Phenotypic and Genetic Variability Among Three *Bacillus Megatherium* Isolates. I. *In Viro* Evoluation of Tri-Calcium Phosphate Solubilizing Potential and Growth Pattern

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Abstract: Three *B. megatherium* isolates namely BM_1, BM_2 and BM_3 were isolated from different soil types. These isolates were evaluated phosphate solubilizing ability *in vitro*. Aleksandrov's medium (AM) supplemented with tri-calcium phosphate at concentration (0.5%). These isolates formed three morphological colony types. Circular colonies < 1 mm with entire margin and dry; flatter colonies > 1 mm irregular entire edge ith gum and convex colonies < 2 mm with entire margin and wet. The isolates have a variable degrees in release of soluble phosphate amount in culture media supplemental with insoluble phosphate. The BM_1 isolate was most powerful P-solubilizer followed by BM_2 isolates detected on AM culture medium. It was observed decreasing in pH due to an increase of total acidity and amount of soluble P in their culture media. The isolates were differed in exopolysaccharides (EPS) production. However, the capacity of P dissolution and viscosity of culture media depending on the quality of EPS produced by *B. megatherium* isolates. The BM_1 isolate grown in AM culture media produced the highest amount of EPS and viscosity compared with BM_2 and BM_3 isolates. Higher amounts of organic acid oxalic citric, tartaric and furmaric were produced by BM_2 and BM_3 in AM culture media accompanied with lowest amount of EPS. Current data showed that, inoculation AM media with 3 isolates of *B. megatherium* having a variable degrees of metabolic effectiveness led to partial degradation of P, resulting in release of higher amounts of soluble P in the culture media. [Journal of American Science 2010;6(8):111-115]. (ISSN: 1545-1003).

Key words: *B. megatherium* isolates, P rock mineral dissolution mechanism ESP, organic acid

1. Introduction

Phosphate is major essential macronutrient required for plant growth to optimize yield. Thus, the use of alternative indigenous resources and minerals such as rock phosphate are growing importance to elleviate the dependence of imported or closely commercial fertilizers (Badr et al., 2006). The use of plant growth promoting rhizobacteria (PGPR) including phosphate solubilizing bacteria as biofertilizers was suggested as a sustainable solution in improve plant nutrient and production (Vessey 2003). Increasing the bioavailability of P in soils with inoculation of PGPR or/and rock material, which may lead to increasing P uptake and plant growth (Sahin et al, 2004; Girgis, 2006 and Eweda et al., 2007).

Bacillus genus is ubiquitous and common soil microorganisms that play an important role in silicate biodegradation during the process of rock disintegration (Liu et al., 2006). The results of such activity involve both geochemical and structural changes in rocks and silicate and the most, powerful phosphate solubilizers. Due to its ability to produce a range of enzymes, solubilization pounded nutrients and degrade organic wastes (Kubo et al., 1994), along with the N2-fixing ability of some strains (Berge et al., 1991). Bacillus sp. seemed to be a good candidate for biofertilizers application in agriculture.

Inoculation with bacteria, which can improve P and K availability in soils by producing organic acids and other chemicals, stimulated growth and mineral uptake of plants (Lucas Garcia et al., 2004).

A considerable number of bacterial species are able to exert a beneficial effect upon plant growth. As the potential of PGPR is realized researches on their application have increased dramatically over the last few decades. A diverse array of PGPR has been shown to enhance growth and plant productivity by different mechanisms (Tilak et al., 2005).

The present study was conducted to evaluate the solubilization potential of selected Bacillus isolates to solubilize various insoluble soil phosphate mineral in vitro.

2. Material and Methods

Microbial isolates :

Bacillus megatherium were isolated from three different soils in Egypt, i.e Sandy; Sandy loam and Clay soil. The physical, mechanical chatracters of collected soils samples were determined according

to Jackson (1973) and Black et al. (1982) in the laboratory of soil Analysis; Soil, Water and Environmental Ins. Agric. Res. Center, Giza, Egypt.

Screening of B. megatherium for their phosphate solubilizing :

Soil samples were serial diluted in dH2O and inoculated on nutrient agar medium at 30ºC for 48 hr. the bacterial growth were harvested then resuspended and rinsed several times with dH2O to remove any remaining culture medium.

Bacterial isolates were screened for their phosphate solubilizing ability on Pikovskaya medium (PVK) (Pikovskayas, 1948) containing (g/l) to glucose, 0.5 (NH4)2 SO4; 0.2 NaCl, 0.1 MgSO4 7H2O; 0.2 KCL, 0.002, MnSO4 H2O, 0.002 FeSO4 8H2O; 0.5 yeast extract and pH was adjusted to 7.0. PVK medium was supplemented with tricalcium phosphate Ca2 (PO4)2, (PVK-TCP) at concentration of 0.5%. Growth experiments were carried out in 100 ml Erlenmeyer flasks containing 25 ml of PVK + TCP medium and inoculated with 1 ml of each isolate suspension (109 cells ml-1). After 48 h. of inoculation at 30ºC with shaking at 100 rpm (The biomass of Bacillus isolates was determined by direct microscopic counting. Cultures were filtered through 0.2 µm Whatman membrane filter, pH was directly measured by pH-meter. And total acidity of culture media was determined according to Helrich (1990).

The concentration of soluble P in the digested solutions were measured using spectrophotometer at 660 nm (Olsen and Sommers, 1982).

The growth curve of each isolate was calculated as follow: Each isolate was grown on 25 ml PVK broth medium in 100 Erlenmeyer flasks and incubated under continuous shaking (150 rpm) for 48 h at 30ºC. The cells of each isolate were periodically harvested by centrifugation at 4ºC from broth culture medium and washed twice with sterile water. The cells yield was determined periodically by using spectrophotometer at 600 nm optical density (Bauch and Lamb Spectronic 21). An aliquot of cellular pellet was resuspended in 0.1 N NaCl and employed for the total protein. The protein content was assayed according to the method of Bradford (1976).

The cultures fluid were treated with 6% (V/V) H2O2 and sterilized at 121ºC for 20 min to decompose the exopolysaccharides (EPS) and release the ions adsorbed by the polysaccharides. Thereafter centrifuged at 10000 rpm for 20 min (Gancel and Novel, 1994).

The total sugars content of EPS was determined by the modified phenol sulfuric acid method (Drapron and Guibot, 1962) using glucose as a standard.

Cultures viscosity was measured with a Brookfield Viscometer model (Hbovll, USA) with spindle 4 at 50 rpm/28ºC.

Determination of organic acids : The organic acids in cultures filtrate were determined by high performance liquid chromatography (HPLC) with ODS column (200 mm, 4.6 mm, 50 mm). The operating conditions consisted of 0.1% H2PO4 as the mobile phase, detector VWD (210 nm) and a constant flow rate of 1.0 ml min, the pH was adjusted to 2 by phosphoric acid and 50 µl of organic acids extract was injected. The organic acids were quantitively determined by comparing the retention times and peak areas of chromatograms standards.

3. Results and Discussion

Data in Table (1) show the soil textures of the tested soil, sandy, sandy loam and clay soil with pH levels ranged from 6.5 to 8.5. Soil salinity showed considerable variations among tested soils. (Lab. Of soil analysis, Agric. Res. Center, Giza).

Characteritsitcsof B. megatherium isolates in vitro :

Three types of colony morphology were observed after 24 hr of growth in nutrient agar, circular colonies <1 mm with entire margin and dry another flatter colonies > 1 mm, irregular edge with gum. Third type convex colonies < 2 mm with entire margin and wet. Three isolates namely BM1, BM2 and BM3.

Similar data was observed by Groudeva and Groudeva (1987), Girgis et al. (2008) and Amer (2008) whom found the varient Bradyrhizobium haponicum and Bacillus spp. Isolates produced punctiform and translucent colonies and markedly distinct from the colony morphology and the other varient showed abundant growth and acid production.

Data present in Table (2) show the differences in pH of media supplemented with insoluble tri-calcium phosphate inoculated with three isolates. For instance the level of pH 4.25, 3.91 and 5.75 in culture media supplemented with tri-calcium phosphate of BM1, BM2 and BM3 isolates respectively. Compared with medium uninoculated medium pH 7.0. The total acidity percentage were increasing due to decrease pH. This effect was observed in culture media supplemented with tri-calcium phosphate of BM2 isolate indicating that, solubilization was higher than BM1 and BM3 isolates that solubilization was higher than BM1 and BM3 isolates. The total acidity percentage recorded 4.75; 5.25 and 3.45 in culture media inoculated with BM1, BM2 and BM3 isolates respectively compared with control 0.30%, The amount of insoluble phosphate were variation among three isolates whereas BM2 isolate gave the higher amount 525.75 followed by BM1, 270.50 and BM3 145.25 mg1-1 compared with uninoculated medium 75.25.

Table (1): Physico-chemical analysis of soils testing.

Soil Typed	EC	Mechanical analysis				Chemical analysis									
		Sand %	Clay %	Silt %	CaCO5	Organic carbon	Total nitrogen	C/N	SL	SU	Ca	Mg	Na	K	PH
Sandy	0.95	42.75	22.12	14.25	3.33	1.9	0.19	5.72	7.0	1.5	2.5	1.2	8.2	0.75	8.21
Sandy loam	1.25	32.85	18.25	5.41	25.15	1.9	0.16	2.01	25.0	9.4	21.7	1.4	70.25	1.5	7.90
Clay soil	0.92	57.25	51.31	2.50	10.27	0.9	0.17	3.25	9.0	2.5	4.3	2.0	9.10	0.9	8.4

The cell densities of B. megatherium isolates were markedly increased in culture medium of BM2 (2.75 x 108); followed by BM3 (1.45 x 108) and BM1 (0.95 x 108) cell ml-1. The high density of biomass was obtained with BM2 isolate. This increase might to attributed the utilization of phosphate by the organism which is followed by effective metabolic activity on the substrate. However, it can be deduced that exopolysaccharide (EPS) may play an important role in the degradation of the tri-calcium phosphate mineral and these results were agreement with the finding by Welch and Ullman (1999), Liu et al. (2006) and Girgis et al. (2008).

Biomass, exopolysaccharide and organic acids: It was found that, the EPS production is common in all tested isolates but showed the variable degrees of metabolic effectiveness between isolates in EPS production, viscosity and biomass density. The variation in the amount of EPS production among 3 isolates were recorded in Table 2. Isolate BM2 grown in culture media supplemented with tri-calcium phosphate produced the highest amount of EPS 154.33 followed by BM1 (95.75) and BM1, 73.25 mg L-1 compared to control (uninoculated) 75.25 mg L-1. The superiority of BM2 isolate could be expected as they produced higher viscosity 24.4 while BM1 and BM3 produced only 12.5 and 14.7 mPa.s, respectively. Therfore, the viscosity of the culture media was related to the quantity of EPS for all tested isolates. The increase of total acidity with high production of EPS may explain that highest concentration of phosphate was detected in insoluble phosphate culture media. These results illustrate the effect of EPS in the degradation of insoluble soil minerals.

Table (2): Variation of three B. megatherium isolates grown on culture media supplemented with tri-calcium phosphate.

Bacterial isolates	PH	Phosphate insoluble (mg L-1)	Total acidity (%)	EPS (mg L–1)	Viscosity (mPa-s)	Cell density (X108 cell mL-1)
Control	7.0	75.25	0.30	75.25	-	-
BM1	4.25	270.50	4.75	73.25	12.5	0.95
BM2	3.91	525.75	5.25	154.32	24.2	2.75
BM3	5.75	145.25	3.45	95.75	14.7	1.45

Control = media uninoculated Eps = Exopolysaccharide

Growth pattern of B. megatherium isolates: The growth rates of isolates were measured by increases in protein content (µg/ml). It is revealed that isolates recorded the highest and lowest rates (Fig. 1) and hence the longest doubling times (Table 3). However, the isolates were shown the amount of protein produced ml-1 medium after 48 h of incubation. According to the growth in PVK medium as recorded in Table (3) the isolates could be classified into three patterns 1- good growth, the maximum amount of protein after 48 hr from incubation was 35.25 for BM3 isolate 2-intermediate growth 33.70 for BM2 isolate and 3-little growth 29.75 µg ml-1 for BM1 isolate.

Results presented in Table (3) and Fig. 1 show the variations in the organic acids quantity produced by isolates in the broth culture media containing tri-calcium phosphate. High copious amounts of organic acids i.e. citric, fumaric, oxalic and tartaric acid accompanied with solubilization of tri-calcium phosphate in culture medium were produced by isolates as well as some unknown organic acids with smaller concentrations were also detected. A high concentration of tartaric acid was in culture medium of PM2 (1875.25 µl-1) accompanied by high amount of EPS (154.32 mg L-1) and high amount of soluble phosphate (525.75 mg L-1). However, this could be explained that the EPS strongly absorb the organic acids under the effects of organic acids the minerals are partially degraded (Table 2).

Table (3): Production of organic acid by three B. megatherium isolates.

Bacteria isolates	Organic acid (ng/ml)				Growth characters			
	Citric	Formic	Oxalic	Tartatic	(1)	(12)	(3)	(4)
BM1	150.75	325.15	275.10	375.75	0.7	1.7	29.75	255.25
BM2	185.21	75.21	35.15	1875.25	0.8	1.45	33.70	282.75
BM3	420.21	150.75	45.15	265.12	0.8	1.65	35.25	305.15

1. Specific growth rate (µ). 2. Doubling time (day)
3. Growth (µg protein/ml). 4. Viability (µ mol INTF/ml)

Recently, Lin et al. (2006) and Girgis et al.(2008) proved that the polysaccharides strongly absorbed the organic acids and were attached to the surface of the mineral, resulting in an area of high concentration of organic acids near the mineral. They stated that the polysaccharides also adsorbed S1O2, which affected the equilibrium between the mineral and fluid phases. In general way, variations in organic acids quality and quantity were shown between the isolates grown in their culture media. The isolates grown in media containing insoluble phosphorus showed variations in the production of organic acids. Perhaps this is an indication that, the solubilizing ability may have a relationship with the type of organic acids produced by the isolates rather than the quantity of acid.

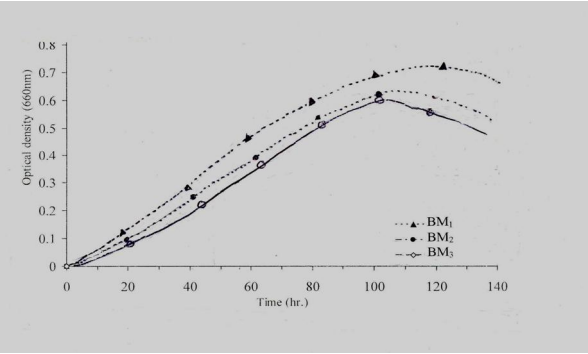

Fig.(1). Growth curves of 3 B. megatherium isolates under stirred conditions in PVK medium.

Several mechanisms have been proposed to explain the phosphate solubilization by microorganisms they are associated with the release of organic and inorganic acids (Richardson, 2001). In addition, the release of phosphatase enzyme that mineralize organic phosphate compounds has also been suggested as another mechanism involved (Marschner, 1997). Since microbial produced organic ligands include metabolic bioproducts, extracellular enzymes, chelates and both simple and complex organic acids. These substances can influence phosphate dissolution rates either by decreasing pH, forming frame work-destabilizing surface complexes or by complexing metals in solution.

The decrease in the pH values with the increase of total acidity in culture media may explain whey higher concentration of phosphate released was detected. Furthermore, linkage was observed between the final pH and the total acidity of the culture media and the amount of soluble phosphate. In this issue, Groudeva and Groudeva (1987) noted that the bacterial action on silicate and aluminosilicate is connected with the formation of mucilaginous capsules consisting of EPS as well as the production of different metabolites such as organic and amino acids. They did not exclude that the bacterial action may also be resulting of an enzymatic nature and that the bacteria are able to utilize energy released from aluminosilicate biodegradation.

4. References

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