated dishes were incubated at 30 °C for 36–48 hours. At the end of the incubation process, the colonies became visible on the surface of the growth medium.

Isolation of bacterial colonies. The colonies were picked with a sterile loop and transferred in slants containing medium with the same composition as the primary medium used for isolation. The slants inoculated by using the streak plating technique were incubated at 30 °C for 48 hours. After incubation, the cultures were examined microscopically, tested for purity and the bacterial cell morphology was analyzed.

Identification of the bacterial strains isolated

In order to identify the bacterial strains isolated, their morphological characteristics, physiological and biochemical properties were examined (Dunca *et al.*, 2004).

The **morphological characterization** involved the analysis of both the macromorphological and micromorphological aspects.

In order to describe the ***macromorphological characters*** of the isolated R_4-UAIC strain, this was inoculated on the growth medium used for isolation and distributed in Petri dishes (using the streak method of inoculation). The following parameters of the grown colonies were analyzed: shape, margins, elevation, consistency, etc.

The ***micromorphological characterization*** of the isolated strains was carried out using microscope slides stained according to Gram's method and examined with a Novex microscope with a magnifying power of 1000 ×.

The Schaeffer-Fulton staining of the bacterial spore is a method used to differentiate the endospores from vegetative cells. Upon microscopic examination of the R_4-UAIC strain, the vegetative cells are red colored while the spores are green.

The ***analysis of the physiological and biochemical properties*** of the bacterial strain isolated (*i.e.* R_4-UAIC) was focused on the following: consumption of carbon sources; casein, starch, and urea hydrolysis; Voges-Proskauer test; citrate uptake; indole formation; nitrate reduction to nitrites; phenylalanine deamination; catalase generation; aerobic growth; resistance to lysozyme; growth at 7 % concentration of NaCl; growth at different temperatures (Sneath *et al.*, 1986).

Detection of soil phosphorus solubilization by the *Bacillus megaterium* R_4-UAIC strain. It is known that phosphorus, an essential element for plant growth, is present in large quantities in the soil, but in inassimilable forms, and its conversion to available compounds occurs as a result of microorganisms' action.

In order to detect the capability of the bacteria to transform the phosphorus organic compounds into forms that can be assimilated by plants Pikovskaya R.E., 1948 (cit. Sylvia, 1999) proposed the use of a special growth medium. After the medium is sterilized, 15 ml of medium are distributed in Petri dishes and let to solidify. The dishes are then inoculated with the bacterial culture under examination by spreading on the surface of the medium to obtain colonies. The dishes are

incubated in a thermostat at 30 °C for 24–36 hours. If the bacterium has the capacity to solubilize the phosphorus in the medium, the medium around the colonies containing $Ca_3(PO4)_2$ clarifies.

RESULTS AND DISCUSSIONS

The ***bacterial strains were isolated*** from samples of soil collected from the Agricultural Research & Development Station in Podul Iloaiei and the Didactic Station of "Ion Ionescu de la Brad" University of Agronomy and Veterinary Medicine Iaşi – the Ezăreni Farm, Iaşi County in June 2004. Various soils cultivated with different crops were used in the study, as follows:

I – free soil cultivated with corn;

II – soil cultivated with wheat;

III – soil cultivated with sun flower, not treated with Extrasol;

IV – soil cultivated with sun flower, treated with Extrasol;

V – rhizosphere soil – corn.

The samples were prepared in the Microbiology Laboratory of the Faculty of Biology Iaşi.

Due to the presence of a large number of microorganisms in the soil, to facilitate the isolation of the strains of *Bacillus* genus, the soil samples to be examined were subject to a thermal pre-treatment. The pre-treatment involved the storage of the previously weighed soil samples at 80 °C for 10 minutes. The method is based on the capacity of *Bacillus* spores to survive at high temperatures while vegetative forms are destroyed (only some thermophile forms can resist). In general, the microorganisms taken from their natural environment and inoculated on nutritive media in laboratory are not considered pure cultures, even if the selective media and the thermal treatments are combined so as to allow the growth of certain species. To overcome these inconveniences, dilutions were prepared from the samples to allow the growth of isolated colonies which were then picked in pure cultures. A number of 15 bacterial strains were isolated in pure cultures from the soil samples examined (*i.e.* I–V).

The ***micromorphological study of the bacterial strains isolated*** consisted in the examination of glass slides stained according to Gram's method using an optical microscope with an immersion lens. The microscopic examination showed that of the 15 bacterial strains isolated the rods forms prevailed. Most of these forms have rounded or truncated ends and parallel margins. Rods are generally sporulated, only few bacteria being non-sporulated. The spores are oval or round shaped, are central or subterminal, and undistorted. The manner in which the rods are grouped on the slides also differs; however, those isolated or grouped in short or long chains prevail. In terms of color, most of the isolated strains are Gram positive, with few exceptions of Gram negative ones (Table 1).

Table 1

Micromorphological examination of the bacterial strains isolated

Crt. No.	Bacterial strain code	Morphological type	Morphological characteristics	Sporulation	Capsule	Color affinity
1.	P_1-UAIC	Rod	Rounded ends, irregular arrangement	–	–	Gram positive
2.	P_2-UAIC	Rod	Rounded ends, irregular arrangement	+	–	Gram positive
3.	Gr_1-UAIC	Rod	Rounded ends, long chain	+	–	Gram positive
4.	Fl_1-UAIC	Rod	Pointed ends, isolated	–	–	Gram negative
5.	Fl_2-UAIC	Rod	–	Completely sporulated	–	Gram positive
6.	Fle_1-UAIC	Rod	–	Completely sporulated	–	Gram positive
7.	R_1-UAIC	Rod	Rounded ends, isolated	+	–	Gram positive
8.	R_2-UAIC	Rod	Rounded ends, isolated	+	–	Gram positive
9.	R_3-UAIC	Rod	Rounded ends, isolated	+	+	Gram positive
10.	R_4-UAIC	Rod	Truncated ends, arranged in long chains	+	+	Gram positive
11.	R_5-UAIC	Rod	Rounded ends, isolated	+	–	Gram positive
12.	R_6-UAIC	Rod	Rounded ends, isolated	+	–	Gram positive
13.	R_7-UAIC	Rod	Rounded ends, isolated	+	–	Gram positive
14.	R_8-UAIC	Coccus	Cluster	–	–	Gram positive
15.	R_9-UAIC	Coccus	Cluster	–	–	Gram positive

Legend: + : present ; – : absent.

Of the 15 bacterial strains isolated and tested in terms of micromorphological characteristics, 13 belong to *Bacillus* genus and 2 to *Staphylococcus* genus.

The microscopic examination of the 15 bacterial strains showed that the R_4-UAIC strain meets the morphological description of the *Bacillus megaterium* bacterium which we aimed to isolate.

In order to identify the species, according to *Bergey's Manual of Systematic Bacteriology*, vol. 2, 1986, the following were determined:

- growth characteristics;
- morphological characters;
- physiological and biochemical properties.

The ***growth characteristics*** of the bacterial strain R_4-UAIC were identified by growing the bacteria on a solid medium (isolation medium), and examining the type, consistency, color, etc. of the colonies using a binocular lens.

After 30 hours of growing on the solid medium at 30 °C, colonies exhibiting characteristic features appeared; the colonies were 1 to 3 mm in diameter, white, glossy, round, with regular margins (Fig. 1).

The ***morphological appearance*** of the bacterial strain R_4-UAIC was examined on fresh cultures (24–36 hours) grown on the isolation medium. From the

morphological point of view, the bacteria have the shape of a straight 1.3 × 3.5 μm rod with parallel margins and truncated ends, arranged in short chains of 2–3 rods or long chains of 3–4 rods (Fig. 2). It can synthesize a capsule containing polypeptides and polysaccharides. In terms of color staining, it is a Gram positive bacterium. It is a sporulated bacterium with round to oblong, intact, central or subterminal spores (Fig. 3 a, b).

The results of the ***physiological and biochemical properties*** examination are indicated in Table 2.

Table 2

Morphological, physiological and biochemical characteristics of the *Bacillus megaterium* R_4-UAIC strain

CHARACTERISTICS	*Bacillus megaterium* R_4-UAIC
Cell diameter > 1 μm	+
Round spores	+
Catalase	+
Anaerobe growth	–
Voges-Proskauer test	–
Acid formation from:	
D-glucose	+
L-arabinose	+
D-xylose	d
Saccharose	d
D-mannitol	d
Gas formation from glucose:	–
Hydrolysis:	
Casein	+
Gelatin	+
Starch	+
Urea	+
Citrate uptake	+
Phenylalanine deamination	d
Nitrate reduction to nitrite	d
Indole (benzopyrole) formation	–
Growth in NaCl 7%	d
Growth at:	
5 °C	d
10 °C	+
30 °C	+
40 °C	d
50 °C	–
55 °C	–
65 °C	–
Resistance to lysozyme	–

Legend: + : 90 % positive strains;
– : 90 % negative strains;
d : between 11 and 89 % of the strains are positive.

The biochemical and physiological characterization was performed by testing the following: uptake of sugars as carbon sources, casein, gelatin, starch, and urea hydrolysis, citrate uptake, indole and acetyl-methyl-carbinole formation, phenylalanine deamination, nitrate reduction to nitrite, catalase generation, anaerobic growth, resistance to lysozyme, growth in a NaCl 7 % medium, growth at various temperatures (5 °C, 10 °C, 30 °C, 40 °C, 50 °C, 55 °C, and 65 °C).

In order to characterize the R_4-UAIC strain in terms of the uptake of sugars as carbon sources, the followings were used: the monosaccharides D-xylose, L-arabinose, D-glucose; the disaccharide saccharose; from alcohols, the mannitol. Two aspects were investigated, namely acid and gas generation. The analysis and investigation of the results showed that the R_4-UAIC strain assimilated glucose, arabinose, xylose, saccharose and mannitol, ferments glucose, hydrolyses casein, gelatin, starch and urea, uses citrate, does not generate indole, reduces nitrates to nitrites, deaminates phenylalanine, grows in a medium containing 7 % of NaCl, grows at 5, 10, 30, 40 °C, but not at 50, 55, 65 °C, does not exhibit resistance to lysozyme, is anaerobe and catalase-positive.

As a result of the examination of the morphological characters, growth characteristics, and physiological and biochemical properties according to *Bergey's Manual of Systematic Bacteriology*, vol. 2, 1986, the R_4-UAIC strain was taxonomically classified in the *Bacillus* genus, *megaterium* species.

To determine the ***capacity of the Bacillus megaterium R_4-UAIC strain to solubilize soil phosphorus***, the test proposed by Pikovskaya (the presence or absence of a clarified medium around the colonies) was used. Clear areas were noticed around the colonies, which show that the *Bacillus megaterium* R_4-UAIC strain isolated from the rhizosphere soil has the capacity to solubilize the soil phosphorus due to the presence of phosphatase in its enzymatic system (Fig. 4).

CONCLUSIONS

The overall analysis of the research studies performed and their results lead to the following conclusions:

1. A number of 15 bacterial strains were isolated (in pure cultures) from 5 samples of cultivated soil.
2. Strain growth and maintenance were carried out by using only the medium from which they were isolated.
3. The micromorphological examination of the 15 bacterial strains isolated showed that 13 belong to the *Bacillus* genus and 2 to the *Staphylococcus* genus.
4. Based on the examination of the morphological characters, growth characteristics and physiological and biochemical properties, the R_4-UAIC

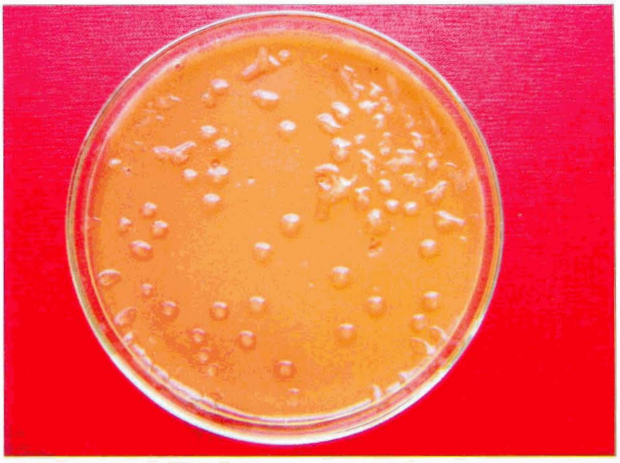


Fig. 1. *Bacillus megaterium* R_4-UAIC – appearance of colonies.

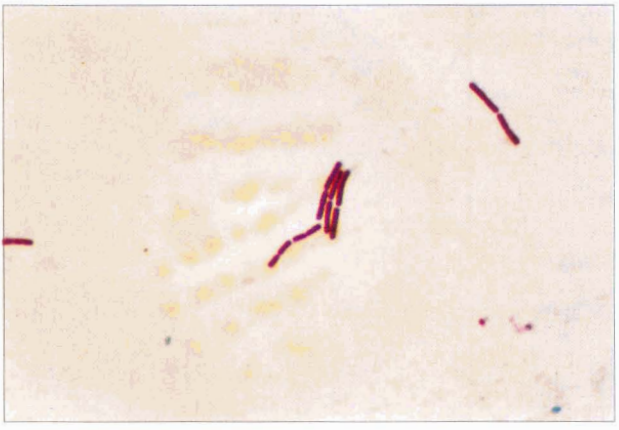
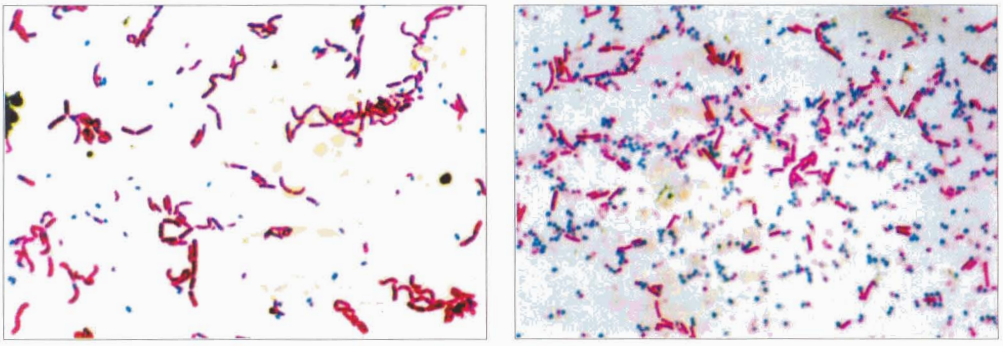


Fig. 2. *Bacillus megaterium* R_4-UAIC – microscopical appearance (× 1000).



a b

Fig. 3 a, b. Spores of *Bacillus megaterium* R_4-UAIC (Schaeffer-Fulton staining).

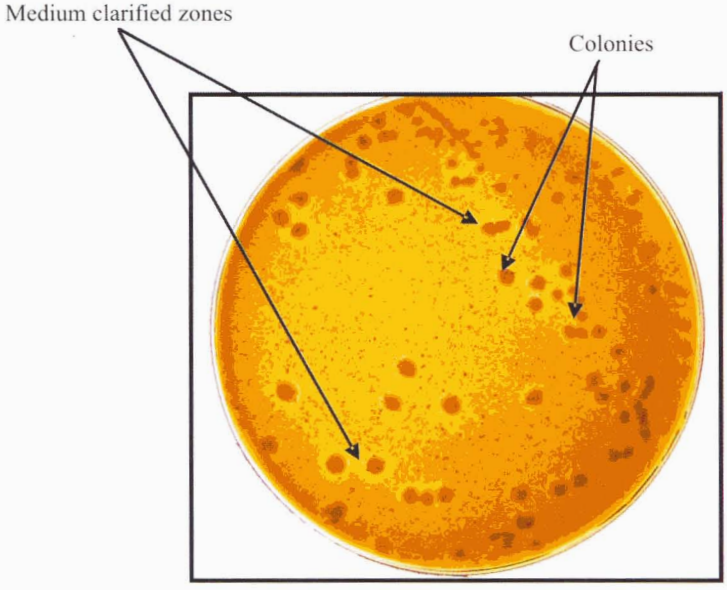


Fig. 4. Determination of the capacity to solubilize inorganic phosphorus in the *Bacillus megaterium* R_4-UAIC strain.

strain was taxonomically classified in the *Bacillus* genus, *megaterium* species.

5. For the submerged growing of the *Bacillus megaterium* R_4-UAIC strain we propose two medium compositions with a view to obtaining industrial bacterial cultures.
6. The *Bacillus megaterium* R_4-UAIC strain has the capacity to solubilize soil phosphorus.

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