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In Vitro Evaluation of Rock Phosphate and Potassium Solubilizing Potential of Some *Bacillus* Strains

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Abstract: The efficacy of 8 rhizobacterial *Bacillus* strains for potassium and phosphate dissolution from insoluble soil minerals were evaluated *in vitro*. Aleksandrov's medium (MA) supplemented with either mica (MA-m) or feldspar (MA-f) and Pikovskaya medium (PVK) containing rock phosphate (PVK-rp) or tri-calcium phosphate (PVK-tcp) at concentration of 0.5% were used to investigate solubilizing ability of tested strains. Final pH, total acidity, soluble K & P, exopolysaccharides (EPS) & viscosity and released organic acids were determined in their culture media. Somaclonal diversity between the most efficient strains using isozyme analysis and PCR-fingerprinting were also detected. Current data showed that inoculation with the selected strains having a variable degrees of metabolic effectiveness led to partial degradation of minerals, resulting in release of higher amounts of soluble K & P in the culture media compared to controls. Their effects were more obvious in culture media supplemented with insoluble forms of P. Strains UBFBc1, UBFBa7 and UBFBa4, UBFBm2 were the most powerful K mobilizes and P solubilizers detected on MA-f, MA-m and PVK-tcp, PVK-rp culture media, respectively. Relationships were observed between the final pH and total acidity and the amount of soluble P in their culture media and this was accompanied by a decrease in pH causing an increase of total acidity of media, while obtained results from K insoluble culture media took contrary view. However, a relationship between the capacity for P & K dissolution and viscosity of culture media depending on the quantity of EPS produced by *Bacillus* strains was also noticed. Differences in EPS production amongst the strains were recorded. Strains UBFBc1 and UBFBa7 grown in MA-f and MA-m culture media produced the highest amount of EPS and viscosities compared to other strains. Variations in organic acids quality and quantity were analyzed. Higher amounts of organic acids i.e., oxalic, fumaric, citric and tartaric, were produced by strains UBFBa4 and UBFBm2 in PVK-tcp and PVK-rp culture media accompanied by lowest amount of EPS. On the other hand, strains UBFBc1 and UBFBa7 in MA-f and MA-m culture media, respectively took contrast trend. Protein fractions, phosphatase isozymes and DNA fingerprint analysis were successfully revealed somaclonal and genetic variations among the 4 closely related *Bacillus* strains. Similarity analysis confirmed the difference in protein pattern and phosphatase confirming the genetic variation between strains.

Keywords: *Bacillus* spp., K & P rock minerals, dissolution mechanism, EPS production, organic acids, somaclonal variations.

INTRODUCTION

Phosphate and potassium are major essential macronutrients required for plant growth and development and they are commonly applied as fertilizer to optimize yield. Thus, the uses of alternative indigenous resources soil minerals such as feldspar and rock phosphate are gaining importance to alleviate the dependence of imported or costly commercial fertilizers (Badr *et al.,* 2006). The use of plant growth promoting rhizobacteria (PGPR), including phosphate solubilizing and potassium mobilizing bacteria as biofertilizers, was suggested as a sustainable solution to improve plant nutrient and production (Vessey 2003). Increasing the bioavailability of P and K in soils with inoculation of PGPR or with combined inoculation and rock materials, which may lead to increasing P uptake and plant growth, was reported by many researchers (Lin *et al.* 2002; Şahin *et al.* 2004; Girgis, 2006 and Eweda *et al*. 2007).

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Bacteria of the genus *Bacillus* are ubiquitous and common soil microorganisms that play an important role in silicate biodegradation during the process of rock disintegration (Han *et al.,* 2006 and 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 (Rodriguez and Fraga, 1999). The metabolic diversity of *Bacillus* spp. in particular together with its low reported incidence of pathogenicity, has led to the fact that many representatives of this group are being used in a wide range of applications. Due to its ability to produce a range of enzymes (Wiwat *et al*., 1999), solubilization pounded nutrients and degrade organic wastes (Kubo *et al*., 1994) along with the N_2-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).

Microbes can enhance mineral dissolution rate by producing and excreting metabolic by-products that interact with the mineral surface. Complete microbial respiration and degradation of particulate and dissolved organic carbon can elevate carbonic acid concentration at mineral surfaces, in soils and in ground water (Barker *et al.,* 1998), which can lead to an increase in the rates of mineral weathering by a proton-promoted dissolution mechanism. Therefore, dissolution of soil minerals by phosphate and silicate dissolving bacteria was monitored in pure culture experiments to determine the effect of these bacteria for releasing K and P.

Experiments revealed that species of *Bacillus* increased the soluble content of K^+ in the culture medium. Vandevivere *et al.* (1994) proposed that *B. mucilaginosus* increases the dissolution rate of silicate and alumino-silicate minerals and releases the K^+ and SiO_2 from the crystal lattice primarily by generating organic acids. However, this hypothesis is controversial and *B. mucilaginosus* is also thought to accelerate the dissolution of a variety of silicates by the production of extracellular polysaccharides (EPS) (Welch and Ulman 1999). The dispute about the mechanism by which *B. mucilaginosus* decomposes silicate minerals and releases K^+ and SiO_2 may have severely limited the use of the organism in agriculture as a form of biological K fertilizer. Recently, Liu *et al.* (2006) proved that the polysaccharides strongly adsorbed the organic acids and attached to the surface of the mineral, resulting in an area of high concentration of organic acids near the mineral. They indicated also that the EPS adsorbed SiO_2 and this affected the equilibrium between the mineral and fluid phases and led to the reaction toward $SiO_2$and K^+ solubilization.

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). Biofertilization with effective representative of the PGPR is shown to save a major part of nutrient requirements of the host plant (Dobbelaere *et al*. 2003).

To study somaclonal variations between microorganisms strains, isolates and tissue culture derived plants, traditional methods, based on morphological karyotypic analysis of metaphase chromosomes, protein fractions and isozyme analysis have been used to determine genetic variations and identify parental hybrids (Sharma, 2003; Swelim 2005 and El-Dougdoug *et al.,* 2007). Rapid and unambiguous identification of marker strains among field isolates has greatly benefited from recent advances in DNA fingerprinting methods based on the polymerase chain reaction (PCR)-Random amplified polymorphic DNA PCR (RAPD-PCR) (Sharma 2003 and Swelim, 2005). The interspersed repetitive sequences PCR (rep-PCR) (Perret and Broughton, 1998) or the fingerprinting of bacterial genomes using ribosomal genes or operons (Schmidt, 1994 and Nakatsu *et al*., 2000) are now routinely used to index prokaryotes. Many of the published oligonucleotide primers, such as those designed for the amplification of 16S rRNA bacterial genes, match most prokaryotic genomic background (Nakatsu *et al*., 2000). Moreover, since PCR amplification requires only minute amount of template DNA, cell typing can be directly performed on nodule and soil extracts-eliminating the need to cultivate the isolated bacteria (Perret and Broughton, 1998).

Therefore this work was conducted to evaluate the solubilization potential of selected *Bacillus* strains to solubilize various insoluble soil minerals such as potassium and phosphate *in vitro*. Detection of somaclonal variations in the most efficient 4 strains of *Bacillus* by isozyme analysis and PCR-fingerprinting were also studied.

MATERIALS AND METHODS

Bacterial Strains:

Eight rhizobacterial *Bacillus* strains i.e., UBFBc10, UBFBa4, UBFBc1, UBFBa7, UBFBc5, UBFBm2, UBFBc7 and UBFBc8 were kindly obtained from the cultures collection of the Biofertilizers Unit, Fac. Agric., Ain Shams Univ., Cairo, Egypt. Prior to experimental use, these strains were initially grown in nutrient broth

at 30°C for 2 days and then harvested by centrifugation at 4000 rpm for 30 min. The bacteria were then resuspended and rinsed several times with distilled water to remove any remaining culture medium, and added at concentration of 10^9 cells ml^{-1} as standard inoculum.

Soil Minerals:

Rock phosphate (RP) (P_2O_5 30.5 %, K_2O 0.31 %, SiO_2 7.9 %, CaO 41.22 %, Al_2O_3 0.41%) and feldspar (K_2O 10.1%, P_2O_5 0.10%, SiO_2 66.12 %, CaO 0.2%, Al_2O_3 17.59%) were obtained from Abo Tartor Mountain, El Kharga region, Western Desert, Egypt. Samples were ground and sieved through 2-mm sieve. The minerals powders were rinsed with distilled water to remove the fine particles.

Screening of Bacillus Strains for Their Phosphate-solubilizing and Potassium Mobilizing Ability:

Bacterial strains were screened for their phosphate-solubilizing ability on Pikovskaya medium (PVK) (Pikovskaya, 1948) containing (g/L): glucose 10, $(NH_4)_2SO_4$ 0.5, NaCl 0.2, $MgSO_4.7H_2O$ 0.1, KCl 0.2, yeast extract 0.5, $MnSO_4.H_2O$ 0.002, and $FeSO_4.7H_2O$ 0.002, and the pH was adjusted to 7.0. PVK medium was supplemented with insoluble phosphates, i.e. rock Phosphate (PVK-rp) or tri-calcium phosphate $Ca_3(PO_4)_2$ (PVK-tcp) at concentration of 0.5%. Modified Aleksandrov's medium (MA) (Zahra, 1969) contained (g/L): Sucrose 5.0, Na_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.5, $CaCO_3$ 0.1, $FeCl_3$ 10 drops of freshly prepared 1% solution, $H_2MoO_4.H_2O$ 0.002, and pH was readjusted to 7.0. The medium was supplemented with insoluble potassium, i.e. powdered mica (MA-m) or feldspar (MA-f) at final concentration of 0.5%.

Dissolution experiments were carried out in 100ml Erlenmeyer flasks containing 25ml of either PVK or modified Aleksandrov's media, and inoculated with 1ml of each strain suspension (containing 10^9 cells ml^{-1}). Control flasks without inoculation were also run, and five replicates were made for each strain. After 8 days of incubation at 30 ±2ºC with shaking at 160 rpm, the biomass of *Bacillus* strains was determined by direct microscopic counting using a haemocytometer slide. Cultures were filtered through 0.2 µm Whatman membrane filter and pH was directly measured by pH-meter. Total acidity of the culture media was determined according to the method described by Helrich (1990). The concentrations of soluble K and P in the digested solutions were measured using a spectrophotometer at 660 nm (Olsen and Sommers, 1982). The cultures fluid were further treated with 6% (v/v) H_2O_2 and sterilized at 121°C for 20 min to decompose the exopolysaccharides (EPS) and release the ions absorbed by the polysaccharides, thereafter centrifuged at 10,000 rpm for 20 min. Separation of capsular polysaccharide from the culture fluid was carried out using the EPS quantification based on the procedure described by Gancel and Novel (1994). The total sugar content of the EPS was determined by the modified phenol-sulfuric acid method (Drapron and Guilbot, 1962) using glucose as a standard. Cultures viscosity was measured with a Brookfield viscometer model (HbovII, USA) with spindle 4 at 50 rpm and 28°C.

Analysis of Organic Acids:

The 4 most effective strains representing the above-mentioned approach i.e., UBFBc1, UBFBa7, UBFBm2 and UBFBa4 were subjected for further examinations.

The organic acids in cultures filtrate fluid were analyzed by high performance liquid chromatography (HPLC) (Hewlett Packard 1050) with ODS column (200 mm 4.6 mm 50m). The operating conditions consisted of 0.1% H_3PO_4 as the mobile phase, detector VWD (210 nm) and a constant flow rate of 1.0 ml min^{-1}, the pH was adjusted to 2 by phosphatic 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 with those of standards.

Bacterial Proteins Extraction and Fingerprint:

Active 24 h liquid cultures of *Bacillus* strains, UBFBc1, UBFBa7, UBFBm2 and UBFBa4 were used to study the genetic variability depending on proteins pattern. Bacterial proteins extraction was performed as described by Wendel and Weeden (1989).

Proteins SDS-PAGE (15 % w/v) was carried out according to the method of Laemmli (1970) modified by Studier (1973). The proteins molecular weights were estimated by comparing with the marker proteins (Promega, USA) lane.

Phosphatase Isozymes (PGP) Electrophoresis:

Disc Polyacrylamide gel electrophoresis (DISC-PAGE) was performed in 10% (W/V) slab gel (Davis, 1964) and gel was stained according to Barker and Hopkinson (1978). After dark blue bands of PGP activity appearance, the zymogram or photograph was recorded instantly due to the highly ephemeral stain.

Isolation of Genomic DNA:

Small scale preparation of *Bacillus* strains genomic DNA (CsCl gradients grade) was produced as performed by Perret and Broughton (1998). DNA concentration was determined by electrophoresis of 5 µg of sample along with lambada DNA marker (Promega, USA) in 0.8 % agarose.

PCR Amplification and Targeted PCR Fingerprinting:

Amplification was performed in 10 µl react mixture containing 20 µl template DNA (25 mg), 0.5 µl Taq DNA polymerase, 3.0 dNTPs (25 mol of each dATP, dCTP, dGTP, dTTP0), 3.0 µl MgCl2 (25 mM), 3.0 µl PCR buffer (10x) and 2 µl random primer (10 pmole) (OPB02 TGATCCCTGG and OPD05 TGAGCGGACA) and 16.8 µl distilled H_2O. The mixture was assembled on ice overlaid with a drop of mineral oil. The amplification was carried out in DNA thermal cycler (M.W.G. BIOTECH Primuse) programmed as follows: one cycle at 94ºC for 30 sec; 36 ºC for 1 min and 72 ºC for 2 min (for denaturation, annealing and extension, respectively), one cycle at 72 ºC for 5 min then 40 ºC for 10 min infinitive.

All PCR products electrophoresis were carried out using a pharmacia GN-100 submarine gel electrophoresis apparatus and 1% agarose gels. Gels documentation was carried out using a charge coupled device camera imaging system with the aid of UVIsoft software to capture the image and calculate band intensities.

RESULTS AND DISCUSSION

Effect of Bacillus Strains on Dissolution of Insoluble Soil Minerals K and P:

The releases of K & P from insoluble soil minerals, i.e. mica or feldspar and tricalcium phosphate or rock phosphate respectively were used to examine *Bacillus* strains ability for mineral dissolution; the acidic effect was indicated by the pH decrease values during these experiments.

Effect of Inoculation with Bacillus Strains on pH, Acidity and Solubilization of K & P:

Generally, compared to the control, the pH of inoculated media supplemented with insoluble soil minerals K and P was decreased as shown in Table (1); this was an indication of acid production. However, there were obvious differences in the recorded pH between the tested strains. For instance, the levels of pH of strains UBFBc1 and UBFBc7 were 4.86 and 5.03 in the MA-m and MA-f culture media respectively, while strains UBFBa4 and UBFBm2 gave the least pH changes of 3.74 and 4.02 in the PVK-tcp and PVK-rp culture media, respectively. Increasing of total acidity percentage was responsible in decreasing the pH. This effect was pronounced in culture media supplemented with tri-calcium phosphate (PVK-tcp) than rock phosphate (PVK-rp), indicating that solubilization was higher in PVK-tcp medium. The total acidity percentage recorded was 6.68% in PVK-tcp medium with strain UBFBa4 followed by 5.24% in the PVK-rp one with strain UBFBm2, respectively. On the other hand, in MA-m and MA-f culture media, the lowest total acidity percentage was recorded in a range of 0.41 to 0.44 respectively.

Table 1: Final pH, soluble K & P and total acidity of 8 *Bacillus*' culture media supplemented with mica, feldspar, tri-calcium phosphate or rock phosphate and incubated for 8 days.

Strains	Insoluble potassium (K)						Insoluble phosphates (P)					
	MA-m[1]			MA-f[2]			PVK-tcp[3]			PVK-rp[4]		
	pH	K (mg l^{-1})	TA[5] %	pH	K (mg l^{-1})	TA %	pH	P (mg l^{-1})	TA %	pH	P (mg l^{-1})	TA %
Control[6]	7.0	40.00	0.11	7.0	45.00	0.30	7.0	50.00	0.25	7.0	37.50	0.22
UBFBc10	6.02	100.25	0.25	6.22	109.17	0.21	4.56	246.81	5.71	6.01	180.55	2.58
UBFBa4	6.17	107.76	0.33	5.83	177.36	0.33	3.74	694.87	6.68	4.72	210.81	5.09
UBFBc1	4.86	186.36	0.41	5.33	236.19	0.38	4.02	582.39	4.98	5.49	97.68	2.89
UBFBa7	5.03	195.26	0.35	5.21	224.68	0.44	4.04	178.76	4.29	4.37	204.69	4.57
UBFBc5	6.35	90.71	0.25	6.07	170.35	0.28	5.92	158.79	3.56	5.93	100.54	2.27
UBFBm2	6.23	86.62	0.26	6.11	186.34	0.32	5.72	139.74	3.09	4.02	225.62	5.24
UBFBc7	5.62	132.27	0.39	5.03	220.18	0.37	5.87	458.67	3.66	5.92	162.67	2.74
UBFBc8	5.62	151.81	0.32	5.04	201.27	0.39	4.49	496.77	5.88	4.97	200.63	4.45

MA-m[1]: Modified Alesandrov's medium supplemented with mica (0.5%).
MA-f[2]: Modified Alesandrov's medium supplemented with feldspar (0.5%).
PVK-tcp[3]: Pikovskaya's medium supplemented with tri-calcium phosphate (0.5%).
PVK-rp[4]: Pikovskaya's medium supplemented with rock phosphate (0.5%).
TA[5]: Total acidity (%)
Control[6]: Uninoculated utilize energy released from aluminosilicate biodegradation.

Bacillus strains showed variable degrees of metabolic effectiveness in both minerals solubilization. Their effects were more revealed in culture media supplemented with insoluble phosphate (PVK-tcp or PVK-rp). In this concern, although strains UBFBa4 and UBFBm2 have a higher capacity to release 694.9 and 225.6 mg P l^{-1} in the PVK-tcp and PVK-rp culture media respectively, they have a lower capacity to liberate K either in MA-m or MA-f culture media. On the other hand, strains UBFBc1 and UBFBa7 were capable of releasing high concentration of soluble K (i.e., 236.2 and 195.3 mg l^{-1} respectively) in MA-f and MA-m culture media. They also have capability to set free soluble P either from PVK-tcp (582.4 mg P l^{-1}) or PVK-rp (204.7 mg P l^{-1}) culture media.

The decrease in the pH values in MA-m or MA-f culture media with increase in their total acidity percentages was not the only direct reason for the release of soluble K. However, no relationships were observed between the final pH and the percentages of total acidity in their culture media and the amount of soluble K. On the other side, the decrease in pH with the increase of total acidity in PVK-tcp or PVK-rp culture media may explain why higher concentration of released P was detected. Furthermore, linkage was observed between the final pH and the total acidity of the culture media and the amount of soluble P. In this issue, Groudeva and Groudev (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

Styriakova *et al.* (2004) reported that the activity of silicate dissolving bacteria played a stimulation role in the release of Si, Fe^{3+} and K^{+} from feldspar and Fe^{+3} oxyhydroxides. The binding of silicate to the bacterial surfaces can thus be described as an outer sphere complex formation as it occurs through electrostatic interaction.

EPS Production, Viscosity and Biomass Density:

The main objective of this experiment was to determine whether the EPS production is common in all tested strains, the effect of EPS in the degradation of insoluble soil minerals, and to select the best strains for further studies. The obtained results listed in Table (2) show variable degrees of metabolic effectiveness between the strains in EPS production, viscosity, biomass density; their effects were clearer with culture media MA-f and MA-m. Variations in the amount of EPS production amongst the strains were recorded. Strains UBFBc1 and UBFBa7 grown in MA-f or MA-m culture media produced the highest amount of EPS (747.1 and 398.6 mg l^{-1} respectively) compared to other strains. On the other hand, the maximum amount of EPS recorded by strains UBFBa4 and UBFBm2 in PVK-tcp and PVK-rp culture media were 148.3 and 92.4 mg l^{-1}, respectively. The superiority of strains UBFBc1 and UBFBa7 could be expected as they produced higher viscosities (139.2 and 71.7 mPa.s, respectively) while strains UBFBa4 and UBFBm2 produced only 20.7 and 13.3 mPa.s, respectively. Therefore, the viscosity of the culture media was related with the quantity of EPS for all tested strains. The increase of total acidity with high production of EPS may explain that highest concentrations of soluble K were detected in insoluble potassium culture media.

Table 2: Exopolysaccharides, viscosity and bacterial density of 8 *Bacillus*' culture media supplemented with mica, feldspar, tri-calcium phosphate or rock phosphate and incubated for 8 days.

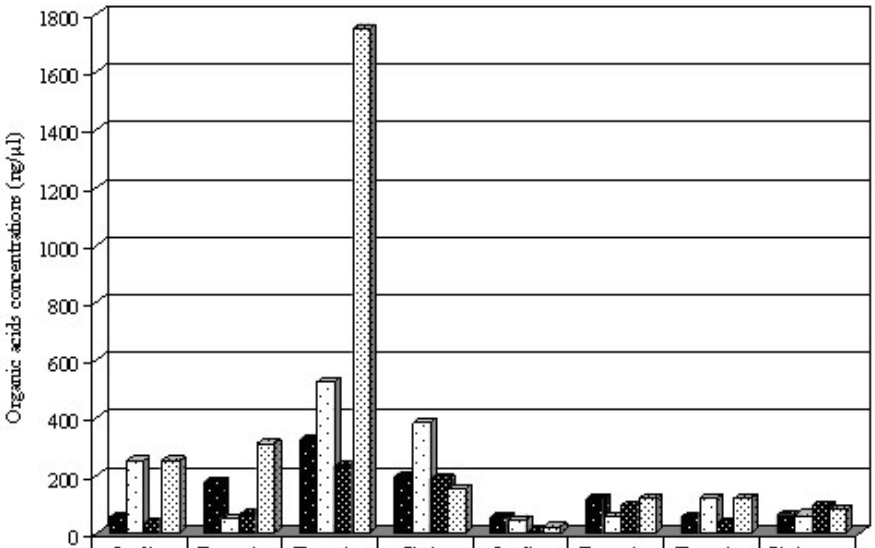
Strains	Insoluble potassium (K)						Insoluble phosphates (P)					
	MA-m[1]			MA-f[2]			PVK-tcp[3]			PVK-rp[4]		
	EPS[5] (mg l^{-1})	Viscosity (mPa.s)[6]	B.density[7] ($x10^7$ cells ml^{-1})	EPS (mg l^{-1})	Viscosity (mPa.s)	B.density ($x10^7$cells ml^{-1})	EPS (mg l^{-1})	Viscosity (mPa.s)	B.density ($x10^7$cells ml^{-1})	ESP (mg l^{-1})	Viscosity (mPa.s)	B.density ($x10^7$ cellsml^{-1})
UBFBc10	165.68	38.64	1.03	200.37	38.54	1.41	69.98	11.39	0.96	60.21	9.48	0.83
UBFBa4	96.68	29.69	1.14	438.65	67.64	2.12	148.25	20.67	2.81	63.34	9.84	2.01
UBFBc1	337.52	60.37	1.86	747.11	139.21	2.62	136.64	18.54	2.52	61.35	9.67	0.77
UBFBa7	398.63	71.69	1.89	182.37	32.96	1.89	80.36	12.92	1.99	84.47	12.27	1.56
UBFBc5	121.59	33.62	1.41	666.24	133.68	1.98	118.48	14.81	1.92	81.22	12.25	0.86
UBFBm2	231.25	40.29	1.74	430.28	73.35	1.49	76.69	11.68	1.01	92.38	13.28	2.61
UBFBc7	159.61	40.58	1.02	249.38	40.95	1.48	69.84	10.24	1.89	64.28	9.64	0.72
UBFBc8	186.95	32.36	1.56	460.89	120.97	1.98	120.24	16.62	2.32	76.68	12.04	1.23

MA-m[1]: Modified Alesandrov's medium supplemented with mica (0.5%).
MA-f[2]: Modified Alesandrov's medium supplemented with feldspar (0.5%).
PVK-tcp[3]: Pikovskaya's medium supplemented with tri-calcium phosphate (0.5%).
PVK-rp[4]: Pikovskaya's medium supplemented with rock phosphate (0.5%).
EPS[5]: Exopolysaccharides.
mPa.s[6]: dynamic viscosity is the pascal-second which is identical to 1 $kg{\cdot}m^{-1}{\cdot}s^{-1}$
B.density[7]: Bacterial density.

Densities of *Bacillus* strains UBFBa4, UBFBc1and UBFBc8 were markedly increased in PVK-tcp cultures media, being 2.81, 2.52 and 2.32 $\times 10^7$ cells ml^{-1} respectively. Inoculation of PVK-rp culture media with UBFBa4, UBFBa7 and UBFBc8 strains gave slightly less counts (2.01, 1.56 and 1.23 $\times 10^7$ cells ml^{-1} respectively). High density of biomass was obtained with strains UBFBc1, UBFBa4 and UBFBc5 (2.62, 2.12 and 1. 98 $x10^7$ cells ml^{-1}) grown in MA-f and MA-m cultures media respectively. These increases might be attributed to the utilization of K by the organism which is followed by effective metabolic activity on the substrate. However, it can be deduced that EPS may play an important role in the degradation of the feldspar mineral and these results were agreement with the finding by Welch and Ullman (1999) and Liu *et al.* (2006).

Organic Acids Production in the Culture Fluid:

Data presented in Fig. (1) show that the bacterial strains produced several organic acids i.e., oxalic, fumaric, citric and tartaric, which can specifically break down mineral structure and extract elements required for metabolism or structure purpose. Variations in the organic acids quantity produced by strains in the fluid culture media were detected; considerable amounts of acids were produced in media supplemented with PVK-tcp. High copious amounts of organic acids, i.e. oxalic, fumaric, citric and tartaric acids accompanied with solubilization of tri-calcium phosphate in culture medium were produced with strains UBFBa4 and UBFBa7. Lower amounts of organic acids were detected in extracts of insoluble K culture media and some unknown organic acids with smaller concentrations were also detected. A high concentration of tartaric acid was produced in PVK-tcp culture medium by strain UBFBa4, being 1748.14 ng μl^{-1}, accompanied by high amount of EPS (148.3 mg l^{-1}). Although, strains UBFBc1 and UBFBa7 produced highest amount of EPS (747.1 and 398.6 mg l^{-1}) and high amounts of soluble K (236.2 and 195.3 mg l^{-1}) from MA-f and MA-m culture media were released respectively, lowest amount of organic acids were detected. However, this could be explained that the EPS strongly absorb the organic acids; under the effects of organic acids the minerals are partially degraded.



	Oxalic PVK-tcp	Fumaric PVK-tcp	Tartaric PVK-tcp	Citric PVK-tcp	Oxalic MA-f	Fumaric MA-f	Tartaric MA-f	Citric MA-f
UBFBc1	56.12	179.69	324.26	198.95	57.91	118.98	55.21	59.28
UBFBa7	253.68	50.6	524.26	385.04	45.98	55.21	121.47	63.94
UBFBm2	34.76	64.42	230.68	189.48	0	95.66	33.98	94.53
UBFBa4	253.68	310.52	1748.14	155.87	23.96	120.25	121.47	82.73

Fig. 1: Organic acids produced by 4 Bacillus strains (UBFBc1, UBFBa7, UBFBm2 and UBFBa4) grown in either PVK medium supplemented with tri-calcium phosphate (PVK-tcp) (0.5%) or Modified Aleksandrov's medium supplemented with feldspar (MA-f) (0.5%) at 8 days of incubation.

Recently, Liu *et al.* (2006) proved that the polysaccharides strongly adsorbed 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 SiO_2, which affected the equilibrium between the mineral and fluid phases and led to the reaction toward SiO_2 and K solubilization. In a general way, variations in organic acids quality and quantity were shown between the strains grown in their culture media. The strains grown in media containing insoluble K and P showed variations in the producing 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 strains rather than the quantity of acid.

Several mechanisms have been proposed to explain the phosphate solubilization by these microorganisms; they are associated with the release of organic and inorganic acids (Richardson, 2001). In addition, the release of phosphatase enzymes that mineralize organic phosphate compounds has also been suggested as another mechanism involved (Marschner, 1997). Since, microbial produced organic ligands include metabolic byproducts, extracellular enzymes, chelates, and both simple and complex organic acids. These substances can influence feldspar dissolution rates either by decreasing pH, forming frame work-destabilizing surface complexes, or by complexing metals in solution (Stillings *et al.,* 1996).

The present study indicated that leaching of K from feldspar by strain UBFBc1 occurs as a result of the participation of both EPS and organic acids produced which can form bidentate complexes with metals ions and tend to be more effective in enhancing dissolution than monodentate ligands formed by acetate or propionate (Welch and Ullman, 1993).

Protein Patterns:

The obtained data listed in Table (3) and Figs. (2 and 3) clearly show that 4 *Bacillus* strains were differed in number and density of protein patterns, i.e. 4, 5, 6 and 8 for UBFBc1, UBFBa7, UBFBm2 and UBFBa4, respectively. The molecular weight of each protein subunits was determined and listed in Table (3). The most prominent similarity between the strains was characterized by a high intensity of protein bands of 12.5 kDa with strains: UBFBc1, UBFBa7 and UBFBm2, 24.2 kDa between strains UBFBc1 and UBFBa4, 48.8 kDa between UBFBa7 and UBFBm2, 30.5, 22.5 KDa between UBFBa4, UBFBa7 and UBFBm2 and 66.5 KDa between UBFBa4 and UBFBm2 strains. Similarity between 4 *Bacillus* strains protein patterns is shown in Fig. (2A). Results indicated that a week similarity between UBFBc1 and UBFBa7, UBFBm2 ($R \leq 0.20$) and UBFBa4 ($R \leq 0.15$). The high similarity was found between UBFBm2 and UBFBa4 $R \leq 0.48$ (Fig. 2B).

Table 3: SDS-PAGE analysis of protein fraction and content of 4 *Bacillus* strains.

Bacillus strains			UBFBc1			UBFBa7			UBFBm2			UBFBa4		
Marker protein														
MW[1]	%M[2]	RF[3]	MW	%M	RF	MW	%M	RF	MW	%M	RF	MW	%M	RF
97.4	1.9	0.042	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	90.5	3.6	0.067
-	-	-	-	-	-	-	-	-	83.4	4.3	0.072	-	-	-
-	-	-	-	-	-	77.5	10.7	0.085	-	-	-	-	-	-
75.2	2.6	0.105	75.2	13.0	0.105	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	66.5	4.1	0.130	66.5	2.4	0.130
58.1	3.1	0.164	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	48.8	8.9	0.318	48.8	3.2	0.318	-	-	-
39.8	1.7	0.347	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	36.2	8.7	0.366	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	32.5	2.8	0.464
-	-	-	-	-	-	30.5	8.5	0.525	30.5	3.1	0.525	30.5	4.3	0.525
29.0	2.8	0.550	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	24.2	17.6	0.635	-	-	-	-	-	-	24.2	2.3	0.635
-	-	-	-	-	-	22.5	10.8	0.690	22.5	5.1	0.690	22.5	3.4	0.690
14.3	5.8	0.724	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	13.5	2.6	0.749
-	-	-	12.5	60.7	0.940	12.5	61.1	0.940	12.5	8.02	0.940	-	-	-
12.0	82.1	0.958	-	-	-	-	-	-	-	-	-	12.0	78.6	0.958

M.W[1]: Molecular weight: KDd
%M[2]: Density of bands.
RF[3]: Relative modality.

Phosphatase Isozymes:

Results of phosphatase isozymes separation are shown in Table (4) and Figs. (4 and 5). Each strain could be characterized by unique set of isozymes. The total number of phosphatase isozymes bands was 5 isozymes for UBFBm2 and UBFBa4 while those of each UBFBa7 and UBFBc1 gave 3 and 2 bands,

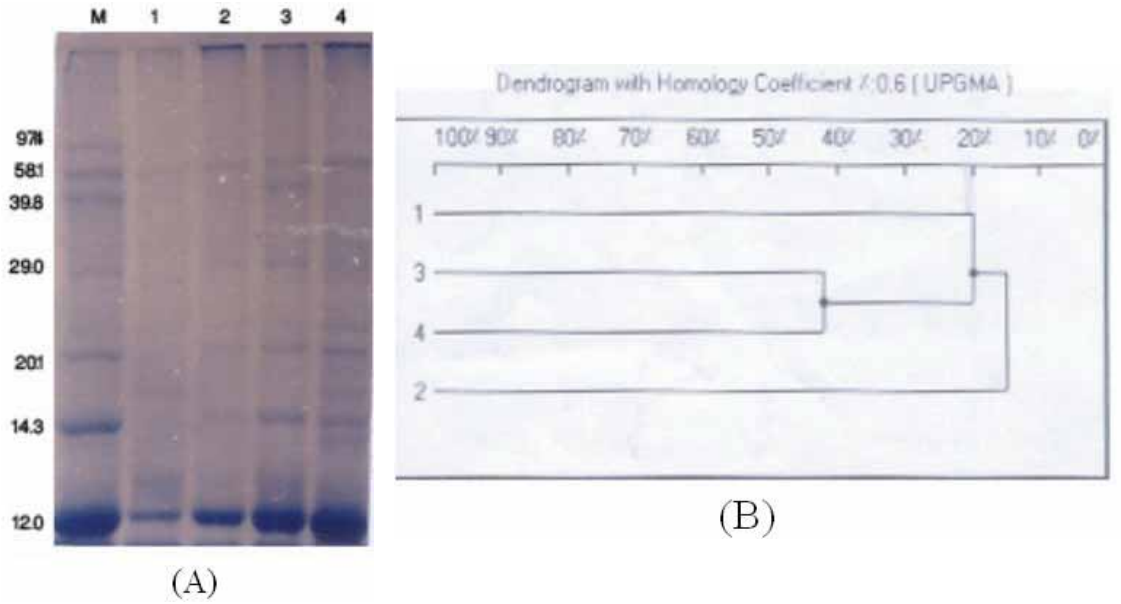


Fig. 2: (A) SDS-PAGE (15%) of protein patterns extracted from 4 *Bacillus* strains UBFBc1, UBFBa7, UBFBm2 and UBFBa4 respectively; M= Marker protein (B) Similarity analysis based on the protein content.

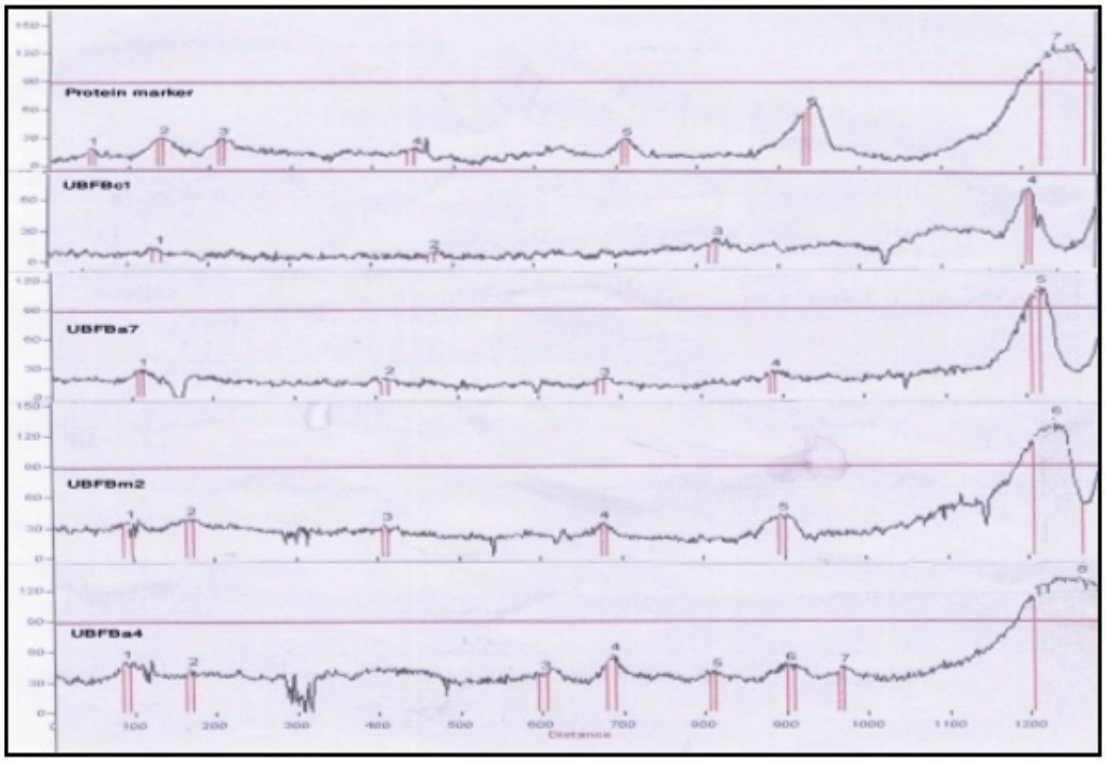


Fig. 3: Diagram illustrate protein fractions of 4 *Bacillus* strains UBFBc1, UBFBa7, UBFBm2 and UBFBa4 respectively and marker protein.

Table 4: DISC-PAGE banding patterns of phosphatase isozymes.

Bacillus strains	UBFBc1		UBFBa7		UBFBm2		UBFBa4	
No. of band	RF[1]	Fraction (%)[2]	RF	Fraction (%)	RF	Fraction (%)	RF	Fraction (%)
1	0.25	14.75	0.25	20.75	0.25	2.6	0.25	14.11
2	0.33	85.25	0.33	69.10	0.36	6.3	0.33	62.52
3			0.46	10.15	0.44	17.8	0.46	10.15
4					0.61	58.5	0.88	5.12
5					0.91	14.8	0.91	8.10

RF[1]: Relative modality.
Fraction (%)[2]: relative to the total protein contents in each *Bacillus* strains.

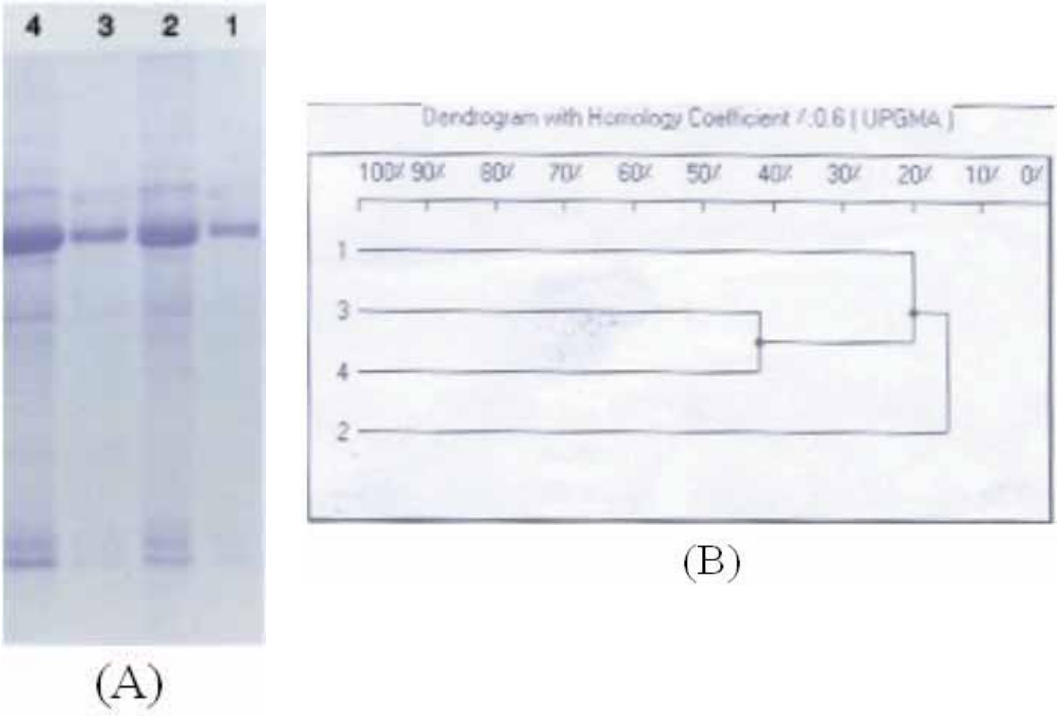


Fig. 4: (A) DISC-PAGE (15%) banding patterns of phosphatase isozymes for Bacillus strains UBFBa7, UBFBc1, UBFBm2 and UBFBa4 respectively. (B) Similarity analysis based on phosphatase isozymes.

respectively (Fig. 4A). Similarity of relationship presented in Fig. (4B) showed a low relation between UBFBa7 and UBFBc1, UBFBm2 & UBFBa4 ($R \leq 0.18$). It was $R \leq 0.33$ between UBFBc1 and both UBFBm2 & UBFBa4, while the higher similarity was found between UBFBm2 and UBFBa4 ($R \leq 0.80$). These results confirm the ability of using protein pattern and isozymes analysis as good tool for studying genetic variability between bacteria.

RAPD-PCR Fingerprints:

DNA sample preparation before RAPD-PCR amplification was found crucial for fingerprint of 4 *Bacillus* strains. The total DNA genome was extracted by using CTAB method. The yield of bacterial genomic DNA was determined spectrophotometrically as 10 µg/0.05 ml of cells suspension. The purity of DNA genome samples as indicated by A_{260}/A_{280} ratio was 1.8 and DNA quantity was evaluated by agarose gel electrophoreses (Fig. 6A).

The reproducibility of RAPD analysis is known to be highly influenced by experimental conditions. It is therefore essential to optimize the PCR conditions to obtain reproducible and interpretable results before going on routine analysis. The PCR conditions for RAPD analysis were optimized by investigating each factor individually. This included genomic DNA quality and concentration, primer annealing and extension temperature as well as denaturation time and temperature. The optimized conditions have been given in detail

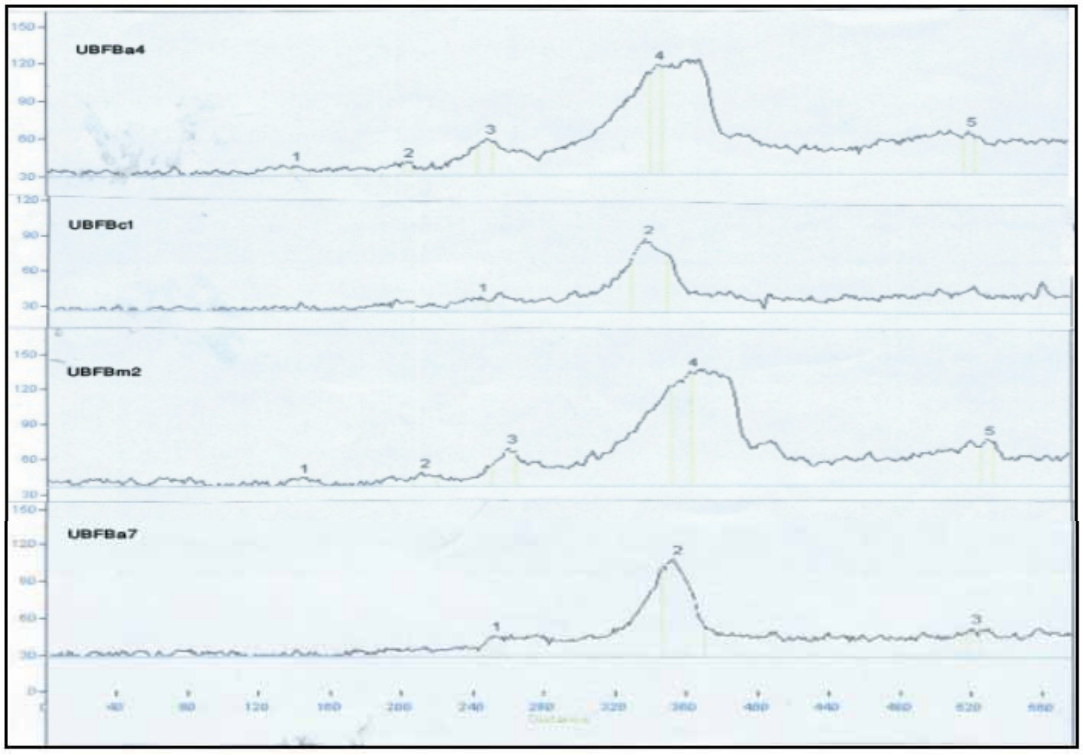


Fig. 5: Diagram illustrates phosphatase isozymes patterns volumes of 4 *Bacillus* strains UBFBc1, UBFBa7, UBFBm2 and UBFBa4 respectively.

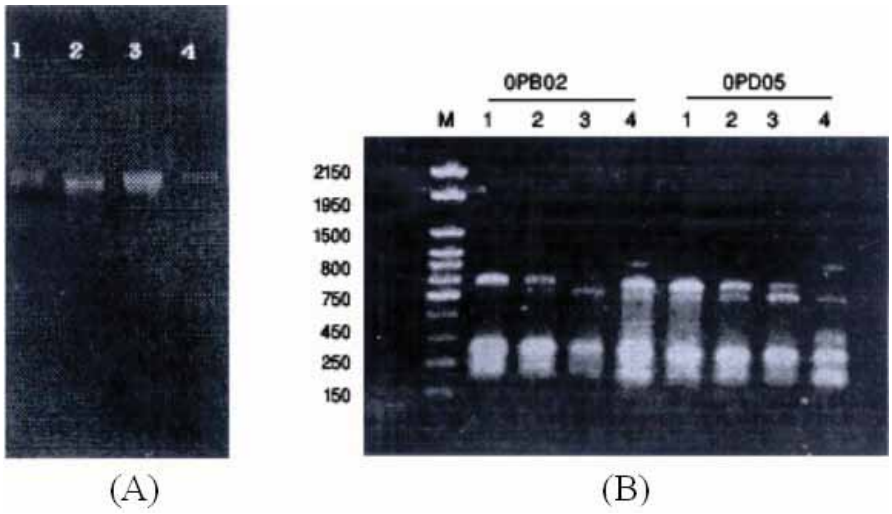


Fig. 6: Agarose gel electrophoresis (1%) showing: (A) The genomic DNA extracted from 4 *Bacillus* strains UBFBc1,UBFBa7, UBFBm2 and UBFBa4, respectively (B) RAPD-PCR products of DNA isolated from the strains using random primers (OPB02 and OPD05) and M= Marker DNA (bp) .

in materials and methods section. It was found that quality of genomic DNA extracted was a good template per PCR amplification. However, treatments of DNA with RN-ase gave sharp and clear amplification products compared with untreated DNA. This may be a result of inactivation of endogenous endonucleases.

Table 5: Comparison between 4 *Bacillus* strains genotype polymorphism (fingerprinting) by number, molecular size (bp) of amplified band using 2 random primers.

Random primer	DNA bands		Size of TAP (pb)	*Bacillus* strains							
				UBFBc1		UBFBa7		UBFBm2		UBFBa4	
	TAF	PAF		PAF	MAF	PAF	MAF	PAF	MAF	PAF	MAF
OPB2											
TGATCCCTGG	7	5	1725	-	-	-	-	2	0	4	0
			1350	1	0	7	0	-	-	0	0
			1075	-	-	0	0	0	0	0	0
			850	-	-	-	-	-	0	0	0
			800	-	-	-	-	-	-	0	0
			650	++++	0	++++	0	0	0	+++	0
			500	+++	0	0	0	0	0	0	0
OPD5											
TGAGCGGACA	7	4	1952	-	-	-	-	-	-	0	0
			1675	0	0	0	0	0	0	-	-
			800	0	0	0	0	0	0	0	0
			650	0	0	-	-	-	-	-	-
			630	0	0	-	-	-	-	0	0
			575	0	0	0	0	0	0	0	0
			450	0	0	0	0	0	0	0	0

Primers	OPB2				OPD5			
Bacillus strains	TAF	MAE	PAF	% of polymorphic	TAF	MAE	PAF	% of polymorphic
UBFBc1	3	2	1	33	6	2	4	66
UBFBa7	4	2	2	50	4	2	2	50
UBFBm2	5	2	3	60	4	2	2	50
UBFBa4	7	2	5	73	5	2	3	60

TAF: Total amplification fragments .
MAF: Monomorphic amplification fragment +
or (common amplified fragment).
PAF: Polymorphic amplification fragment ++
or (specific amplification fragment. +++
% PAF = $\frac{\text{No. of polymorphic bands}}{\text{Total amplified fragments}}$ x100 ++++

Castiglione *et al.* (1994) also reported similar observations. Decreasing of annealing temperature lower than 35ºC led to generation of very crowded RAPD patterns, while higher annealing temperature gave insufficient amplification products.

Using RAPD-PCR, polymorphism among the different strains of *Bacillus* were detected using different random primers (12 random primers were screened) RAPD analysis. Primers giving best results of amplification (expressed as average number of bands per primer). For the reproducibility of RAPD patterns, two independent experiments were performed for each primer. Out of the 10 random primers that were screened in RAPD analysis for their ability to produce sufficient amplification products, 2 random primers namely OPB02 and OPD05 were more stable and reproducible and gave sufficient polymorphism among strains. Therefore, we focused our efforts on these primers. The distribution of the polymorphe bands among the 4 *Bacillus* strains are summarized in Table (5) and Fig. (6B). The results revealed that by using the primer OPB2, 1, 2, 3 and 5 PAF (Polymorphic amplification fragment) bands as well as % polymorphic 33, 50, 60 and 73% were detected in UBFBc1, UBFBa7, UBFBm2 and UBFBa4 DNA respectively. With the primer OPD5; 4, 2, 2 and 3 PAF bands as well as % polymorphic 66, 50, 50 and 60% were detected in UBFBc1, UBFBa7, UBFBm2 and UBFBa4 DNA respectively (Table 5).

The results of the present study gave preliminary informative DNA-based markers for 4 *Bacillus* strains identification. Optimizations of physical experimental conditions of PCR amplification are also a prerequisite for the performance of RAPD analysis, this increase of reproducibility and efficiency of RAPD as a molecular marker technique. Two random primers gave reproducible and very stable peculiar, while the other primers did not always give the exact fingerprints for the strains. Accordingly, it may be suggested to use bulked DNA samples of different species to eliminate intraspecific variations.

It is concluded that distinct RAPD fingerprints among the different bacterial strains were obtained when suitable primers were used and PCR conditions were optimized. During the past four years, numerous publications demonstrated the utility of RAPD markers for the analysis of the genetic diversity among bacterial isolates, fungi and plants (Perret and Broughton, 1998; Shaker *et al*., 2000 and Swelim, 2005). In the case of DNA based markers absence, the protein fingerprint and isozyme for distinguishing detection of somaclonal variations can be used instead of DNA fingerprint (Ali and Metwally, 1992 and Shaker *et al*., 2000).

The molecular mechanism underlying somaclonal variations have been attributed to chromosome breakage, single base changes and ones in copy number of repeated sequences and alteration in DNA methylation patterns (Kaeppler and Philips, 1993 and Munthali *et al*., 1996). The polymorphism in the amplification products may be due to either from changes in the sequence of the primer binding site (e.g. point mutations) or from changes which after the size or prevent the successful amplification of the target DNA (e.g. insertation, deletions, inversions) as suggested by Rani *et al.* (1995). Genetic variations among subcultural bananas plants were also reported by Ali and Metwally (1992) and El Dougdoug *et al.* (2007) using in this context isozyme analysis. Therefore, the present investigation was undertaken to employ RAPD analysis as a simple molecular marker tool for the analysis of 4 selected *Bacillus* strains genetic variations. Genetic variability among the strains can be used to gain respect and precise information about genetic similarities and dissimilarities. Numerous of researches proved that the sensitivity of protein fingerprint, isozyme analysis or DNA-fingerprint was sufficient enough to detect genetic change in many species of bacteria and fungi (Perret and Broughton, 1998; Sharma, 2003 and Swelim, 2005).

In conclusion, use of low grade, locally available soil minerals such as mica, feldspar, tri-calcium phosphate and rock phosphate, for both neutral and alkaline soils in combination with selected efficient strains of phosphate dissolving and potassium mobilizing bacteria as biofertilizers are urgently needed to replace chemical fertilizer and reducing the cost of crop production. An application of rock P and K minerals with co-inoculation of bacteria that dissolve them might provide continuous supply of soluble P and K used for increasing soil productivity to improve sustainability of agricultural production. According to the observations during this study a relationship seems to be established between final pH, total acidity, EPS and organic acids produced depending on the type of bacterial strains and solubilizing ability. Therefore, biochemical and molecular marker can be successfully used to detect somaclonal variations among the bacterial strains. The frequency of genetic variability was detected in *Bacillus* strains dependant on variations in growth and available potassium and phosphorus *in vitro*.

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