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Full Length Research Paper

Solubilization of inorganic phosphate and production of organic acids by bacteria isolated from a Moroccan mineral phosphate deposit

Ilham Mardad[1,2]*, Aurelio Serrano[2] and Abdelaziz Soukri[1]

[1]Laboratory of Physiology and Molecular Genetics (LPGM), Department of Biology, University Hassan II, Faculty of Sciences Ain Chock, Km 8 route d'El Jadida, B.P. 5366 Mâarif. Casablanca, Morocco.
[2]Instituto de Bioquímica Vegetal y Fotosíntesis (CSIC-Universidad de Sevilla), Centro de Investigaciones Científicas Isla de la Cartuja, Américo Vespucio 49, 41092-Sevilla, Spain.

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Three efficient inorganic-phosphate solubilizing bacteria (PSB) were isolated from a phosphate rock deposit of a Moroccan mine. The phosphate solubilization index of these isolates, determined in National Botanical Research Institute's phosphate (NBRIP) medium supplemented with tribasic calcium phosphate, ranging from 2.8 to 4.4. The medium pH dropped from 7.0 to 3.5 units after growth under continuous agitation for seven days. PSB6, the most efficient PSB, closely related to *Enterobacter hormaechei* subsp. steigerwaltii strain NM23-1, permitted the recovery of the maximum soluble orthophosphate concentration in the medium (505 mg/L) after a growth period of 60 to 72 h. PSB4 and PSB5 strains were identified as *Enterobacter* sp. Strain TSSAS2-48 and *Bacterium* DR172. The capacity to solubilize inorganic phosphate by these PSB can be attributed to the secretion of organic acids, to determine their presence in the cultures supernatant, reverse-phase high performance liquid chromatography was performed. The presence of 9 identified and three unidentified organic acids was consequently demonstrated. Gluconic acid was strongly produced by all strains, extending to 55.4 mM by PSB6, 46.4 mM by PSB5, and 44.9 mM by PSB4. Besides succinic, acetic, glutamic, oxaloacetic, pyruvic, malic and fumaric acids, a newly detected and identified organic acid was the alpha-ketoglutaric acid. To the best of our knowledge this is the first report mentioning alpha-ketoglutaric acid production by PSB strains.

Key words: Biosolubilization; Inorganic-phosphate solubilizing bacteria (PSB); Enterobacter; Organic acids; Gluconic acid; α-Ketoglutaric acid.

INTRODUCTION

Phosphorus (P) is an essential element classified as a macronutrient because of the relatively large amounts required by plants. It is in fully oxidized state as phosphate, but invariably forms a large number of insoluble chemical complexes with calcium, iron and aluminium, thus generating insoluble phosphate salts present in the soil, and converted to unavailable forms with low solubility. The P-content in average soils is about 0.05% (w/w) but only 0.1% of the total P is available to plants (Zou et al., 1992). It is recognized that the availability of phosphate in soils is a major factor limiting the productivity of many ecosystems (Daniels et al., 2009). Therefore, a large quantity of soluble forms of P is applied as fertilizers to achieve maximum plant productivity. However, the soluble forms of P fertilizers are easily precipitated into insoluble forms -$CaHPO_4$, $Ca_3(PO_4)_2$, $FePO_4$ and $AlPO_4$- and are not efficiently taken up by plants, this lead to an excess application of P fer-

*Corresponding author. E-mail: mardadilham@yahoo.fr Tel: +212660768709. Fax: +212522230674.

Abbreviations: PSB, Phosphate solubilizing bacteria; **NBRIP,** National Botanical Research Institute's phosphate.

fertilizers to crop land (Omar, 1998) since most of them are rapidly converted into insoluble forms (Reddy et al., 2002) or washed away into fresh and ground waters, making a regular but unfortunately polluting application required (Shigaki et al., 2006). Therefore, the necessity to develop economical and eco-friendly technologies to solve this problem is steadily increasing (Vassilev and Vassileva, 2003).

Fortunately, various kinds of bacteria (Rodríguez and Fraga, 1999; Harris et al., 2006; Zaidi et al., 2009; Khan et al., 2010) and fungi (Whitelaw, 2000; Wakelin et al., 2007) have been isolated and characterized for their ability to solubilize unavailable phosphate (P) to available forms. Such transformations increase P availability and promote plant growth (Rodriguez and Fraga, 1999; Whitelaw, 2000; Harris et al., 2006).

Microbial biodiversity in the soil plays a significant role in the metabolism of complex molecules, supports plant nutrition and offers countless new genes, biochemical pathways, antibiotics, metabolites and other useful molecules for agronomic productivity.

Recently, phosphate solubilizing microorganisms have attracted the attention of agriculturists as soil inocula to improve the plant growth and yield (Rodriguez and Fraga, 1999; Whitelaw, 2000; Harris et al., 2006). Phosphate solubilizing bacteria (PSB) actively participate in soil P cycle, increasing the bioavailability of soil P for plants.

There are various kinds of PSB characterized that belong to different phylogenetic groups: *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aerobacter*, *Flavobacterium*, *Mesorhizobium*, *Azotobacter*, *Azospirillum* and *Erwinia* (Sperber, 1958; Goldstein, 1986; Rodriguez and Fraga, 1999).

PSB use different mechanisms to bring about the insoluble forms of the phosphate into soluble forms, but it is generally believed that the major mechanism of the mineral phosphate solubilization is the release of microbial metabolites such as organic acids (Cunningham and Kuiack 1992; Singh and Amberger, 1997; Gadd, 1999; Whitelaw, 2000; Lin et al., 2006), which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms (Kpomblekou and Tabatabai, 1994; Chen et al., 2006).

These organic acids of low molecular weight are produced by microorganisms found in the rhizosphere. The organic acid concentration in soil solution is typically low, varying from 1 to 50 mM (Strobel, 2001). The production of organic acids by the PSB has not been studied in detail, so it is necessary to generate more knowledge on this subject.

The PSB belong to the plant growth promoting rhizobacterias (PGPRs) and are capable of solubilizing inorganic phosphate from different compounds, such as dicalcium phosphate, tricalcium phosphate and rock phosphate.

Moroccan phosphate mines constitute an unexplored ecological niche likely to shelter a population of microorganisms well equipped to solubilize insoluble phosphate. The objective of this study was to isolate, identify and characterize some phosphate-solubilizing bacteria form this peculiar biotope and attempt to investigate the solubilization mechanism by analysis of organic acids produced by these PSB.

MATERIALS AND METHODS

Collection of rock phosphate samples

Soil samples were collected locally from rock phosphate deposits (rock phosphate stockpiles) of mines located at Khouribga city (Morocco) (Operated by 'Office Chérifien des Phosphates', OCP Morocco). Samples of approximately 250 g were aseptically collected from the studied areas of the phosphate layer, stored at 4°C and processed within 48 h.

Isolation of total bacteria solubilizing tribasic calcium phosphate

The soils were then suspended in sterile saline solution and were shaken for 6 h. The serially diluted soil samples were placed on standard National Botanical Research Institute's phosphate (NBRIP) agar medium (glucose 10 g, $MgCl_2$. 6 H_2O 5 g, $MgSO_4$. $7H_2O$ 0.25 g, KCl 0.2 g and $(NH_4)_2SO_4$ 0.1 g, dissolved in 1 L distilled water) supplemented by 5 g of tribasic calcium phosphate (TCP) as sole phosphorus source for selectively screening the bacteria which have the ability to release soluble inorganic phosphate from TCP (Nautiyal et al., 2000). The pH was adjusted to 7.0 ± 0.2 and uninoculated plates served as controls. After three days of incubation at 30°C, phosphate solubilizing bacteria developed clear zones around colonies. Colonies surrounded by clear zones were picked and streaked onto NBRIP plates. The plates were again incubated under the same conditions to confirm their abilities to solubilize phosphate.

Phosphate solubilization in solid medium

Abilities of the isolated bacteria to solubilize TCP were investigated. For this, the sterilized NBRIP agar medium was poured into sterilized Petri plates, containing insoluble $Ca_3(PO_4)_2$ at 5 $g.l^{-1}$ as the only source of phosphate. After solidification of the media, the plates were inoculated with the isolated bacteria, incubated at 30°C for two weeks and assayed visually. The solubilization index was determined by measuring the halo (clear zone) diameter and the colony diameter, using the following formula (Edi-Premono et al., 1996).

$$SI = \frac{\text{Colony diameter + Halozone diameter}}{\text{Colony diameter}}$$

Those bacteria which solubilize TCP on plates (clear halos surrounding colonies) were further cultivated and monitored in liquid NBRIP-BRB media. All assays were replicated three times.

Phosphate solubilization in liquid medium

In brief, the isolated bacteria presenting large halo zones on solid NBRIP media were used to measure phosphate solubilization in liquid medium. The *in vitro* phosphate solubilizing capacity of each strain was determined on NBRIP-BPB medium containing bromophenol blue as pH indicator (Mehta and Nautiyal, 2001).

The isolates were grown in NBRIP medium without agar, containing a pH indicator (Bromophenol Blue 0.025 $mg.ml^{-1}$) for seven days at 30°C with continuous agitation. Erlenmeyer flasks (500 ml) containing 200 ml of NBRIP liquid medium were inoculated with 2 ml of bacterial suspension (2×10^9 $cfu.ml^{-1}$). At several time interval points of incubation, aliquots of cultures were aseptically taken from each flask to follow the bacterial growth, the pH of the medium as well as the concentration of released soluble phosphorus.

Genomic DNA extraction, 16S rDNA sequencing

DNA extraction

Bacterial strains were cultivated in 10 ml of Luria Bertani Broth (LB) at 30°C in agitation for 18 h. 1 ml of the culture was placed in microtubes and pelleted by centrifuging at 12300 × *g* for 2 min. For total DNA extraction, the GenElute Bacterial Genomic DNA Kit (Sigma) was used following the manufacturer's instructions. The DNA extracted from each strain was visualized by electrophoresis in 1% agarose gels using Tris-EDTA-acetate (TAE) 1X buffer containing ethidium bromide. Visualization was carried out using a Shimadzu ultraviolet (UV)-visible spectrophotometer.

Amplification and sequencing of the 16S rDNA from the selected strains

The region of the 16S ribosomal gene (rRNA) of the genomic DNA extracted from each bacterial strain was amplified by polymerase chain reaction (PCR). The amplification of the approximately 1500 bp DNA fragment was performed with the highly conserved 16S rDNA universal primer pair, fD1 and rP2 (Weisburg et al., 1991), with the following sequences: fD1: 5'-AGAGTTTGATCCTGGCTCAG-3' and rP2: 5'-ACGGCTACCTTGTTACGACTT-3'. Each 70 µL PCR reaction contained about 1.5 µg of purified genomic DNA, 200 µmoL of each deoxynucleotide triphosphate (dNTP), 7 µL of 10 High Fidelity buffer with 4.2 µL of 25 mM Mg^{2+}, 250 nM of each primer, and 5 U Taq polymerase, brought to the final volume with deionized sterile water (Millipore Milli-Q, Bedford, MA) filtered through a 0.22 µm nylon filter. All PCR thermocycling was carried out using a BioRad S1000 Thermocycler (Bio-Rad Laboratory, CA) and analyzed using the ABI 3130xl genetic analyzer (Applied Biosystems, CA). Amplification of 16S rDNA using the fD1/rP2 primer set was achieved with a thermocycling program that consisted of an initial denaturation at 95°C for 3 min 30 s, followed by 35 cycles of denaturation at 95°C for 1 min and 10 s and annealing at 56°C for 50 s, extension at 72°C for 2 min and a final extension at 72°C for 6 min 10 s. The PCR products were then separated on a 1% agarose SeaKem ME agarose (FMC BioProducts, Rockland, ME) gel containing 2 µL of ethidium bromide for visualization on a UV light box.

PCR products were purified using the PCR purification kit: ExoSAP-IT (GE Healthcare Bio-Sciences Corp) according to the manufacturer's recommendations. Concentrations of purified products were determined using a NanoDrop 8000 UV spectrophotometer (Thermo Scientific NanoDrop Products, Wilmington, DE).

Sequencing was carried out using the universal and reverse oligonucleotides by automated DNA fluorescent sequencing, purification was done using the BigDye X Terminator purification kit (Applied Biosystems, CA), and products were analyzed using the ABI 3130xl genetic analyzer. The sequences were obtained from the CNRST-UATRS Unit (CNRST: Centre National Pour La Recherche Scientifique et Technique)/(UATRS : Unités d'Appui Technique à la Recherche Scientifique).

Sequence data were aligned and compared with available sequences of bacterial lineage in the National Center for Biotechnology Information GenBank (http://www.ncbi.nlm.nih.gov/) using BLAST.

Analytical methods

Cell growth was estimated by the measurement of the absorbance at 660 nm. Samples from cultures grown in medium with insoluble phosphate were previously diluted 1:1 (v/v) using 1 N HCl to dissolve the residual insoluble phosphate and measured against a blank identically treated (Rodriguez et al., 2000). Orthophosphate content in the supernatant was determined colorimetrically using the molybdate blue normalized AFNOR method (AFNOR, 1994). Values obtained with the uninoculated controls were always subtracted from their respective treatments.

Change in pH of the culture broth was recorded by a JENWAY 3505 pH meter equipped with a glass electrode. Control flasks were not inoculated. All experiments were performed in triplicates.

Organic acids analysis by high performance liquid chromatography (HPLC)

HPLC reverse-phase chromatography was used for the analysis of organic acids produced by PSB strains in broth medium. Supernatant was taken from bacterial cultures that had been centrifuged at 13000 rpm for 15 min.

Samples were filtered through 0.2 µm filter (Millipore, GTBP) and 20 µl of filtrates were injected to a HPLC Waters Lc Module I Plus equipped with a UV- 210 nm detector. The organic acid separation was carried out on an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad Laboratories, Inc.). The operating conditions consisted of 5 mM H_2SO_4 as mobile phase at a constant (isocratic) flow rate of 0.6 mL/min and the column was operated at 35°C. Retention time (RT) of each signal was recorded at a wavelength of 210 nm.

The software used for HPLC analyses was the Millennium 32, and the organic acids detected were identified by comparing their retention times and the peak areas of their chromatograms with those of standards.

RESULTS AND DISCUSSION

The elemental composition of the rock phosphate mineral originating from the Khouribga phosphate mine (RPK) was determined using scanning electron microscopy (Stereoscan 260, Cambridge, England) and consisted of O, 56.53%; Ca, 16.35%; P, 9.37%; F, 2.42%; Al, 2.03%; Mg, 1.94%; Na, 1.81%; S, 0.77%; Fe, 0.60%; Sn, 0.12%. Preliminary selection of solubilizing bacteria isolated from Khouribga phosphate deposit was based on their ability to release inorganic orthophosphate from TCP as sole phosphorus (Nautiyal et al., 2000). From the 60 bacteria

Table 1. Solubilization Index (SI) of the selected PSB strains during 14 days of incubation.

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14
PSB 4	3.0	3.2	3.6	3.54	3.72	3.66	3.83	4.0	4.0	3.92	4.04	4.14	4.14	4.0
PSB 5	2.8	3.0	3.18	3.36	3.5	3.58	3.66	3.61	3.61	3.76	3.64	3.66	3.60	3.50
PSB 6	2.8	3.2	3.4	3.54	3.5	3.66	3.83	4.0	4.08	4.16	4.33	4.23	4.38	4.4

Solublization index for each PSB based on colony diameter and clear zones formed by solubilizing suspended tricalcium phosphate. PSB, phosphate solubilizing bacteria. SI = colony diameter + halozone diameter)/colony diameter.

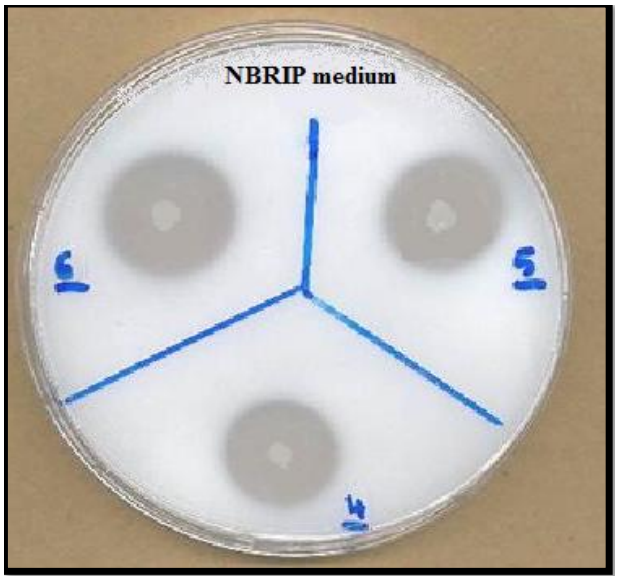

Figure 1. Solubilization halos by the three isolated bacterial strains (4, PBS4; 5, PBS5; 6, PBS6) on solid medium NBRIP after 14 days incubation (This experiment was done in three section (Y) petridish).

isolated, three bacterial strains were chosen and thus screened efficiently and solubilized TCP was selected for additional analysis. These isolates were designated as PSB4, PSB5 and PSB6.

Solubilization index

The screened bacteria were able to solubilize TCP on solid culture state by forming clear halozone, with different degree of solubilization, depending on the type of organism involved. However, all the strains maintained their phosphate solubilizing activity after being re-sown five consecutive times in NBRIP medium. Table 1 shows the strains solubilization indices assessed in NBRIP agar medium (Figure 1).

Generally, the halozone increased concomitantly with the colony diameter, but in our case some fluctuations in solubilization index were observed during the observation period. The P solubilization in solid medium suggested a two-phase process with a rapid halo formation in the first 24 h forming about 75% of the halozone and a subsequent slow expansion during the rest of the incubation period. This kinetic is due to the fact that those bacteria were in a state of phosphate deficiency and starvation which explains the rapid assimilation of TCP within the first hours of incubation.

Results show that PSB6 was the most efficient phosphate solubilizer on NBRIP plates with a solubilization index of 4.40. It is followed by strains PSB4 and PSB5 respectively, with solubilization indices of 4.14 and 3.76 (Table 1), suggesting that this magnitude of TCP solubilization is a function of the studied bacterium.

Phosphate solubilization and pH

In this study the pH in the supernatant of the bacterial incubations was observed to decrease during the release experiments while being almost stable in the control incubation. The obtained results (Figure 2) show that the pH of the culture media of the three selected bacterial strains, in the presence of TCP, gradually decreased during the first few days of incubation up to values in the range 3.5 to 4.0 that were subsequently maintained.

All PSB strains, when inoculated in NBRIP broth containing TCP as the only source of phosphate, started to grow exponentially (Figure 3). The pH of the medium started to drop immediately after the cells were inoculated and continued to decrease until the stationary phase (Figures 2 and 3). A colour change from blue to green-yellow in the NBRIP-BRB broth indicated that the bacterial pH dropped significantly up to 3.5 for PSB6, and to 3.66 and 3.85 for PSB4 and PSB5, respectively as compared to the uninoculated control, for which pH remained almost constant at 7.1±0.2 (Figure 2).

The essential part of this acidification could be attributed to the consumption of glucose from the growth media and to the production of the organic acids. Similar results were extrapolated from previous studies (Trivedi and Sa 2008; Rodriguez et al., 2004).

The reduction in the quantity of soluble phosphate after reaching the highest level of production during incubation can be explained as auto-consumption of soluble phosphate by the growing bacterial population (Rodriguez et al., 2000). Phosphorous release ability could be evaluated from the levels of this element liberated, which was either dissolved in supernatant in the form of orthophosphate or was assimilated by microorganisms to form cell biomass. The maximum soluble orthophosphate concentration in the medium released from TCP was recorded for

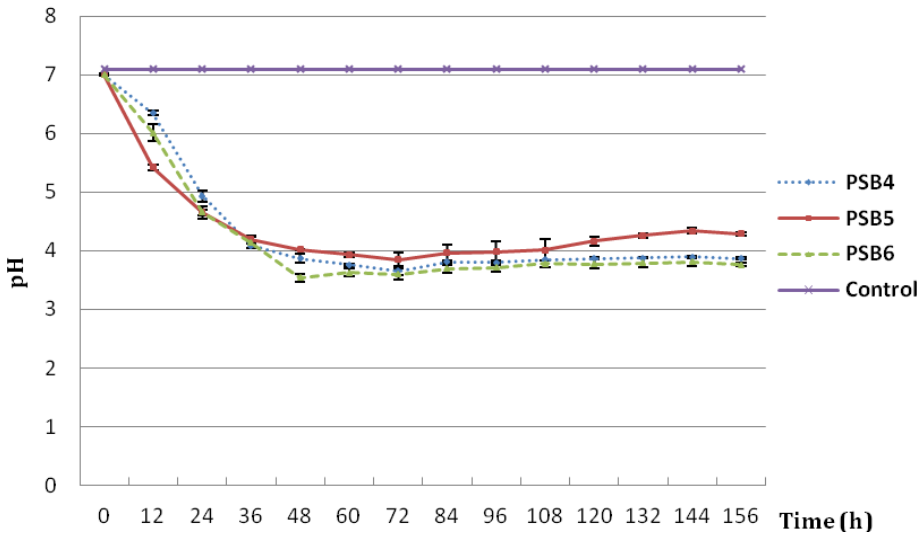

Figure 2. Change in pH of liquid NBRIP medium by the three PSB strains during seven days of incubation. Data are representative of three independent experiments. Un-inoculated medium served as a control.

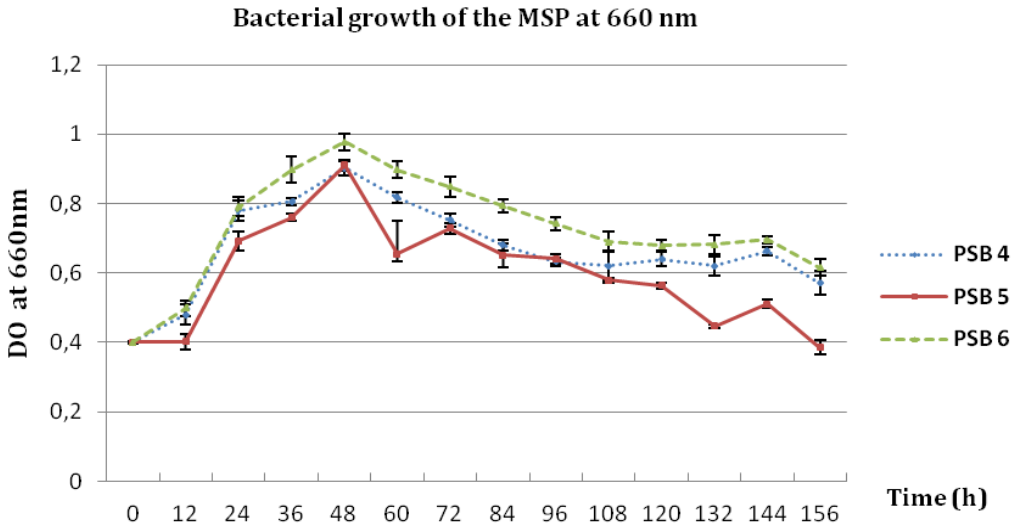

Figure 3. Growth of the three PSB in NBRIP liquid medium during seven days of incubation. Data are representative of three independent experiments.

PSB6 (505 mg/L) after a growth period of 60 to 72 h, followed by PSB4 and PSB5, respectively, 450 mg/L after the same time of incubation, and 423 mg/L after 72 to 84 h inoculation (Figure 4).

In the blank treatment, values obtained from the uninoculated control were always subtracted from their respective treatments. No significant decrease in pH was observed. Indeed, in all cases, a clear relationship could be established between bacterial growth, supernatant acidification and orthophosphate solubilization from TCP. The specific bacteria with inorganic phosphate solubilization abilities were subjected to genomic identification by 16S rRNA gene sequencing.

Taxonomic and phenotypic characterization of PSB strains isolated from Moroccan phosphate stock piles.

The nucleotidic sequence of the 16S rRNA gene of PSB6 showed 99% identity with the homologous sequence reported in GenBank for the gamma-proteobacterium *Enterobacter hormaechei* subsp. *steigerwaltii* strain NM23-1 (NCBI accession number HM218110.1). The nucleotidic sequences of PSB4 and PSB5 strains allowed them to be identified as *Enterobacter* sp. (100% identity to strain TSSAS2-48, NCBI accession number GQ284539.1) and *Bacterium* sp. (99% identity to DR

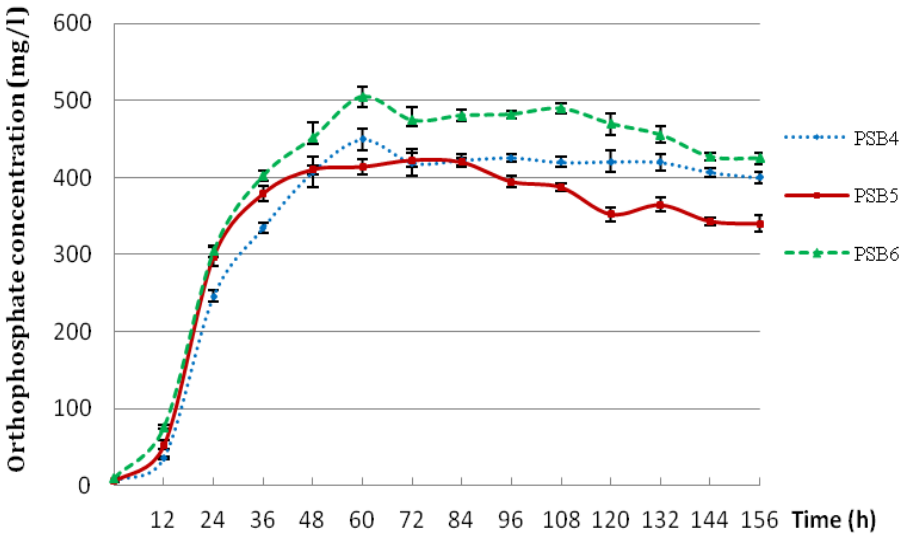

Figure 4. Solubilization of orthophosphate from insoluble TCP in broth culture by the isolated PSB strains. Data are representative of three independent experiments. All the concentration curves presented a trend of rapid increase during the first 36 h, followed by a decrease to the original level where it remains almost stable and then decreases again to different levels at the end of the incubation time.

172, NCBI accession number EU603515.1), respectively.

The three PSB strains belonged to the same family, *Enterobacteriaceae*. Isolations of bacterial strains belonging to this family have already been obtained from various soils and found to have inorganic phosphate (IP) solubilizing abilities (Vassilev et al., 1999).

Organic acid production

It has been well established that the most important mechanism used by microorganisms for solubilizing TCP is through acidification of the medium via biosynthesis and release of a wide variety of organic acids (Rodríguez and Fraga 1999).

Organic acids production by the Moroccan mines PSB strains was investigated in NBRIP broth culture. HPLC reverse phase chromatography analyses of the culture filtrates revealed the presence of several organic acids which confer the capacity to solubilize insoluble TCP to these strains (Table 2). These organic acids can either dissolve phosphate as a result of anion exchange or can chelate Ca, Fe or Al ions associated with the insoluble phosphates (Gyaneshwar et al., 2002).

Our results indicate that the most quantitatively produced organic acid was gluconic acid (GA) for all PSB strains, reaching concentrations of 55.4 mM by PSB6, 46.4 mM by PSB5 and 44.9 by PSB4 (Table 2). The highest concentration of total organic acids was monitored for PSB6 with 83.287 mM (consisting mainly of GA, 55.41 mM, acetic acid (11.92 mM) and succinic acid (9.16 mM).

Previous studies on organic acids production by microorganisms (Illmer and Schinner, 1992; Whitelaw et al., 1999) have shown that GA was not produced by the strains used in their experiments. Our findings differ from those of Illmer and Schinner (1995) who reported small quantities of GA produced by the fungus *Aspergillus niger* and Alam et al. (2002) who reported citric and oxalic acid as the most common organic acids produced by maize rhizosphere microorganisms. GA production as a result of the activity of periplasmic or cell-membrane bound NADP-dependent glucose dehydrogenase (GDH) is one of the best studied mechanisms by which phosphate solubilizing microorganism (PSM) liberate P from poorly soluble mineral phosphate (Goldstein, 1995).

Furthermore, Liu et al. (1992) suggested that solubilization is as the result of acidification of the periplasmic space due to the direct oxidation of glucose (not phospholytic oxidation) or other aldose through the action of quinoprotein glucose dehydrogenase (PQQGDH). Glucose is converted to gluconic acid which generates a transmembrane proton usable for bioenergetics and transport functions of the membrane, while the GA protons is available for solubilizing phosphates (Liu et al., 1992). Besides GA, succinic, malic and glutamic acids were also produced in high amounts by the three PSB strains. The acetic acid was produced by the two species of *Enterobacter*, but not by the unclassified bacterial strain DR172, whereas fumaric acid was secreted by *Enterobacter hormaechei* and *Bacterium* sp.

After two successive oxidations in periplasmic space, glucose became 2-ketogluconic acid or 2.5-diketogluconic (Anderson et al., 1985), but interestingly, α-ketoglutaric acid (KGA) was identified as a newly produced acid by all three PSB strains (Table 2). To our knowledge, this is the first time that this organic acid has been

Table 2. Organic acids produced by the PSB strains isolated from a phosphate mines deposit[1].

Bacterial strain	Total organic acids (mM)	Glutamic acid	α-keto glutaric acid	Gluconic acid	Pyruvic acid	Oxaloacetic acid	Malic acid	Succinic acid	Fumaric acid	Acetic acid	Unknown organic acids
PSB 4	75.056	1.964 ± 0.074	1.042 ± 0.052	44.910 ± 1.922	0.5 ± 0.036	0.460 ± 0.008	2.371 ± 0.201	6.721 ± 0.230	ND	17.086 ± 0.210	a, b, c
PSB 5	61.478	1.950 ± 0.088	1.341 ± 0.082	46.435± 1.824	0.481 ± 0.081	0.371 ± 0.068	2.513 ± 0.171	8.298 ± 0.084	0.087 ± 0.009	ND	a, b, c
PSB 6	83.287	2.181 ± 0.067	1.260 ± 0.075	55.41 ± 1.732	0.503 ± 0.076	0.356 ± 0.053	2.450 ± 0.223	9.163 ± 0.165	0.060 ± 0.004	11.92 ± 0.166	a, b, c

[1]Organic acids concentrations in culture media were determined by HPLC reverse-phase chromatography. Maximal values in each case are shown in bold. Data are means values of three independent determinations. Standard errors were less than 5% in all cases. a, b, c. Unknown organic acids eluted at the following retention times (min): 7.6±0.1, 18.7±0.1, 21.5±0.1. ND, not detected.

reported to be produced by PSB. HPLC analysis of the culture filtrate revealed two major peaks. These two peaks were identified as GA and KGA by comparing the retention time with those of authentic standards.

During the initial period of growth the concentration of KGA was lower compared to that of gluconic acid, but it continuously increased during the growth period until 10 days of incubation when the GA had totally disappeared leaving one major peak corresponding to the KGA (Figure 5).

The KGA is of particular interest due to its broad biotechnological and industrial applications, e.g., it is used for the production of an agent that protects humans or animals from oxidative stress by increasing their antioxidant capacities (Moser et al., 2007). Glutamic, oxaloacetic and pyruvic acids were also produced at lower concentrations by all three PSB strains (Table 2). Three unknown acids were detected but none of them did match with the standards used for the identification of organic acids produced by these PSB strains.

Correlation between different studied parameters

Statistically, our results exhibit a strong negative correlation ($r = -0.975$; $p<0.01$) between the culture pH and the release of soluble P which indicates that the decrease in pH brings inorganic P into the solution for those bacterial strains as reported in previous studies (Chen et al., 2006; Hwangbo et al., 2003). This consolidates the hypothesis of organic acid involvement in the solubilization of insoluble P by those isolates (Trivedi and Sa, 2008; Elizabeth et al., 2007; Chen et al., 2006; Hwangbo et al., 2003; Vazquez et al., 2000).

In addition, cell densities and levels of solubilized P were also positively correlated ($r = 0.65$; $p<0.01$) whereas a negative correlation ($p<0.01$, $r = -0.64$) between cell densities and the pH of the culture medium was observed. Also solubilization index was highly correlated with the soluble P liberated ($r = 0.821$; $p<0.01$) which comes along with the finding of Edi-Premono et al. (1996)–and Kumar and Narula (1999). However, the solubilization index does not significantly correlated with the cell density ($r = 0.258$, $p> 0.05$). This is due to the fact that some PSB show fluctuations in their behaviors during the incubation, and also because a part of the liberated orthophosphate was assimilated by PSB to grow and to form microbial biomass.

The release of soluble orthophosphate from TCP by microorganisms usually involves the production of organic acids and a decrease in culture medium pH (Carrillo et al., 2002; Illmer and Schinner 1995; Puente et al., 2004). The main mechanism of inorganic P solubilization is through a decrease in pH (Illmer and Schinner, 1992; Rodriguez and Fraga, 1999). However, another mechanism is the alteration of the solubility product by acids producing chelates with Ca^{2+} accompanying release of orthophosphate from insoluble phosphate compounds (Vazquez et al., 2000; Nautiyal et al., 2000; Puente et al., 2004).

The pH and organic acids were found to be negatively correlated with each other ($p<0.01$, $r = - 0.853$), and also highest positive correlation was noted between organic acids secreted and the orthophosphate produced ($p<0.01$, $r = 0.93$). This is due to the fact that perhaps organic acids are the main factor responsible for P solubilization, which was similar to the result given by Nahas (1996) and Whitelaw et al. (1999), indicating that acid production was the key of P solubilization mechanism in many cases.

It could be extrapolated that the massive production of organic acid leads to acidification of microbial cells and their surroundings, and then orthophosphate is released from mineral phosphate by proton substitution for Ca^{2+}. It is a fact that our strain produced GA which is a property exhibited by PSM members of the *Enterobacteriaceae* family (Buch et al., 2008; Hameeda et al., 2006; Lin et al., 2006) in the presence of glucose (precursor for synthesis of gluconic acid).

For the first time this study reports phosphate solubilizing bacterial strains isolated from extracted Moroccan Rock phosphate stockpiles which exhibit high *in vitro* capacity of inorganic phospha-

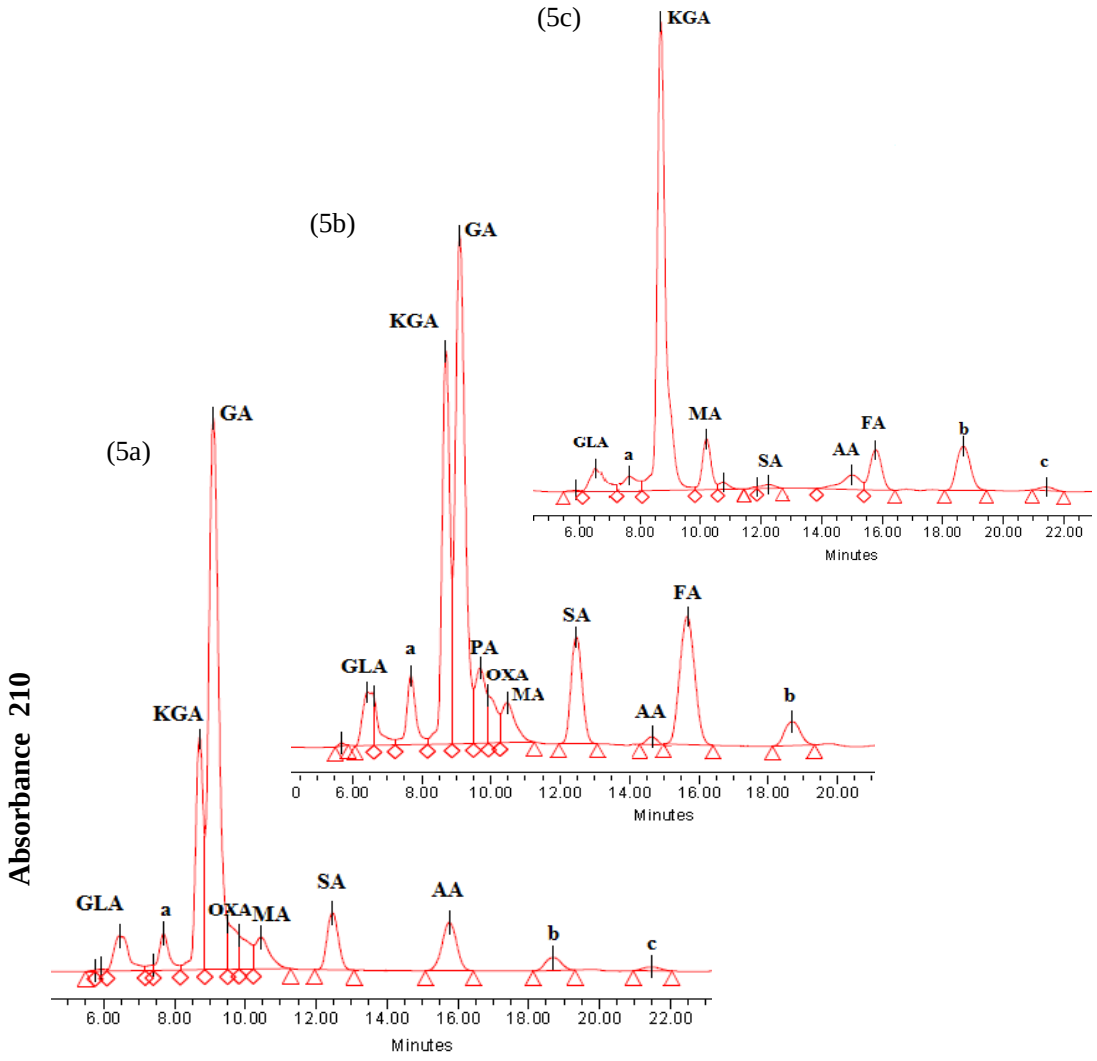

Figure 5. Identification by HPLC of organic acids produced by *Enterobacter hormaechei* (PSB6) in broth medium containing NBRIP medium with TCP as inorganic phosphate during the incubation.(5a: day 1 of incubation, 5b: day 5 of incubation and 5c: day10 of incubation) (GA gluconic acid; GLA:glutamic acid; KGA: ketoglutaric acid; PA: pyruvic acid; OXA: oxaloacetic acid; MA: malic acid; SA:succinic acid; AA: acetic acid; FA: fumaric acid; a,b and c: 3 Unknown organic acids).

te solubilization in culture medium NBRIP with TCP as the only source of P. All together our results have shown that a sharp decrease in pH was recorded concomitantly with the TCP solubilization. This suggested massive production of organic acids during the metabolism of glucose, a mechanism that is considered responsible for solubilizing insoluble orthophosphate (Hwangbo et al., 2003; Ben et al., 2009). This hypothesis was confirmed by HPLC reverse phase chromatography analyses, which revealed that the solubilization of the $Ca_3(PO_4)_2$ (as well as of rock phosphate, data not shown) by the PSBs was most likely attributed to secretion of organic acids into the culture medium.

In the current study, we have demonstrated that PSB strains isolated from the Moroccan Phosphate Stock piles are certainly capable of producing a number of organic acids and specially a high secretion of GA and other acids when growing on poorly soluble phosphate from TCP, at the same time there is a medium pH lowering and release of soluble orthophosphate.

These facts together with halozone formation on plates, indicate phosphate-solubilizing activity by *Enterobacter hormaechei* subsp. *steigerwaltii* strain NM23-1, *Bacterium DR 172* and *Enterobacter* sp. The PSB have proved to be useful for promoting plant growth (Vessey, 2003) and in developing inoculants that improve plant phosphate nutrition and allow plants to use soil stocks of phosphorus.

In fact, in addition to the beneficial effects of PSB strains on plants, inoculation and inoculants production technologies are already available, and this kind of bacteria are generally perceived as environmentally friendly.

Nucleotide sequence data reported are available in the GenBank databases under their accession numbers.

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REFERENCES

AFNOR (1994). Recueil des normes Françaises « Eau, Méthodes d'essais ». 2 édition, Afnor, Paris, France, pp 342.

Alam S, Khalil S, Ayub N, Rashid M (2002). In vitro solubilization of inorganic phosphate by phosphate solubilizing microorganism (PSM) from maize rhizosphere. Intl. J. Agric. Biol. 4: 454-458.

Anderson S, Marks CB, Lazarus R, Miller J, Stafford K., Seymour S, Light D, Rastetter W, Estell D (1985). Production of 2-keto-L-gluconate, an intermediate in L-ascorbate synthesis, by a genetically modified *Erwinia herbicola*. Science 230: 144-149.

Ben Farhat M, Farhat A, Bejar W, Kammoun R, Bouchaala K, Bejar S, Chouayekh H (2009). Characterization of the mineral phosphate solubilizing activity of *Serratia marcescens* CTM 50650 isolated from the phosphate mine of Gafsa. Arch Microbiol. 191: 815–824.

Buch A, Archana G, Naresh KG (2008). Metabolic channelling of glucose towards gluconate in phosphate solubilizing *Pseudomonas aeruginosa* P4 under phosphorus deficiency. Res. Microbiol. 159: 635–642.

Carrillo AE, Li CY, Bashan Y (2002). Increased acidification in the rhizosphere of cactus seedlings induced by *Azospirillum brasilense*. Naturwissenschaften. 89: 428–432.

Chen YP, Rekha PD, Arun AB, Shen FT, Lai WA, Young CC (2006). Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Appl. Soil. Ecol. 34: 33–41.

Cunningham JE, Kuiack C (1992). Production of citric and oxalic acids and solubilization of calcium phosphate by *Penicillium bilaii.* Appl. Envir. Microbiol. 58: 1451–58.

Daniels C, Michan C, Ramos JL (2009). New molecular tools for enhancing methane production, explaining thermodynamically limited lifestyles and other important biotechnological issues. Microb. Biotechnol. 2: 533–536.

Edi–Premono M, Moawad MA, Vleck. PLG (1996). Effect of phosphate solubilizing Pseudmonas putida on the growth of maize and its survival in the rhizosphere. Indonesian J. Crop. Sci. 11: 13–23.

Elizabeth P, Miguel S, Ball Maria M, Andrés YL (2007) Isolation and characterization of mineral phosphate-solubilizing bacteria naturally colonizing a limonitic crust in the south-eastern Venezuelan region. Soil Biol. Biochem. 39:2905–2914

Gadd G (1999). Fungal production of citric and oxalic acid: Importance of metal specification, physiology and biogeochemical processes. Adv. Microb. Physiol. 41: 47-92.

Goldstein AH (1986). Bacterial solubilization of mineral phosphates: historical perspective and future prospects. Am. J. Altern. Agric. 1: 51-57.

Goldstein AH (1995). Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by gram negative bacteria. Biol. Agric. Hortic. 12: 185-193.

Gyaneshwar P, Kumar N, Parekh LJ, Poole PS (2002). Role of soil microorganisms in improving P nutrition of plants. Plant Soil 245: 83–93.

Hameeda. B, Reddy Y, Rupela OP, Kumar GN, Reddy G (2006). Effect of carbon substrates on rock phosphate solubilization by bacteria from composts and macrofauna. Curr. Microbiol. 53: 298–302.

Harris JN, New PB, Martin PM (2006). Laboratory tests can predict beneficial effects of phosphate-solubilizing bacteria on plants. Soil. Biol. Biochem. 38: 1521-1526.

Hwangbo H, Park RD, Kim YW, Rim YS, Park KH, Kim TH, Suh JS, Kim KY (2003). 2-ketogluconic acid production and phosphate solubilization by *Enterobacter intermedium*. Curr. Microbiol. 47: 87–92.

Illmer P, Schinner F (1992). Solubilization of inorganic phosphates by microorganisms isolated from forest soils. Soil. Biol. Biochem. 24: 389–395.

Illmer P, Schinner F (1995). Solubilization of inorganic calcium phosphate - solubilization mechanisms. Soil Biol. Biochem. 27: 257–263.

Khan MS, Zaidi A, Ahemad M, Oves M, Wani PA (2010). Plant growth promotion by phosphate solubilizing fungi – current perspective. Arch Agron Soil Sci. 56(1): 73–98.

Kpomblekou K, Tabatabai MA (1994). Effect of organic acids on release of phosphorus from phosphate rocks. Soil Sci. 158: 442–453.

Kumar V, Narula N (1999). Solubilization of inorganic phosphates and growth emergence of wheat as affected by *Azotobacter chroococcum* mutants. Biol. Fert. Soils 28: 301–305.

Lin TF, Huang HI, Shen FT, Young CC (2006). The protons of gluconic acid are the major factor responsible for the dissolution of tricalcium phosphate by *Burkholderia cepacia* CC-Al74. Bioresour. Technol. 97: 957–960.

Liu TS, Lee LY, Tai CY, Hung CH, Chang YS, Wolfram JH, Rogers R, Goldstein AH (1992). Cloning of an *Erwinia herbicola* gene necessary for gluconic acid production and enhanced mineral phosphate solubilization in *Escherichia coli* HB101: nucleotide sequence and probable involvement in biosynthesis of the coenzyme pyrroloquinoline quinone. J. Bacteriol. 174: 5814-5819.

Mehta S, Nautiyal CS (2001). An efficient method for qualitative screening of phosphate-solubilizing bacteria. Curr. Microbiol. 43: 51-56.

Moser PM, Greilberger J, Maier A, Juan H, Bücherl-Harrer C, Kager E (2007). Verwendung von alpha-Ketoglutarsäure und 5-Hydroxy-methylfurfural zur Reduktion von oxidativem Stress. C. Y. L. P. GmbH. Patent EP1842536 A1.

Nahas E (1996). Factors determining rock phosphate solubilization by microoganisms isolated from soil. World J. Microbiol. Biotech. 12: 567–572.

Nautiyal CS, Bhadauria S, Kumar P, Lal H, Mondal R, Verma D (2000). Stress induced phosphate solubilization in bacteria isolated from alkaline soils. FEMS Microbiol. Lett. 182: 291–296.

Omar SA (1998). The role of rock-phosphate-solubilizing fungi and vesicular–arbuscular–mycorrhiza (VAM) in growth of wheat plants fertilized with rock phosphate. World J. Microbiol. Biotechnol. 14: 211–218.

Puente ME, Bashan Y, Li CY, Lebsky VK (2004) Microbial populations and activities in the rhizoplane of rock- weathering desert plants. I. Root colonization and weathering of igneous rocks. Plant Biol. 6:629–642.

Reddy MS, Kumar S, Babita K, Reddynd MS (2002). Biosolubilization of poorly soluble rock phosphates by Aspergillus tubingensis and Aspergillus niger. Biores. Technol. 84: 187–189.

Rodríguez H, Fraga R (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol. Adv. 17: 319–339.
Rodriguez H, Gonzalez T, Goire I, Bashan Y (2004). Gluconic acid production and phosphate solubilization by the plant growth-promoting bacterium Azospirillum spp. Naturwissenschaften. 91: 552–555.
Rodriguez H, Gonzalez T, Selman G (2000). Expression of a mineral phosphate solubilizing gene from Erwinia herbicola in two rhizobacterial strains. J. Biotechnol. 84: 155–161.
Shigaki F, Sharpley AN, Prochnow LI (2006). Animal-based agriculture, phosphorus and management and water quality in Brazil: options for the future. Sci. Agric. 63: 194–209.
Singh CP, Amberger A (1997). Organic acids and phosphorus solubilization in Straw composted with rock phosphate. Bioresour. Technol. 63: 13–16.
Sperber J (1958). The incidence of apatite-solubilizing organisms in the rhizosphere and soil. Austr. J. Agric. Res. 9: 778-781.
Strobel BW (2001). Influence of vegetation on low-molecular-weight carboxylic acids in soil solution: A review. Geoderma 99: 169-198.
Trivedi P, Sa T (2008). Pseudomonas corrugata (NRRL B-30409) Mutants Increased Phosphate Solubilization, Organic Acid Production, and Plant Growth at Lower Temperatures. Curr Microbiol. 56(2):140-144.
Vassilev N, Vassileva M (2003). Biotechnological solubilization of rock phosphate on media containing agro-industrial wastes. Appl. Microbiol. Biotech. 61: 435–440.
Vassilev N, Toro M, Vassileva M, Azcon R, Barea JM (1999). Rock phosphate solubilization by immobilized cells of Enterobacter sp. in fermentation and soil conditions. Bioresouc. Technol. 61: 29-32.
Vazquez P, Holguin G, Puente ME, Lopez-Cortes A, Bashan Y (2000). Phosphate-solubilizing microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon. Biol. Fertil. Soils 30: 460–468.
Vessey JK (2003). Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255: 571-586.
Wakelin SA, Gupta VV, Harvey PR, Ryder MH (2007). The effect of *Penicillium* fungi on plant growth and phosphorus mobilization in neutral to alkaline soils from southern Australia. Can. J. Microbiol. 53: 106–115.
Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991). 16s ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173: 697–703
Whitelaw MA (2000). Growth promotion of plants inoculated with phosphate-solubilizing fungi. Adv. Agron. 69: 99–151.
Whitelaw MA, Harden TJ, Helyar KR (1999). Phosphate solubilization in solution culture by the soil fungus *Penicillium radicum*. Soil Biol. Biochem. 32: 655-665.
Zaidi A, Khan MS, Ahemad M, Oves M, Wani PA (2009). Recent advances in plant growth promotion by phosphate-solubilizing microbes. In: Mohammad Saghir Khan; Almas Zaidi and Javed Musarrat (Editors). Microbial strategies for crop improvement. Springer-Verlag, Berlin. pp: 23-50.
Zou K, Binkley D, Doxtader KG (1992). New methods for estimating gross P mineralization and mobilization rates in soils. Plant Soil 147: 243–250.