The Open Agriculture Journal

Content list available at: https://openagriculturejournal.com

RESEARCH ARTICLE

Isolation and Characterization of Phosphate Solubilizing Bacteria from Phosphate Solid Sludge of the Moroccan Phosphate Mines

Fatima Z. Aliyat[1], Mohamed Maldani[1], Mohammed El Guilli[2], Laila Nassiri[1] and Jamal Ibijbijen[1,*]

[1]*Environment and Soil Microbiology Unit, Faculty of Sciences, Moulay Ismail University, 11201, Meknes, Morocco.*
[2]*National Institute of Agricultural Research, Regional Center for Agricultural Research, 293, Kenitra, Morocco.*

Abstract:

Background:

Although phosphorus (P) reserves in the soil are important, orthophosphate ions are the only forms of P that can be used by plants. The specificity of phosphate solubilizing bacteria (PSB) to convert insoluble forms of P to an available form Pi is an important trait to reactivate immobilized phosphorus. Therefore, the use of PSB as inoculants increases the P uptake by plants.

Objective:

The present study aims at isolating and selecting the phosphate solubilizing bacteria from Phosphate Solid Sludge (PSS) in order to improve the agronomic efficacy of P fertilizers.

Materials and Method:

The strains isolated from 7 samples of phosphate sludge were tested for their ability to solubilize phosphate in NBRIP medium with Tricalcium Phosphate (TCP) as the sole source of phosphorus, strains with a high solubility index (PSI) were selected. The isolates obtained have been studied for their ability to solubilize TCP quantitatively, and their phosphate solubilizing activity was estimated by the vanadate-molybdate method. The production of Indol-1-Acetic Acid (IAA), Siderophore, and Hydrogen Cyanide (HCN) by the isolates was tested. The best strains have been identified based on 16S rRNA gene sequence comparisons.

Results:

27 strains were isolated on selective medium NBRIP from Phosphate solid sludge. They all showed significant different abilities to solubilize P. CB19 was the most efficient strain in NBRIP agar with SI= 4.79; BM11 was the most efficient strain in NBRIP liquid medium releasing 174.33µg/ml soluble P. Based on 16S rRNA gene sequence comparisons, four genera were identified: *Pseudomonas, Serratia, Pantoea* and *Enterobacter.*

Conclusion:

The PSB isolates varied in their Phosphate solubilizing efficiency, IAA production, Siderophore, and HCN.

Keywords: Biofertilizer, Phosphate solid sludge, Phosphate solubilizing bacteria, PGPR, Phosphate solubilization, Solubility index.

1. INTRODUCTION

Phosphorus (P) is one of the major essential macronutrients for growth and plant development. It is involved in key plant metabolic processes such as storage of cellular energy, photosynthesis, and respiration [1]. The majority of agricultural

soils contain a high reserve of P. However, the amount of soluble P in soil is generally very low (0.4-1.2 g/kg) [2, 3]. The important phosphate reserve in the soil is habitually accumulated as a consequence of regular applications of P fertilizers. According to FAO [4], more than 175.5 million tons of chemical fertilizers are used in agriculture to obtain the best yield of crops and therefore cover increasing habitat needs. More than 80% of P fertilizer, applied to the soil, becomes unavailable for plant via sorption or precipitation by reaction

* Address correspondence to this author at the Environment & Soil Microbiology Unit, Faculty of Sciences, Moulay Ismail University, B.P. 11201 Zitoune, Meknes, Morocco, Tel: +212 70 13 50 02 Fax: +212 535 53 68 08 E-mail: j.ibijbijen@fs.umi.ac.ma,

with Ca^{2+} in calcareous soils, Al^{3+} and Fe^{3+} in acidic soils [5].

At present, the main objective of soil phosphorus management is to optimize agricultural production and minimize phosphorus losses in soils. Insoluble P compounds can be solubilized by organic acids, phosphatase enzymes and complexing agents produced by soil microorganisms [6]. Hence, among the solutions of soil phosphorus management are the use of Phosphate solubilizing microorganisms as soil inoculums to enhance chemical fertilization and thereby improve plant growth and yield [7, 8]. For several decades, agricultural microbiologists have studied the ability of some bacteria to dissolve insoluble P [9]. Currently, most bacteria named PSB belong to the genera: *Pseudomonas, Serratia, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aerobacter, Flavobacterium, Acinetobacter, and Pantoea* [9 - 13].

Morocco covers a significant share in global phosphate reserves; it contains 75% of phosphate reserves on the planet, and it is the leading exporter of phosphate and its derivatives. The country's global market share is more than 30% [14]. Phosphate deposits in Morocco are located in three areas - Khouribga area (Oulad Abdoun Plateau), Gantour area (Youssoufia area) and Laayoune-BouCraa [14]. The phosphate processing activity generates enormous solid sludge. The chemical characterization of PSS has been the subject of several works in the past, and they consist generally of clay, but the phosphate material is found there. The presence of phosphorus in these PSS means a loss of phosphates during treatment. OCP Group aims to develop phosphate as part of a vision of sustainable agricultural development and respect for the environment. Consequently, the search for appropriate solutions to the pollution risk posed by this PSS and the recovery of some of the lost phosphorus becomes necessary.

In this context, the objectives of the present study are to isolate and characterize phosphate-solubilizing strains from Phosphate solid sludge and assess their PGPR traits in order to evaluate the phosphate solid sludge.

2. MATERIALS AND METHODS

2.1. Sample Collection And Analyses

PSS samples were collected in seven different locations (Table **1**) in Khouribga region from the rhizosphere of *Tamarix* sp. (S-BT), *Nicotiana glauca* (S-BN), *Atriplex halimus* (S-AT), *Medicago Arabica* (S-BM), *Acacia Cyclops* (S-ACY), and one sludge sample was collected far from the vegetation (S-CB and S-AC). The samples were collected at a depth of 10 to 40 cm and they were mixed to obtain a representative sample. The samples were transported to the laboratory and conserved at 4 °C for further analysis.

Physico-chemical characteristics of collected PSS samples such as moisture content, pH, and organic matter, and the enumeration of total Bacteria were also determined (Table. **2**). The pH was measured in 1:2.5 soil: water suspension. Organic matter was determined by the dichromate-oxidation method [15]. Total bacteria were enumerated by the plate counts method. 1 g dry weight of samples suspended in 9.0 ml of phosphate buffer saline (pH=7.2) was used to provide a

dilution series from 10^{-1} up to 10^{-7}.

2.2. Selection and Purification of Phosphate Solubilization Bacteria

One gram of each PSS samples was suspended in 9.0 ml of phosphate buffer saline (pH 7.2) and serial dilutions from 10^{-1} up to 10^{-6} were realized. 0.1 ml suspension was spread on NBRIP solid medium (10 g/l D-glucose, 5 g/l $MgCl_2$ $6H_2O$, 0.25 g/l $MgSO_4$ $7H_2O$, 0.2 g/l KCl, 0.1 g/l $(NH_4)_2SO_4$), amended with 5.0 g/L Tricalcium Phosphate (TCP) as a sole source of P [16]. It was incubated at 30°C for 7 days. The bacterial colonies with a clear halo zone were selected and purified three times on NBRIP solid medium. The purified bacterial isolates were maintained on nutrient Agar Plates and stored at 4°C, and a copy of each isolate was stored as a glycerol 40% stock at -30°C.

2.3. Inoculum Preparation

The purified isolates were grown in the NBRIP medium. Each isolate was inoculated into test tubes containing 3 ml of NBRIP liquid medium and incubated for 24 h at 30°C to obtain an inoculum ~ 2×10^8 CFU/ml.

2.4. Qualitative Estimation of Phosphate Solubilization Bacteria

Qualitative estimation of P solubilization was done by measuring the P solubilizing index (PSI). 10 μl of inoculum of each isolate was spotted on the NBRIP solid medium amended with 5.0 g/L TCP as a sole source of P and incubated at 30 °C for 14 days. The experiments were performed in four replicates and the sterile medium served as a control. PSI was calculated according to formula PSI = C+H/C, (C = Colony diameter; H = Halo zone diameter) [17].

2.5. Quantitative Estimation Of Phosphate Solubilization Bacteria

Quantitative estimation of P solubilization was determined by vanado-molybdat phosphoric method [18]. 50 ml of NBRIP broth medium adjusted to pH=7.0±0.2 before autoclaving was taken in 250 ml flask; then, it was inoculated with 200 μl of fresh inoculum and incubated in shaking condition at 120 rpm/min at 28°C for 7 days. Autoclaved and uninoculated NBRIP broth medium served as control. 2 ml of the cultures were taken and centrifuged at 10,000 rpm for 15 min. The content of soluble P was estimated by colorimetry; using the molybdenum blue method, optical density was measured on UV–VIS spectrophotometer at 882 nm. The pH of the NBRIP medium was also measured with a digital pH meter. All treatments were in triplicate.

2.6. Plant Growth-Promoting Traits of Phosphate Solubilization Bacteria

2.6.1. Siderophore Production

Siderophore production of each isolate was determined on Chrome-Azurol S (CAS) medium following the Universal Chemical Assay [19]. Bacterial strains (24 h fresh bacterial) were spotted on CAS plates and incubated at 28°C for 4 days.

The production of siderophore was determined by the development of orange halo around the colonies. The experiments were performed in four replicates.

2.6.2. Indole-3-Acetic Acid Production

Based on their capacity to solubilize P, the selected PSBs were analyzed for IAA production. 200 μl of fresh bacterial cultures were inoculated in 30 mL of LB broth containing 0.1% L-tryptophan, and they were incubated in incubator Shaker at 28°C and 140 rpm/min for 72 h in dark. The bacterial cultures were centrifuged at 10,000 rpm for 10 min. After centrifugation, 2ml of supernatant was mixed with Salkowski reagent. After 30 min in dark, for pink color development, the optical density was measured at 530 nm using UV spectrophotometer. The amount of IAA produced was calculated by the standard graph of pure indole acetic acid [20].

2.6.3. Hydrogen Cyanide Production

Selected isolates were streaked on nutrient agar amended with 0.44% glycine. After that, a soaked filter paper in 2% Sodium carbonate and 0.5 ml picric acid solution was placed in the lid of the agar plate and sealed with parafilm. The plates were incubated for 4 days at 28°C. The turn of the yellow color to the brown one was considered as positive [21].

2.7. Identification of Isolated Strains

Nine isolates were selected based on their performance in TCP solubilization and their PGP trait. Crude DNA extractions from these selected isolates were prepared, and DNA Extraction was performed using the PureLink® Genomic DNA

Mini Kit (Invitrogen, K1820-01) following the steps described by the manufacturer, adapted for Gram-negative bacteria. PCR amplification of the 16S rDNA of the bacterial strains was performed using the DreamTaq PCR Master Mix (Invitrogen), consisting of 22 mM Tris-HCl (pH 8.4), 55 mM KCl, 1.65 mM MgCl 2, 220 μM dGTP, 220 μM dATP, 220 μM dTTP, 220 μM dCTP, 22 U recombinant Taq DNA Polymerase / ml.

The universal primers 27F (sense) (5'AGAGTTTGAT CCTGGCTCAG-3') and 1492R (antisense) (5'-ACGGTTAC CTTGTTACGACTT-3') were used to amplify a 1500pb fragment, which corresponds to the genes of the bacterial 16S rDNA. PCR products were purified with the PureLink™ Quick Gel Extraction & Purification combo kit (Invitrogen K220001), following the manufacturer's recommendations. Two-way sequencing was performed using primers 27F and 1492R. It was carried out according to the Sanger method adapted by the Big Dye Terminator V3 sequencing kit. Finally, it is the ABI3730 DNA sequences that allowed the automatic analysis of sequence reactions. The crude electropherograms were analyzed by MEGA7 software [22]. The consensus sequence from the sense and antisense raw sequences was obtained for each strain; then, it was compared to other sequences using the BLAST server (blast.ncbi.nlm.nih.gov) to determine their phylogenetic affiliation.

2.8. Statistical Analysis

Data were analyzed using SPSS 20 software, and the results were expressed as the means ± standard deviation of three replicates. Data were examined by ANOVA, and post-hoc mean comparison was performed by Duncan's multiple range test at $p \leq 0.05$.

Table 1. Samples site location.

Location	Latitude	Longitude	Altitude (m)
L 1	32.75493458	-6.85403284	683
L 2	32.75526446	-6.85307395	686
L 3	32.75578241	-6.85418146	679
L 4	32.75619226	-6.85484674	683
L 5	32.75639581	-6.85474465	684
L 6	32.75636841	-6.85437991	684
L 7	32.75533956	-6.85338013	689

Table 2. Physicochemical and microbiological analyzes.

Samples	pH	Moisture (%)	Organic Matter (%)	Total Bacteria (UFC/mL)
S-BN	7.84 ± 0.2	29.97 ± 1.02	7.84 ± 0.49	9.9×10^5
S-BM	7.1 ± 0.34	18.69 ± 0.9	7.1 ± 0.2	1.35×10^6
S-BT	7.84 ± 0.25	20.66 ± 0.90	2.97 ± 0.08	1.42×10^6
S-ACY	7.62 ± 0.11	10.13 ± 0.45	1.93 ± 0.09	2.06×10^6
S-CB	7.72 ± 0.09	13.80 ± 0.87	0.02 ± 0.004	$1,48 \times 10^5$
S-AC	7.67 ± 0.5	10.04 ± 0.19	2.69 ± 0.08	4.87×10^6
S-AT	7.77 ± 0.67	6.08 ± 0.80	7.77 ± 0.23	1.75×10^6

3. RESULTS

3.1. Selection and Purification of Phosphate Solubilization Bacteria

Data on the physicochemical analysis of the samples of the PSS are represented in Table **2**. The distribution of total PSB isolated from seven phosphate solid sludge samples is shown in Table **3**. PSBs isolation and selection were achieved in NBRIP solid medium. The strains were selected according to morphological difference colonies and TCP solubilization halos. Although the halo formation around the colonies provided the first qualitative indication of PSB, the colonies which were developed without forming a halo were also isolated to test their performance in the solubilization. As a first step, 150 isolates were selected; these isolates were transplanted on NBRIP medium 3 times. The isolates that possessed the ability to form a halo were selected. Out of 150 isolates, 27 strains were selected from the different PSS samples as follows: where 29.63% were isolated from S-BM, 22.22% from S-CB, 22.22% from S-AT, while 18.51%, 3.7%, 3.7% were obtained from S-BN, S-AC, S-ACY respectively, and no PSB isolates were selected from S-AT sample (Table **3**).

3.2. Qualitative Estimation of Phosphate Solubilization Bacteria

A significant difference was observed in all strains tested ($P \leq 0.05$) Fig. (**1**). The soluble phosphate in NBRIP sold medium ranged from $2.27 \leq SPI \leq 4.79$, and the appearance of the halo zone in all strains was remarked after the 3rd day of incubation. Maximum PSI was noticed in CB19 with 4.79±0.57, followed by BT125 (4.12±0.49), BM28 (4.11±0.25) and ACY13 (4.02±0.12). Moderate solubilization index was observed in BT3S17 (2.27±0.24) and CB32 (2.27±0.14).

3.3. Quantitative Estimation of Phosphate Solubilization Bacteria

The amounts of Soluble-P and pH variation are presented in Fig. (**2**). The TCP solubilization in NBRIP liquid medium, by the strains tested, was accompanied by a significant decrease in pH. The soluble-P concentration in the medium ranged between 101.91 μg/ml and 174.33 μg/ml with significant variations among different isolates ($p \leq 0.05$). Maximum TCP solubilization in NBRIP liquid medium was observed in BM11 (174.33±12.5μg/ml), followed by BT125 (159.48±1.8 μg/ml), BM 28 (159.48±1.7 μg/ml) and CB13 (158.73±3.6 μg/ml), and Modest P solubilization was observed in CB32 (101.91±9.6 μg/ml). In the control, 12±1.6 μg/ml of P was solubilized in the culture medium.

3.4. Plant Growth-Promoting Traits of Phosphate Solubilization Bacteria

3.4.1. Siderophore production

Siderophore production was carried out on solid CAS blue agar; the development of orange halo around the colonies represents iron chelation by the isolate in the medium. The totality of the tested isolates confirmed the secretion of siderophore (Table **4**). 48.14% of the isolates showed a high production of siderophore, while 29% of the isolates showed a medium performance, and 22.22% showed a poor efficiency.

3.4.2. IAA Production

All selected isolates demonstrated an important production of IAA with a significant difference between strains ($p \leq 0.05$) (Table **4**). Highest IAA production was reported in BT3S171 with 43.80±0.39 μg/ml, followed by BM218 (20.53±0.03 μg/ml) and BM215 (15.96±0.20 μg/ml), and the lowest production of IAA was detected in BN313 with 1.37±0.09 μg/ml.
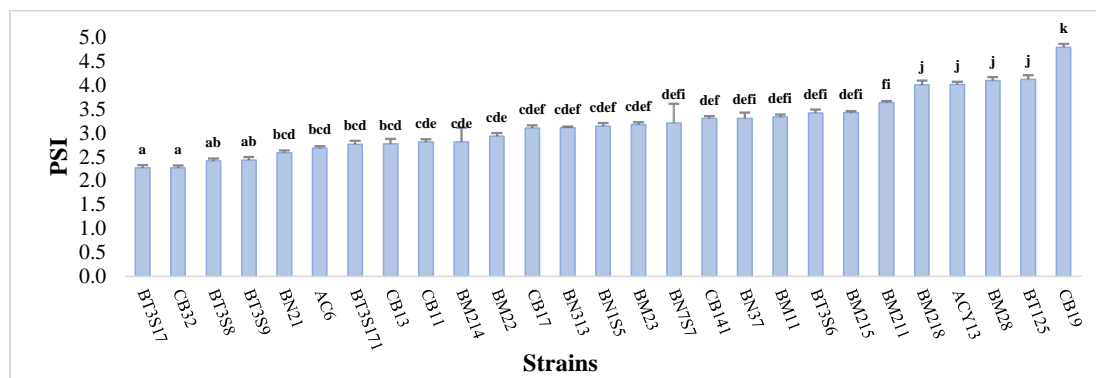

Fig. (1). Selected PSB strains on the basis of phosphate solubilization index (PSI). n = 4 expressed with the standard error of means. Values superscripted by different letters are significantly different (P≤0.05).
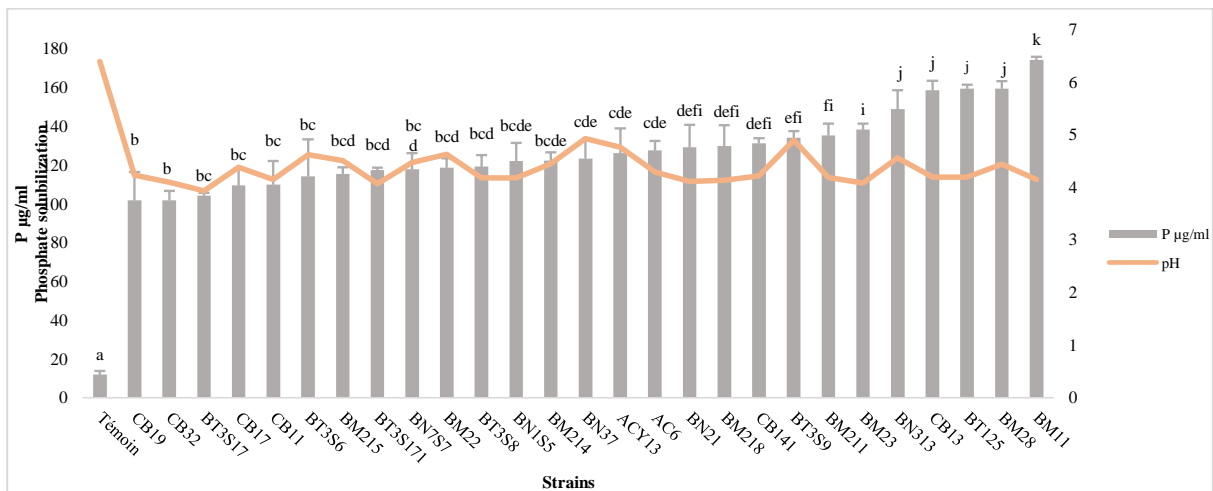
Fig. (2). Quantitative estimation of P solubilization in NBRIP broth medium by selected PSB accompanying with pH variation. Values are the mean of n = 3, expressed with the standard error of means. Values superscripted by different letters are significantly different (P≤0.05).

Table 3. Percentage of PSB strains isolated from the different PSS.

Samples	Isolates	Strains %
S-BN	BN313, BN1S5, BN7S7, BN21, BN37	18.51
S-BM	BM11, BM28, BM22, BM23, BM21, BM214, BM215, BM218	29.63
S-BT	BT125, BT3S17, BT3S171, BT3S6, BT3S8, BT3S9	22.22
S-ACY	ACY13	3.7
S-CB	CB17, CB11, CB19, CB141, CB13, CB32	22.22
S-AC	AC6	3.7
S-AT	-	0

Table 4. IAA, Siderophore and Hydrogen cyanide productions by the selected PSB.

Strains	IAA (µg/mL)	Siderophore	Hydrogen Cyanide
Control	0.12 ± 0.01^a	-	-
BT125	3.90 ± 0.04^{igk}	+++	++
BM218	20.53 ± 0.03^r	+	-
BM28	7.69 ± 0.03^n	++	-
BM211	2.66 ± 0.03^{bcdefig}	+	-
BN37	11.47 ± 0.01^p	++	-
BM11	2.05 ± 0.08^{bcd}	++	-
BN313	1.37 ± 0.09^b	+++	-
CB13	8.73 ± 0.19^n	++	-
CB141	2.53 ± 0.04^{bcdefi}	+++	-
BN21	3.40 ± 0.04^{fig}	+++	-
BT3S6	1.64 ± 0.05^{bc}	++	-
BM22	11.49 ± 0.61^o	+	-
AC6	4.77 ± 0.16^{lm}	+++	+
ACY13	3.12 ± 0.13^{cdefig}	++	-
BM23	4.06 ± 0.04^{gkl}	+	-
BN1S5	5.71 ± 0.05^m	+	+++
CB17	2.86 ± 0.33^{fig}	+++	+++
BT3S9	2.76 ± 0.03^{cdefig}	+++	-

(Table 4) contd.....

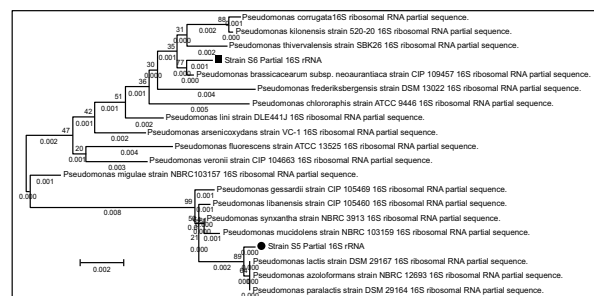|			
BT3S8	3.22 ± 0.05 defig	+++	++
BN7S7	8.19 ± 0.15 n	+	+++
CB19	3.17 ± 0.03 efig	++	-
BT3S17	1.90 ± 0.03 bcde	+++	-
BT3S171	43.80 ± 0.39 s	+++	-
CB32	3.12 ± 0.05 efig	+++	-
CB11	4.99 ± 0.13 klm	+++	-
BM214	2.38 ± 0.01 bcdef	++	-
BM215	15.96 ± 0.20 q	+++	-

IAA values are the mean of n = 3, expressed with standard error of means. Values superscripted by different letters are significantly ($P \le 0.05$). Symbols: - =Negative; + = Moderate; ++ = High; +++= Very high

Table 5. Sequence comparison results of 16S rDNA with BLAST database (blast.ncbi.nlm.nih.gov).
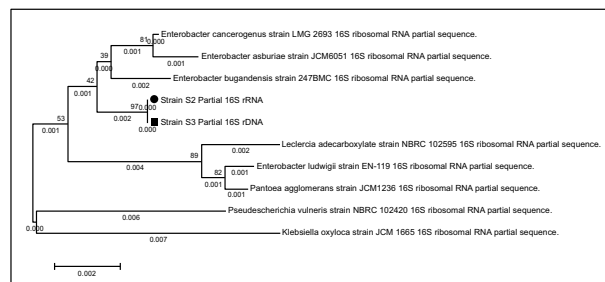
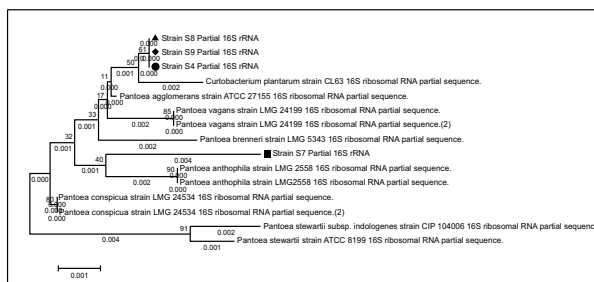| Strain	Gram	16S rRNA Gene Sequence Lengh	Accession N°	Species	% Gene Identity
BM11	Gram	1350bp	NR_024644.1	*Serratia rubidaea strain JCM1240*	99.70%
BM28	Gram	1350bp	NR_148649.1	*Enterobacter bugandensis strain* 247BMC	99.33%
BM215	Gram	1300bp	NR_148649.1	*Enterobacter bugandensis strain* 247BMC	99.23%
CB19	Gram	1350bp	NR_114505.1	*Pantoea agglomerans strain ATCC 27155*	98.00%
BN313	Gram	1350bp	NR_156986.1	*Pseudomonas lactis strain DSM 29167*	99.85%
BT125	Gram	1350bp	NR_116299.1	*Pseudomonas brassicacearum subsp. Neoaurantiaca strain* CIP 109457	99.78%
BT3S171	Gram	1300bp	NR_104928.1	*Pantoea stewartii subsp. Indologenes strain* CIP 104006	98.59%
CB13	Gram	1300bp	NR_114505.1	*Pantoea agglomerans strain ATCC 27155*	98.00%
BM218	Gram	1350bp	NR_114505.1	*Pantoea agglomerans strain ATCC 27155*	98.00%

Phylogenetic tree showing the relationship between strain BM11 (S1) and the most closely related species of the genns *Serratia sp.*

Phylogenetic tree showing the relationship between strains BN313 (S5) and BT125 (S6) and the most closely related species of genns *Pseudomonas sp.*

Phylogenetic tree showing the relationship between strain BM28 (S2) and strains BM215 (S3) and the most closely related species

Phylogenetic tree showing the relationship between CB19 (S4), BT3S171 (S7), CB13 (S8) and BM28 (S9) strains and the most related species

Fig. (3). Phylogenetic tree based on 16S rRNA gene sequences. The relationships among the PSB strains and between representatives of other related species were shown.

3.4.3. Hydrogen Cyanide Production

Out of the 27 isolates, only six isolates (22.2%) showed HCN production after 4 days of incubation (Table **4**).

Maximum HCN production was observed in BN1S5, BN7S7 and CB17, followed by BT125 and BT3S8. AC6 was the least efficient isolate in HCN production.

3.5. Identification and Characterization of the Isolated Strains

The identifications of PSBs strains, based on 16S rRNA sequences, are presented in Table **5**. Among 27 bacterial strains, nine strains (BM11, BM28, BM215, BM218, CB13, C19, BN313, BT125, BT3S171) were selected for molecular characterization, based mainly on their solubilization capacity and also on their PGP traits: IAA secretion, siderophore production and the HCN released from genetic analyses; we identified four genera: *Pseudomonas, Serratia, Pantoea* and *Enterobacter*. Based on the analysis of the 16S rDNA partial sequence, the strain BM11 was identified as *Serratia rubidaea*. Strains BM28 and BM215 were identified as *Enterobacter bugandensis*. Moreover, three strains were identified as *Pantoea agglomerans*. Strains CB19, CB13, and BM218, BT3S171 were identified as *Pantoea stewartii subsp. Indologenes*. BT125 was identified as *Pseudomonas brassicacearum supsp.neoaurantiaca,* and strain BN313 was identified as *Pseudomonas lactis* (Fig. **3**).

4. DISCUSSION

The objective of our study was to isolate PSB from phosphate solid sludge samples, based on the NBRIP medium with TCP as the sole source of phosphorus. From 150 isolates, we selected 27 strains qualified as phosphate solubilizers, after evaluating their ability to solubilize TCP and their PGP traits. We chose 9 isolates for molecular characterization.

It has been found that P is mandatory for plant growth, and its deficiency limits plant development. Even though chemical fertilizers are added to the soils, plants can only use low phosphatic fertilizer quantity because of the immobilization of P. Thus, the selection of highly efficient PSB is very important; it will practically raise P in plant rhizosphere. Various PSB have been isolated from different soil and rhizosphere [23]. As a consequence, PSB can be considered as a plant growth-promoting rhizobacteria, which are commonly regarded as biofertilizers [24, 25]. Also, the presence of PSB in soils will be judged as a positive indicator of using the bacteria as biofertilizers for crop production [23, 26]. In our study, the selected isolates showed high efficiency of solubilization, the soluble-P concentration in the NBRIP medium ranged between 101.91 µg/ml and 174.33 µg/ml. This result is in accordance with previous studies, the solubilization of TCP in their experiments was from 41.20 to 119.95 µg/ml in the NBRIP medium and from 30 to 246 µg/ml respectively [27, 28]. Phosphate solubilization ability has been considered as a principal criterion for isolating highly efficient PSB strains from phosphate solid sludge, but we have also focused on other PGP criteria. Numerous studies have been conducted to isolate effective PGPR according to various criteria, such as IAA and siderophore production or HCN production [29 - 31]. IAA, siderophore and HCN production are an indicator of PGP Rhizobacteria [29, 32]. IAA production by bacteria isolated from the soil has previously been reported [33 - 35] as an important phytohormone, and as a regulatory molecule in plant development [32]. In the present study, all the isolates produced IAA within the range of 43.80 µg/ml – 1.37 µg/ml (p ≤ 0.05), which indicated a significant variability among isolates

for IAA production. Moreover, another important trait of PGPR is the production of siderophores which influences plant growth. They are produced by numerous types of bacteria in response to iron deficiency, and they play a vital role in determining the competitive fitness of bacteria to colonize plant roots and to compete for iron with other microorganisms in the rhizosphere [36]. In our study, all strains produced siderophore with a significant difference between the strains; HCN production by rhizobacteria has been postulated to play an important role in the biological control of pathogens. HCN is volatile, a secondary metabolite that inhibits the growth of pathogen due to inhibitor of metal enzymes, particularly cytochrome C oxidases in an electron transport system [37]. In this research, it has been found that from 27 isolates, only 22.2% produced HCN. These results closely correlate with the study [27] which reports that 83.33% of the strains produce IAA, while 66.66% of strains have HCN and siderophore producers. The [38] study also reveals that 95% of their isolated PSB strains are IAA producers, 100% of strains produce siderophores and 67.5% of strains are HCN producers. In the literature, numerous bacterial strains are considered as PSBs, and among them, those from genera are *Pseudomonas, Serratia, Pantoea, Alcaligenes, Enterobacter, Acinetobacter, Arthrobacter, Azospirillum, Bacillus, Burkholderia, Erwinia, Flavobacterium* and *Paenibacillus*. Our selected strains belong to four genera which have been deemed as PSB: *Pseudomonas, Serratia, Pantoea* and *Enterobacter*. These results are congruent with those of [10, 39 - 42].

The use of PSB as biofertilizers would strengthen the use of chemical fertilizers and promote plant growth. Taken together, the selection of an efficient PSB strain as inoculants and qualifying them as biofertilizers should be based not only on laboratory experiments and greenhouse trials but also on field experiments. Further studies should focus on practical applications of PSB in the field.

CONCLUSION

A total of 27 strains were isolated in this study, among which 9 were identified; they belong to the genus: *Pseudomonas, Serratia, Pantoea,* and *Enterobacter*. Results showed that P solubilization by PSB in the NBRIP media performed differently and most of the strains displayed plant growth-promoting traits, thereby indicating that they have a role in enhancing plant growth. Further studies should be carried out, namely the production of organic acids by those strains. We can also test them on crops as biofertilizers in greenhouse.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

CONFLICT OF INTEREST

The author declares no conflict of interest, financial or otherwise.

REFERENCES

[1]   Awasthi R, Tewari R, Nayyar H. Synergy between plants and p-solubilizing microbes in soils: effects on growth and physiology of crops. Int Res J Microbiol 2011; 2: 484-503.

[2]   Fernández VP, Guzmán CA, Peirce TM, McBeath M, Khayet L, McLaughli MJ. Effect of wheat phosphorus status on leaf surface properties and permeability to foliar-applied phosphorus. Plant Soil 2014; 384: 7-20.
[http://dx.doi.org/10.1007/s11104-014-2052-6]

[3]   Joe MM, Deivaraj S, Benson A, Henrya AJ, Narendrakumar G. Soil extract calcium phosphate media for screening of phosphate-solubilizing bacteria. Agric Nat Resour (Bangk) 2018; 52(3): 305-8.
[http://dx.doi.org/10.1016/j.anres.2018.09.014]

[4]   IMF O, UNCTAD W.. Price Volatility in Food and Agricultural Markets: Policy Responses. Rome: FAO 2011.

[5]   Gyaneshwar P, Kumar GN, Parekh LJ, Poole PS. Role of soil microorganisms in improving P nutrition of plants. Plant Soil 2002; 245: 83-93.
[http://dx.doi.org/10.1023/A:1020663916259]

[6]   Illmer P, Schinner F. Solubilization of inorganic calcium phosphates-solubilization mechanisms. Soil Biol Biochem 1995; 27: 257-63.
[http://dx.doi.org/10.1016/0038-0717(94)00190-C]

[7]   Adhikari P, Pandey A. Phosphate solubilization potential of endophytic fungi isolated from *Taxus wallichiana* Zucc. Roots. Rhizosphere 2019; 9: 2-9.
[http://dx.doi.org/10.1016/j.rhisph.2018.11.002]

[8]   Kumar A, Kumar A, Devi S, Patil S, Payal C, Negi S. Isolation, screening and characterization of bacteria from rhizospheric soils for different plant growth promotion (PGP) activities: An *in vitro* study Recent Res Sci Technol 2012; 4(1): 01-5.

[9]   Castagno LN, Estrella MJ, Sannazzaro AI, Grassano AE, Ruiz OA. Phosphate-solubilization mechanism and *in vitro* plant growth promotion activity mediated by *Pantoea eucalypti* isolated from *Lotus tenuis* rhizosphere in the Salado River Basin (Argentina) J Appl Microbiol 2011; 110(5): 1151-65.
[http://dx.doi.org/10.1111/j.1365-2672.2011.04968.x]   [PMID: 21299771]

[10]   Chen YP, Rekha PD, Arun AB, Shen FT, Lai WA, Young CC. Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Appl Soil Ecol 2006; 34: 33-41.
[http://dx.doi.org/10.1016/j.apsoil.2005.12.002]

[11]   Peix A, Ramírez-Bahena MH, Velázquez E. Historical evolution and current status of the taxonomy of genus *Pseudomonas*. Infect Genet Evol 2009; 9(6): 1132-47.
[http://dx.doi.org/10.1016/j.meegid.2009.08.001] [PMID: 19712752]

[12]   Hu XJ, Li ZJ, Cao YC, Zhang J, Gong YX, Yang YF. Isolation and identification of a phosphate-solubilizing bacterium *Pantoea stewartii subsp. stewartii g6*, and effects of temperature, salinity, and pH on its growth under indoor culture conditions. Aquacult Int 2010; 18(6): 1079-91.
[http://dx.doi.org/10.1007/s10499-010-9325-8]

[13]   Chauhan H, Bagyaraj DJ, Selvakumar G, Sundaram SP. Novel plant growth promoting rhizobacteria—Prospects and potential. Appl Soil

Ecol 2015; 95: 38-53.
[http://dx.doi.org/10.1016/j.apsoil.2015.05.011]

[14]   Hakkou R, Benzaazoua M, Bussière B. Valorization of phosphate waste rocks and sludge from the moroccan phosphate mines: Challenges and perspectives. Procedia Eng 2016; 138: 110-8.
[http://dx.doi.org/10.1016/j.proeng.2016.02.068]

[15]   Walkley A, Black IA. An examination of Degtjareff method for determining soil organic matter and a proposed modification of the chromic acid titration method. Soil Sci 1934; 37: 29-37.
[http://dx.doi.org/10.1097/00010694-193401000-00003]

[16]   Nautiyal CS. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 1999; 170(1): 265-70.
[http://dx.doi.org/10.1111/j.1574-6968.1999.tb13383.x]   [PMID: 9919677]

[17]   Premono ME, Moawad AM, Vlek PLG. Effect of phosphate-solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. Indones J Crop Sci 1996; 11: 13-23.

[18]   Murphy J, Riley JP. A modified single solution method for the determination of phosphate in natural waters. Anal Chim Acta 1962; 27: 31-6.
[http://dx.doi.org/10.1016/S0003-2670(00)88444-5]

[19]   Schwyn B, Neilands JB. Universal chemical assay for the detection and determination of siderophores. Anal Biochem 1987; 160(1): 47-56.
[http://dx.doi.org/10.1016/0003-2697(87)90612-9] [PMID: 2952030]

[20]   Gordon SA, Weber RP. Colorimetric estimation of indoleacetic acid. Plant Physiol 1951; 26(1): 192-5.
[http://dx.doi.org/10.1104/pp.26.1.192] [PMID: 16654351]

[21]   Bakker AW, Schippers B. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas spp*-mediated plant growth-stimulation. Soil Biol Biochem 1987; 19: 451-7.
[http://dx.doi.org/10.1016/0038-0717(87)90037-X]

[22]   Kumar S, Stecher G, Tamura K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 2016; 33(7): 1870-4.
[http://dx.doi.org/10.1093/molbev/msw054] [PMID: 27004904]

[23]   Majeed A, Abbasi MK, Hameed S, Imran A, Rahim N. Isolation and characterization of plant growth-promoting rhizobacteria from wheat rhizosphere and their effect on plant growth promotion. Front Microbiol 2015; 6: 198.
[http://dx.doi.org/10.3389/fmicb.2015.00198] [PMID: 25852661]

[24]   Chung H, Park M, Madhaiyan M, Seshadri SJ, Song H. Cho, Sa T. Isolation and characterization of phosphate solubilizing bacteria from the rhizosphere of crop plants of Korea. Soil Biol Biochem 2005; 37: 1970-4.
[http://dx.doi.org/10.1016/j.soilbio.2005.02.025]

[25]   Pathak D, Lone R, Khan S, Koul KK. Isolation, screening and molecular characterization of free-living bacteria of potato (*Solanum tuberosum* L.) and their interplay impact on growth and production of potato plant under Mycorrhizal association. Sci Hortic (Amsterdam) 2019; 252: 388-97.
[http://dx.doi.org/10.1016/j.scienta.2019.02.072]

[26]   Deshwal VK, Singh SB, Kumar P, Chubey A. Isolation and characterization of *Pseudomonas* strains from potatoes rhizosphere at Dehradun Valley. India. Int J Basic Appl Sci 2013; 2(2): 53-5.

[27]   Batool S, Iqbal A. Phosphate solubilizing rhizobacteria as alternative of chemical fertilizer for growth and yield of *Triticum aestivum* (Var. Galaxy 2013). Saudi J Biol Sci 2019; 26(7): 1400-10.
[http://dx.doi.org/10.1016/j.sjbs.2018.05.024] [PMID: 31762601]

[28]   Rfaki A, Nassiri L, Ibijbijen J. Isolation and characterization of phosphate solubilizing bacteria from the rhizosphere of faba bean (*Vicia fab*a L.) in Meknes Region, Morocco. Br Microbiol Res J 2015; 6(5): 247-54.
[http://dx.doi.org/10.9734/BMRJ/2015/14379]

[29]   Verma JP, Yadav J, Tiwari KN, Jaiswal DK. Evaluation of plant growth promoting activities of microbial strains and their effect on growth and yield of chickpea (*Cicer arietinum* L.) in India. Soil Biol Biochem 2014; 70: 33-7.
[http://dx.doi.org/10.1016/j.soilbio.2013.12.001]

[30]   Grobelak A, Napora A, Kacprzak M. Using plant growth-promoting rhizobacteria (PGPR) to improve plant growth. Ecol Eng 2015; 84: 22-8.
[http://dx.doi.org/10.1016/j.ecoleng.2015.07.019]

[31]   Djayaa L. Hersantia, Istifadaha N, Hartatia S, Joni IM. *In vitro* study of plant growth promoting rhizobacteria (PGPR) and endophytic

bacteria antagonistic to *Ralstonia solanacearum* formulated with graphite and silica nano particles as a biocontrol delivery system (BDS) Biocatal Agric Biotechnol 2019; 19: 101-53.
[http://dx.doi.org/10.1016/j.bcab.2019.101153]

[32]   Kumar A, Maurya BR, Raghuwanshi R. Isolation and characterization of PGPR and their effect on growth, yield and nutrient content in wheat (*Triticum aestivum* L.). Biocatal Agric Biotechnol 2014; 3: 121-8.
[http://dx.doi.org/10.1016/j.bcab.2014.08.003]

[33]   Mirza MS, Ahmad W, Latif F, Haurat J, Bally RP. Normand, Malik KA. Isolation, partial characterization, and the effect of plant growth-promoting bacteria (PGPB) on micro-propagated sugarcane *in vitro* Plant Soil 2001; 237: 47-54.
[http://dx.doi.org/10.1023/A:1013388619231]

[34]   Park M, Kim C, Yang J, *et al.* Isolation and characterization of diazotrophic growth promoting bacteria from rhizosphere of agricultural crops of Korea. Microbiol Res 2005; 160(2): 127-33.
[http://dx.doi.org/10.1016/j.micres.2004.10.003] [PMID: 15881829]

[35]   Zhang J, Wang PC, Fang L, Zhang QA, Yan CS, Chen JY. Isolation and characterization of phosphate-solubilizing bacteria from mushroom residues and their effect on tomato plant growth promotion. Pol J Microbiol 2017; 66(1): 57-65.
[http://dx.doi.org/10.5604/17331331.1234993] [PMID: 29359698]

[36]   Siddiqui ZA. PGPR: Prospective biocontrol agents of plant pathogens.PGPR: Biocontrol and Biocontrol. Springer, Netherlands 2006; pp. 112-42.

[37]   Crowley DE. Microbial siderophores in the plant rhizosphere.Iron Nutrition in Plants and Rhizospheric Microorganisms. Springer, Netherlands 2006; pp. 169-98.
[http://dx.doi.org/10.1007/1-4020-4743-6_8]

[38]   Shakeela S, Padder SA, Bhat ZA. Isolation of phosphate solubilizing rhizobacteria and endorhizobacteria from medicinal plant *Picrorhiza kurroa* and their optimization for tricalcium phosphate solubilization. Pharma Inn 2017; 6(6): 160-70.

[39]   Halder AK, Mishra AK, Bhattacharyya P, Chakrabartty PK. Solubilization of rock phosphate by *Rhizobium* and *Bradyrhizobium.* J Gen Appl Microbiol 1990; 36: 81-92.
[http://dx.doi.org/10.2323/jgam.36.81]

[40]   Illmer P, Schiner F. Solubilization of inorganic phosphates by microorganisms isolated from forest soils. Soil Biol Biochem 1992; 24: 389-95.
[http://dx.doi.org/10.1016/0038-0717(92)90199-8]

[41]   Panda B, Rahman H, Panda J. Phosphate solubilizing bacteria from the acidic soils of Eastern Himalayan region and their antagonistic effect on fungal pathogens. Rhizosphere 2016; 2: 62-71.
[http://dx.doi.org/10.1016/j.rhisph.2016.08.001]

[42]   Behera BC, Yadav H, Singh SK, *et al.* Phosphate solubilization and acid phosphatase activity of *Serratia* sp. isolated from mangrove soil of Mahanadi river delta, Odisha, India. J Genet Eng Biotechnol 2017; 15(1): 169-78.
[http://dx.doi.org/10.1016/j.jgeb.2017.01.003] [PMID: 30647653]

[http://dx.doi.org/10.1007/1-4020-4152-7_4]