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Molecular Characterization of Some Phosphate Solubilizing Microorganisms

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ABSTRACT

One of the most important macronutrients to plants is phosphorus, which increases the biological activities in plants. Phosphorus is found as insoluble and unavailable form for plant. Some microbes of the soil can transform insoluble phosphate shape to a more suitable shape for plants' requirements. The current study used some microorganisms as bacteria and fungus that had the ability to convert phosphorus. Different phosphate microorganisms have been isolated, screened, identified; and the intrinsic antibiotic resistance was tested. One hundred bacterial isolates were isolated from *Curcuma*, *Ocimum* and rhizospheres of *Eruca* plants and tested for phosphate solubilization. The results showed nine bacterial isolates (R1B2, R2W22, R3B28, R4B31, R5C33, R6B34, R7 C35, R8 B40, and C15) out of 100 investigated bacterial isolates, which had a potential of phosphate solubilization ability based on Pikovasky (PVK) and National Botantial Research Institute Phosphate (NBRIP). The media was based on clear halo zone, and the nine isolates of the bacteria were determined using biochemical (API 20) test. Based on the results, the identified isolates of bacteria were: *Enterobacter cloacae* (R1B2, R2W22, R3B28, R4B31, R5C33, R6B34, R6 C35, R7 C35 and R8 B40), *Klebsiella pneumonia* (C15), *Bacillus cereus* (R2W22) and *Bacillus megaterium* (R3B28). We found eight selected bacteria (R1B2, R2W22, R3B28, R4B31, R5C33, R6B34, R7C35 and R8B40 and C15) which were confirmed by 16S rRNA sequence. These sequences were submitted to the NCBI GeneBank under accession number MK064178 for *E. cloacae* R1B2, (MK064181) for *E. cloacae* R4B31, (MK064182) for *E. cloacae* R5C33 (MK064183) for *E. cloacae* R6B34, (MK064184) for *E. cloacae* R7C35, (MK064185) for *E. cloacae* B40R8, MK064179 for *B. cereus* R2W22 and MK064180 for *B. megaterium* R3B28. Intrinsic antibiotic resistance test was carried out for 8 phosphate solubilizing bacteria (R5C33, R7C35, R2W22, R8B40, R3B28, and C15) using 9 antibiotics. The obtained data revealed that *B. cereus* (R2W22), *E. cloacae* (R5C33, R7C35, and R6B34), were resistant against six antibiotics. While *E. cloacae* (R8B40 and R1B2) was resistance against eight antibiotics. *B. megaterium* (R3B28) had resistance for three antibiotics. Seven isolates of fungi were isolated from soil samples and screened for phosphate solubilization. The data showed *Aspergillus niger* (LA) had high phosphate solubilization and identified based on 18S rRNA sequencing. The sequence data were submitted to Gene Bank. Isolates of bacteria and fungi could be used as potent bio-fertilizer for phosphate.

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Introduction

Phosphorus is very important macronutrient to plants, where dry plant weight contains about 0.2% of phosphorus. It is an important element for phospholipids, nucleic acids, ATP, controlling reactions of key enzymes and metabolic pathway regulations [1]. The phosphorus nutrition affects nitrogen fixation in legumes, quality of crop, and resistance against many plant diseases [2]. Richardson [3] reported that, phosphorus in soil was found in both organic and mineral form, which resemble from 20 to 80% in organic material and from 10 to 30% in applied fertilizers (P) available for plants. Mclaughlin *et al.*, [4] and Holford, [5] reported that, more than 80% of it is not used by plant because of its precipitation, adsorption, or

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conversion to organic P. In addition, total P is in the insoluble form as reported by [6, 7]. Using phosphorus fertilizing led to increase the plant problems, more cost, and define a new alternative strategy to provide phosphorus to plant and improve crop production [8].

Microbes activity is important in cycling of phosphorus in the soil [9]. Microorganisms in soil which convert insoluble phosphates to soluble forms are called phosphate-solubilizing microorganisms (PSM) [10, 11], which provide P to plants and increase the growth performance.

Activity of those microorganisms could be tested on media containing (dicalcium and tricalcium) phosphate, phosphoric rock, hydroxyapatite, and the minerals contain P (insoluble) similar to sole conditions [12]. Activity of covered phosphate solubilization was assessed by formation of halos solubilization (light zones) around microbes [13].

Reddy *et al.*, [14] tested solubilization of phosphates from rock by two isolates of *A. tubingensis*, *A. niger*, isolated from rhizospheric soils, the results showed increase P solubilization percent by *A. tubingensis* (AT1) strain as it used 2% of rock phosphate.

An aim of the present study was to identify solubilizer isolates of bacteria and fungi by morphological, biochemical and molecular characteristics using 16S rRNA for bacteria and 18S rRNA for fungi.

Materials and Methods

Collection and isolation of bacterial isolates:

Three rhizospheric soil samples were collected from *Cucumis*, *Ocimum*, and *Eruca* plants. The samples were taken from different regions located at Kilo 14 district, Jeddah (Saudi Arabia). One gram of rhizosphere soil sample was placed in the sterile falcon tubes containing 9 ml distilled water. The serially diluted soil samples (10^{-5} and 10^{-7}) were placed on nutrient agar medium and incubated at 30°C for 48 h [15].

Media:

Agar medium was used for bacteria and Potato Dextrose Agar (PDA, Oxoid) was used for fungal cultivation (Pikovskaya medium) (PVK) [16], and National Botanical Research Institute's phosphate growth medium (NBRIP) [17] were used for detection of microbe's phosphate solubilizing. Muller- Hinton medium Muller- Hinton medium was used for the disk diffusion assay method.

Screening of solubilizing phosphate bacterial isolates using two media (PVK) and (NBRIP). One hundred bacterial isolates were screened on (PVK) and (NBRIP) media supplemented with $Ca_3 (PO_4)_2$ to detect phosphate solubilizing bacteria. Isolates of bacteria and fungi were incubated for 7 days at 30 ºC [18] the clear zone diameter and colony diameter were measured on 1st, 2nd, 3rd, 4th and 7th days. The formula that was used for solubilization index (SI) = $\frac{\text{(Colony diameter+Halo zone)}}{\text{colony diameter}}$ [19].

Study of morphological and biochemical characteristics of isolates:

Gram stain and the method of Sallisaw were used to study morphological characteristic of high phosphate solubilizing bacterial isolates. Biochemical tests were carried out by using API 20-[20].

Amplification of the 16S rRNA from the selected strains:

Genomic DNA samples from eight isolated phosphate solubilizing bacteria were extracted using Gene lute TM Bacterial Genomic Kit (Sigma A1drich- USA). The regions of the 16S ribosomal genes (rRNA) of the genomic DNA extracted were amplified by polymerase chain reaction (PCR). The amplification of the approximately 1492 bp DNA fragment were performed with the highly conserved 16S rDNA universal primer pair, fD1 and rP2 [21], with the following sequences: fD1: 5'AGA GTT TGA TCM TGG CTC AG-3' and rP2: 5'-TAC GGY TAC CTT GTT ACG ACTT-3'.

Intrinsic antibiotic resistance test (IAR):

Intrinsic antibiotic resistance test was used to study the bacterial resistance or sensitivity to antibiotics. Plates of culture medium were incubated by spreading bacterial culture on agar surface of the Mueller-Hinton agar medium (MH). Filter paper disc containing different concentration of antibiotics, 100 µg/ml of: Levofloxacin (Lev), Tobramycin (TMN), Tigecycline (TGC), Vancomycin (VA) and Clindamycin (CM) while 50 µg/ml Fusidic acid (FA), ampicillin/sulbactam (SAM) 20 µg/ml and Taxo A (A), and 10 µg/ml Metronidazole (MET) were then placed on plates. The plates were incubated at 30ºC after inoculation.

Fungal isolation and identification:

To isolate and detect fungal phosphate solubilization, soil samples were collected from King Abdul Aziz University region, and the fungal isolates were morphologically identified [22].

Fungal molecular identification using 18S rRNA:

Chromosomal DNA of *A. niger* (LA) was amplified with primers derived from the cDNA sequence. The 18S rRNA gene was amplified using PCR. Two oligonucleotide fungal primers were used for amplification, NS1 (forward) 5' GTA GTC ATA TGC TTG TCT C 3' and NS8 (reverse) 5' TCC GCA GGTCA CCT ACG GA 3'. PCR was performed in a final volume of 30 $\mu\ell$ with 20 ng of genomic DNA as the template and *EF-Taq* (Sol Gent, Korea) as follows: initial denaturation at 95 °C for 2 min., 35 cycles of 95 °C for 1min., 55°C, and 72 °C each for 1min. and final elongation for 10 min. at 72 °C.

The products of amplification were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reactions were performed using a PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95 °C (5 min.), 5 min on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). The sequence was searched against BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). GenBank accession numbers for sequences were obtained.

Optimizing physiological conditions (PH & temperature) for phosphate solubilization of *A. niger* (LA):

A. niger (LA) strain cultures were checked for optimization for phosphate solubilization at two different temperatures (28 and 40ºC) and pH (5, 6, 7, and 8) on PVK medium plates. Two methods (fungal disk & spore suspension) were used.

Fungal disk:

A. niger (LA) was grown on PVK medium at 28ºC for 5 days. Six mm-diameter wells were punctured in the medium using sterile cork borer. *A. niger* (LA) grown on PVK plates inverted inside the well on PVK plates and incubated at 28ºC for 4 days with continuous observation for zone diameter. PVK plates not inoculated were maintained as control according to [23, 24].

Spore suspension method:

Fresh speculated *A. niger* (LA) culture prepared on PVK medium was selected for preparation of spore suspension. Twenty ml sterile water was spread in aliquots on a culture plate and the fungal colony surface was lightly scratched off using a sterilized spreader [25]. A sterile glass bottle with Whatman No. 42 filter paper was used to filter cultures. Spore counts were taken using a hemocytometer and the suspension was adjusted to have approximately 1×10^7 spores per 1 ml. 20 µl of spore suspension was added to each side of the hemocytometer. The average number of conidia calculated in one cell of hemocytometer was $4{,}37 \times 10^7$ conidia /ml.

Results:

Collection of samples and isolation of bacteria:

One hundred isolates were obtained, of which 40 were from *Cucumis sativus*, 20 from *Eruca sativa* and 40 from *Ocimum basilicum*; they were preserved at 4°*C*. These isolates were screened for phosphate solubilization.

Screening of phosphate solubilizing bacteria using (PVK) and (NBRIP) media

Phosphate solubilizing bacteria (PSB) showing clear zone were selected. The result indicated that 9 isolates (R1B2, R2W22, R3B28, R4B31, R5C33, R6B34, R7C35, R8B40, and C15) showed a clear zone. Screening data of PVK and NBRIP media is shown in Fig. 1. Seven isolates out of 9 showed a clear zone using PVK medium. Results of solubilization index (SI) on PVK showed 3, 2.7, 2.6, 2.5 for (R1B2) (R4B31) (R7C35) (R5C33, R840 and R2W22), respectively and no clear zone with (C15) and *(*R3B28) (Table 1). Table 2 showed that SI was 4, 3.6, 3.5, 3.3, 2.8 and 2.6 for (R3B28) (C15) (R1B2 and R5C33) (R840) (R7C35, R4B31, and R6B34) (R2W22), respectively on NBRIP medium. The isolates (C15) and *(*R3B28) illustrated clear zone on NBRIP and not on PVK media.

SI was ranged from (2.5 to 2.2), (2.5 to 2.1), (2.6 to 2.4), (2.3 to 2.7), (2.5 to 2.1), and (2.4 to 2.2) for (R2W22), (R5C33), (R7C35), (R4B31), (R8B40), and (R6B34) on PVK medium, respectively; SI for (R1B2) isolate in seventh day was 3 (Fig. 2). Also, SI was ranged from (2.6 to 2.2), (2.4 to 3.5), (2.8 to 2.5), (3.6 to 2.4), (3 to 3.3), (2.8 to 2.4), (3.3 to 2.4), (4 to 2.2), and (2.7 to 2.5) for (R2W22), (R5C33), (R7C35), (C15), (R1B2), (R4B31), (R8B40), *(*R3B28), and (R6B34) on NBRIP medium, respectively (Fig. 1).

Fig. 4 shows comparisons of SI between two media. Results indicated that all isolates possess phosphate solubilization activity on NBRIP medium except (R840) isolate that possess high phosphate solubilization activity on PVK. Also, higher SI was observed for (R2W22), (R6B34), and (R7C35) isolates after 1 day of incubation on both media, for *(*R3B28) isolate at the first day on NBRIP medium, for (R840) isolate at the first and second days on both media, for (R5C33) isolate at the first and seventh days of incubation on both media, and for (R4B31) at the 7th and 1st days of incubation on both media. The results in 3rd day of incubation showed higher SI on NBR medium for (C15) and after 7 and 3 days of incubation on PVK and NBRIP media for (R1B2).

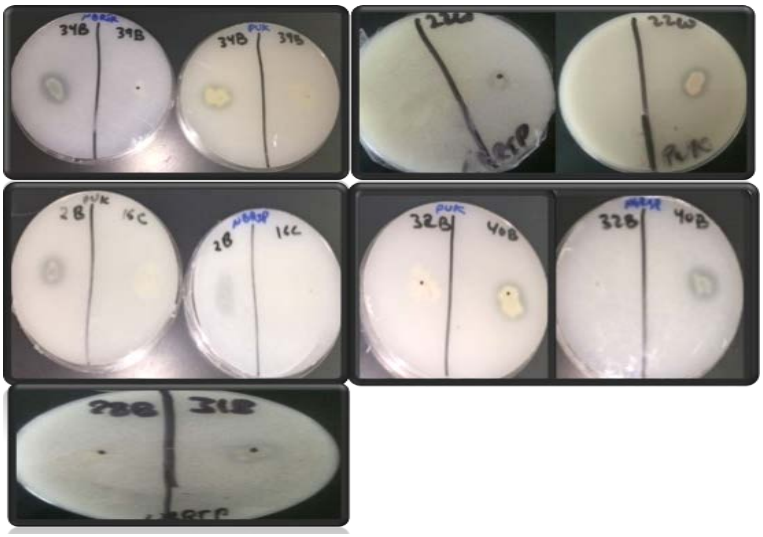

Fig. 1: The phosphate solubilizing zone formed on PVK and NBRIP media containing $Ca_3(PO_4)_2$ by bacterial isolates.

Table 1: Phosphate solubilization of bacterial isolates on (PVK) agar medium after incubation at 30ºC up to 7 days.

Isolates code	One day (PVK)		SI	Two days		SI	Three days		SI	Four days		SI	Seven days		SI
	Zone diameter cm	Colony diameter		Zone diameter	Colony diameter		Zone diameter	Colony diameter		Zone diameter	Colony diameter		Zone diameter	Colony diameter	
Eruca sativa (R2W22)	0.6	0.4	2.5	0.8	0.6	2.4	0.9	0.7	2.3	1	0.8	2.3	1.1	0.9	2.2
Cucumis sativus (R5C33)	0.6	0.4	2.5	0.8	0.7	2.4	1	0.9	2.3	1.2	1	2.2	1.2	0.9	2.1
Cucumis sativus (R7C35)	0.5	0.3	2.6	0.7	0.5	2.5	1	0.8	2.5	1.2	0.8	2.4	1.3	0.9	2.4
Cucumis sativus (C15)	-	-		-	-		-	-		-	-		-	-	
Ocimum basilicum (R1B2)	-	-		-	-		-	-		-	-		0.4	0.2	3
Ocimum basilicum (R4B31)	0.8	0.6	2.3	1	0.8	2.3	1.3	1	2.4	1.3	1	2.5	1.7	1	2.7
Ocimum basilicum (R8B40)	0.4	0.3	2.3	0.6	0.4	2.5	0.7	0.6	2.2	0.8	0.7	2.1	1	0.8	2.1
Ocimum basilicum (R3B28)	-	-		-	-		-	-		-	-		-	-	
Ocimum basilicum (R6B34)	0.7	0.5	2.4	0.9	0.7	2.3	1.1	0.9	2.2	1.2	1	2.2	1.6	1.3	2.2

Table 2: Phosphate solubilization of phosphate solubilizing bacteria on (NBRIP) medium up to seven days of incubation at 30ºC.

Isolates code	(NBRIP) One day		SI	Two days		SI	Three days		SI	Four days		SI	Seven days		SI
	Zone diameter	Colony diameter		Zone diameter	Colony diameter		Zone diameter	Colony diameter		Zone diameter	Colony diameter		Zone diameter	Colony diameter	
Eruca sativa (R2W22)	0.5	0.3	2.6	0.7	0.5	2.5	0.8	0.6	2.4	0.9	0.7	2.4	1	0.6	2.2
Cucumis sativus	0.7	0.5	2.4	0.8	0.6	2.4	0.9	0.8	2.5	1.1	0.9	2.9	1.5	0.6	3.5

(R5C33)															
Cucumis sativus (R7C35)	0.7	0.4	2.8	0.8	0.5	2.7	0.9	0.6	2.6	1	0.7	2.6	1.2	0.7	2.5
Cucumis sativus (C15)	-	-	-	-	-	-	0.8	0.3	3.6	0.9	0.5	2.8	1	0.7	2.4
Ocimum basilicum (R1B2)	-	-	-	0.4	0.2	3	0.5	0.2	3.5	0.6	0.3	3.3	0.7	0.3	3.3
Ocimumbasilicum (R4B31)	0.7	0.4	2.8	0.9	0.5	2.8	1	0.6	2.6	1.1	0.7	2.6	1.4	1	2.4
Ocimum basilicum (R8B40)	0.7	0.3	3.3	0.9	0.4	3.3	1	0.5	3	1.1	0.7	2.7	1.1	0.8	2.4
Ocimumbasilicum (R3B28)	0.3	0.1	4	0.5	0.3	2.6	0.5	0.4	2.3	0.6	0.5	2.2	0.7	0.5	2.2
Ocimumbasilicum (R6B34)	0.5	0.3	2.7	0.7	0.4	2.8	0.8	0.5	2.6	0.9	0.6	2.5	1.4	0.8	25

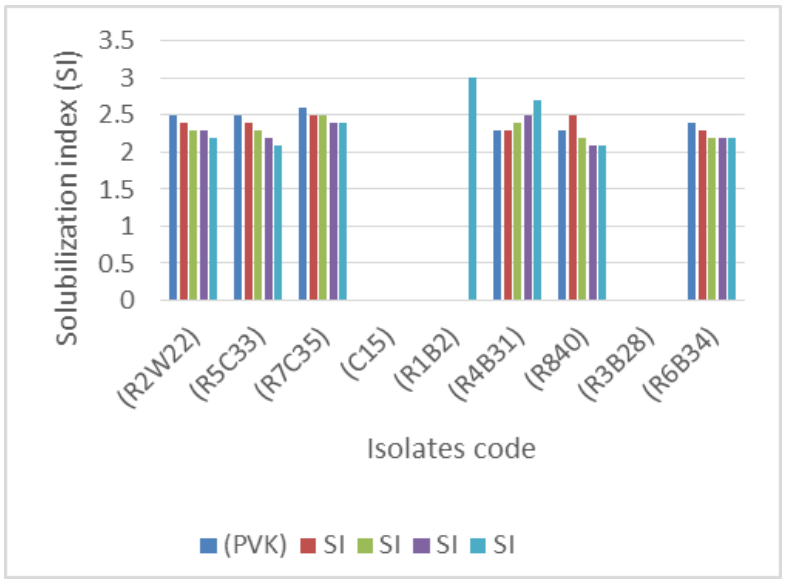

Fig. 2: Phosphate solubilizing isolates on Pikovasky agar medium (PVK) after 7 days of incubation at 30^0C.

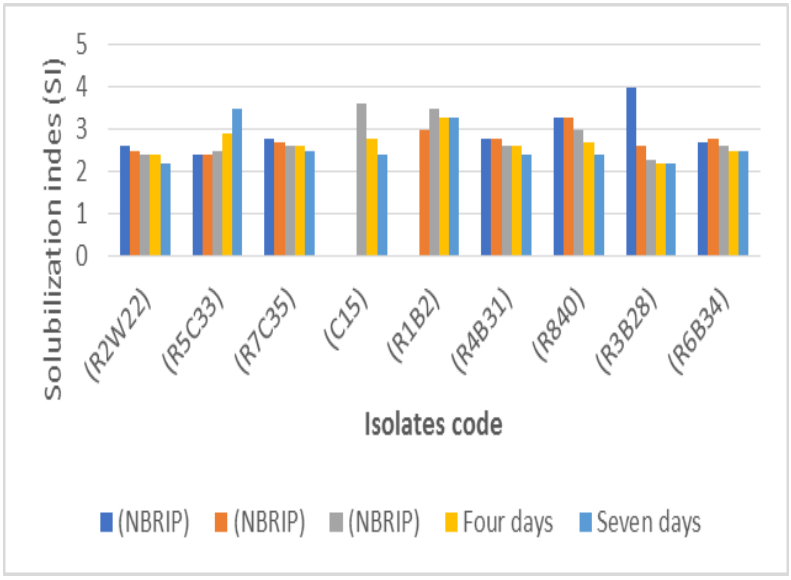

Fig. 3: Phosphate solubilizing bacteria on (NBRIP) medium after 7 days of incubation at 30ºC.

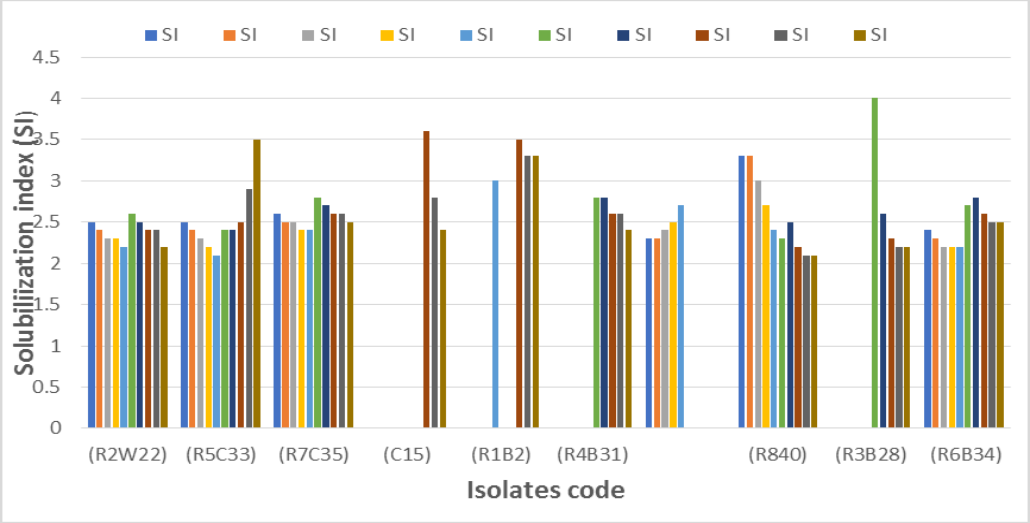

Fig. 4: Index of phosphate solubilization on both PVK and NBRIP media after 7 days of incubation

Biochemical and molecular identification of phosphate solubilizing bacterial isolates (PSB):

Gram staining was used, nine phosphate solubilizing bacterial isolates inoculated on the glass slide of the smear. The results showed that, 7 out of 9 phosphate solubilizing bacteria were gram-negative (R1B2, R5C33, R7C35, R8B40, R4B31, R6B34, and C15) and two (R2W22 and R3B28) were gram-positive. Based on the results of biochemical tests using API-20 system, isolates were identified as *Enterobacter cloacae* (R5C33, R7C35, R8B40, R1B2, R4B31, and R6B34), *Klebsiella pneumonia* (C15), *Bacillus cereus* (R2W22) and *Bacillus megaterium* (R3B28). The identification of eight selected bacterial isolates (R1B2, R2W22, R3B28, R4B31, R5C33, R6B34, R7C35 and R8B40, and C15) was confirmed by 16S rRNA gene sequence data. The sequences were submitted to the NCBI GeneBank (www.ncbi.nlm.nih.gov) under accession numbers MK064178 for *E. cloacae* R1B2, MK064181 for *E. cloacae* R4B31, MK064182 for *E. cloacae* R5C33, MK064183 for *E. cloacae* R6B34, MK064184 for *E. cloacae* R7C35, MK064185 for *E. cloacae* B40R8, MK064179 for *B. cereus* R2W22, and MK064180 for *B. megaterium* R3B28 (Table 3). The results of the phylogenetic tree based on 16S rRNA sequence analysis are shown in figures 5-8.

Table 3: BLAST search for the DNA sequence in NCBI (GenBank).

Name	Strain code	GenBank accession number	Identity %
E. cloacae (R1 B2)	R1B2	MK064178	99
B. cereus (R2W22)	R2w22	MK064179	99
B. megaterium (R3B28)	R3B28	MK064180	99
E. cloacae (R4B31)	R4B31	MK064181	99
E. cloacae (R5 C33)	R5C33	MK064182	99
E. cloacae (R6B34)	R6B34	MK064183	99
E. cloacae (R7 C35)	R7C35	MK064184	99
E. cloacae (R8B40)	R8B40	MK064185	99

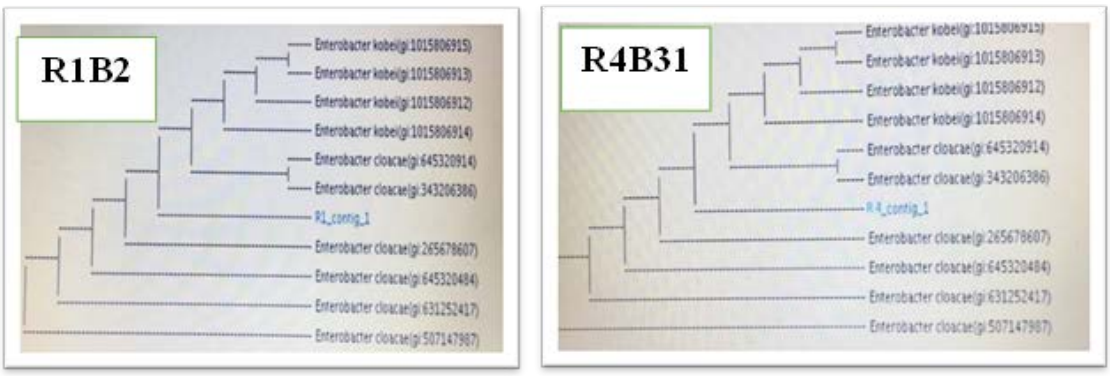

Fig. 5: Phylogenetic tree based on 16S rRNA sequence analysis and the relation between *E. cloacae* (R1B2, R4B31) and the most closely related bacterial species.

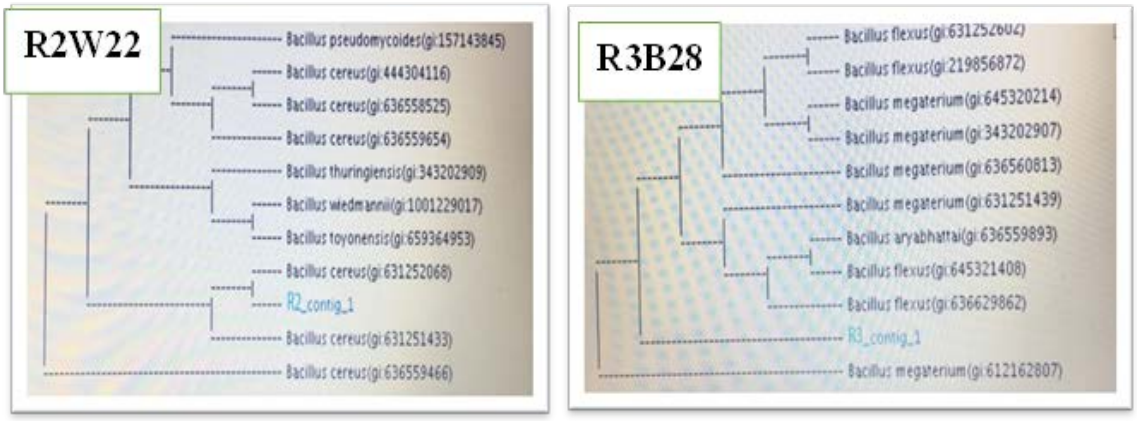

Fig. 6: Phylogenetic tree based on 16S rRNA sequence analysis and the relation between *B. cereus* (R2W22), *B. megaterium* (R3B28) and the most closely related bacterial species.

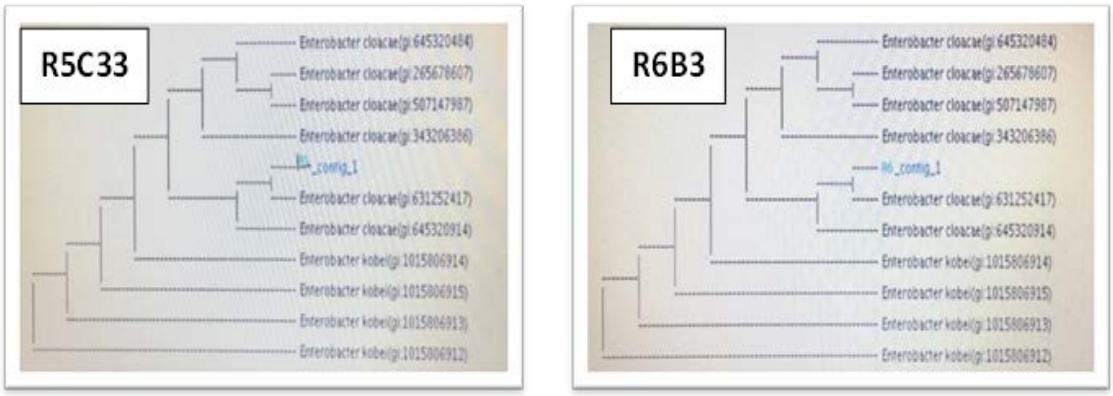

Fig. 7: Phylogenetic tree based on 16S rRNA sequence analysis and the relation between *E. cloacae* (R5C33, R6B34) and the most closely related bacterial species.

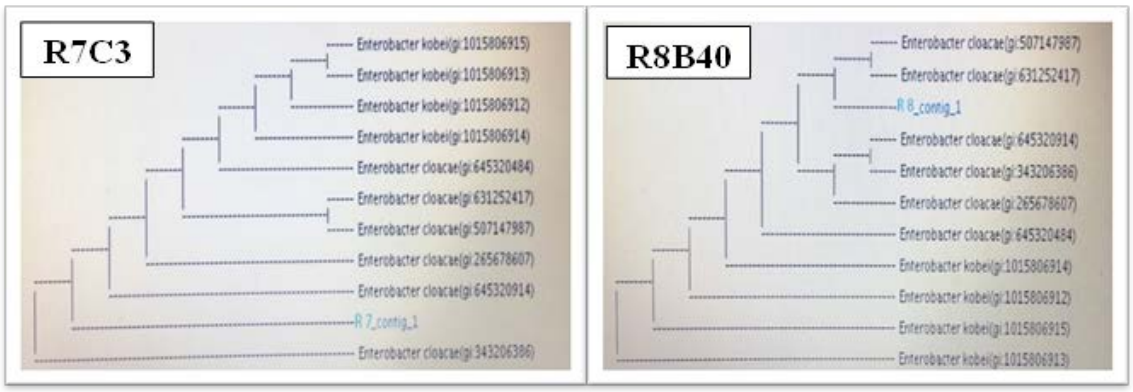

Fig. 8: Phylogenetic tree based on 16S rRNA sequence analysis and the relation between *E. cloacae* (R7C35, R8B40) and the most closely related bacterial species.

Intrinsic antibiotic resistance patterns of phosphate solubilizing bacteria (PSB):

Intrinsic antibiotic resistance (IAR) study of high PSB strains was carried out for 8 (R1B2, R2W22, R3B28, R4B31, R5C33, R7C35 and R8B40, and C15) PSB using 9 antibiotics. The results indicated that all strains were sensitive to Levofloxacin (Lev) and resistant to Taxo (A) and Clindamycin except (*E. cloacae* (R1B2); six strains were resistant to 6 antibiotics; *B. cereus* (R2W22), *E. cloacae* (R5C33, R4B31, and R7C35) were resistant to 6 antibiotics (Table 4). *E. cloacae* (R1B2) was resistant to 7 antibiotics and *B. megaterium* (R3B28) was resistant to 3 antibiotics. *E. cloacae* (A36) showed resistance to three antibiotics: ampicillin, neomycin and rifampin and sensitivity to five antibiotics: chloramphenicol, streptomycin, kanamycin, tetracycline and amikacin [26]. *B. cereus* showed resistance to 6 antibiotics: Vancomycin (VA), Clindamycin (CM), Fusidic acid (FA), ampicillin/sulbactam (SAM), Taxo A (A), and Metronidazole (MET), and sensitivity to Tobramycin (TMN) and Levofloxacin (Lev). The gram-positive bacterium, *B. cereus* was resistant to ampicillin,

tetracycline, chloramphenicol, erythromycin, kanamycin, and streptomycin [27]. *B. megaterium* (R3B28) strain showed resistance to 3 antibiotics: Metronidazole (MET), Taxo A (A) and Clindamycin (CM). Timmusk *et al.,* [28] reported that five strains of *B. megaterium* were sensitive to all used antibiotics (ampicillin, chloramphenicol, kanamycin, and tetracycline).

Isolation and screening of phosphate solubilizing fungi:

Soil samples were collected to isolate fungi and detect phosphate solubilization. Isolates identified as 7 strains of fungi: one isolate of *Aspergillus niger* (LA), three isolates of *A. flavus*, one of *Alternaria* sp., one isolate of *Penicillium* sp., and one isolate of *Fusarium sp*. Screening of fungal isolates for phosphate solubilization indicated that one (*Aspergillus niger* (LA) out of 7 isolates of fungi showed clear zone and solubilize phosphates using PVK medium.

Table 4. Sensitivity (S) and resistance (R) of PSB isolates for antibiotics.

Bacterial strains	LEV (µg)	MET (mcg)	VA (µg)	FA (µg)	TMN (µg)	TGC (µg)	A (µg)	SAM (µg)	CM (µg)	No. of antibiotic resistance
	Diameter of the inhibition zone (mm)									
B. cereus (R2W22)	(S)	(R)	(R)	(R)	(S)	(I)	(R)	(R)	(R)	6
E. cloacae (R5C33)	(S)	(R)	(R)	(R)	(I)	(I)	(R)	(R)	(R)	6
E. cloacae (R7C35)	(S)	(R)	(R)	(R)	(I)	(S)	(R)	(R)	(R)	6
Klebsiella pneumonia (C15)	(S)	(R)	(R)	(R)	(I)	(S)	(R)	(R)	(R)	6
E. cloacae (R4B31)	(S)	(R)	(R)	(R)	(S)	(I)	(R)	(R)	(R)	6
E. cloacae (R8B40)	(S)	(R)	(R)	(I)	(I)	(R)	(R)	(R)	(R)	6
E. cloacae (R1B2)	(R)	(R)	(R)	(R)	(I)	(R)	(R)	(R)	(R)	8
B. megaterium (R3B28)	(S)	(R)	(I)	(I)	(I)	(I)	(R)	(I)	(R)	3

Levofloxacin (Lev), Tobramycin (TMN), Tigecycline (TGC), Vancomycin (VA) and Clindamycin (CM) while 50 µg/ml Fusidic acid (FA), ampicillin / sulbactam (SAM) 20 µg/ml and Taxo A (A), and 10 µg/ml Metronidazole (MET). Diameters of the inhibition zone were measured inclusive of the diameter of the disc, according to which the isolates were considered Sensitive (>21mm), Intermediate (16-20) and Resistant (<15) (48).

Molecular identification of Fungi

One fungal isolate (LA) was selected to be identified using 18S rRNA gene sequencing. The molecular method confirmed the isolate to be *A. niger* with 100% identity to 18S rRNA gene sequences of *A. niger* in NCBI database. The accession number for *A. niger* (LA) is MK070508. The results of the phylogenetic tree based on 18S rRNA sequence analysis are shown in Fig. 9.

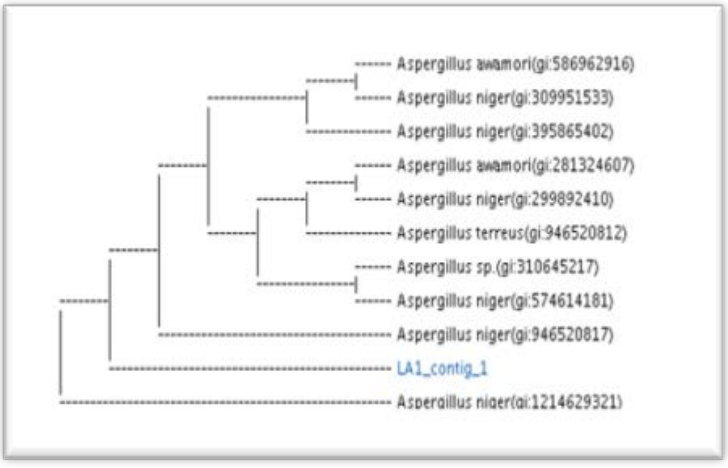

Fig. 9: Phylogenetic tree based on 18S rRNA sequence analysis and the relation between *A. niger* (LA) and the most closely related fungal species.

Fungal disk method for qualitative estimation of *A. niger* (LA) phosphate solubilization:

Colony and zone diameters (cm) were observed on PVK medium for *A. niger* (LA), after two, three and seven days, then the index was calculated. The results showed that after two days of incubation at 28 ˚C and pH of 5, 6, 7, and 8, SI was 3.1, 2.4, 2.3, and 2.3, respectively. SI after three days was 2.2 at all pH values (5 6, 7, and 8). After two days of incubation at 40 ˚C, SI values were 2.6, 2.3, 2.3, and 2.2 at pH of 7, 5, 6 and 8, respectively. The results showed that the maximum clearing zone was

at 28ºC and pH 7, and phosphate solubilization was greater at 28 ˚C than 40 ˚C. The best results were observed after two days, while there was a decline in third day (Table 5 and Fig. 10).

Spores suspension method for qualitative estimation of *A. niger* (LA) for phosphate solubilization:

After two days of incubation at 28 ˚C, SI values were 2.7, 2.5, 2.4, and 2.3 at pH 7, 5, 6, and 8, respectively. SI after three days were 2.3, 2.2, 2.2 and 2.1 at pH 7, 5, 8, and 6, respectively (Table 7 and Fig. 12). The results indicated that after two days of incubation at 40 ˚C, SI values were 2.3, 2.3, 2.2, and 2.1 at pH 7, 8, 6, and 5, respectively. SI after three days was 2.1 at all pH values (5, 6, 7, and 8) (Table 8 and Fig. 13).

Table 5: Solubilization index measurement of *A. niger* (LA) at 28 ˚C under different pH (5, 6, 7 and 8).

28 ˚C	Zone diameter	Colony diameter	SI 2days	Zone diameter	Colony diameter	SI 3days
pH 5	2	1.5	2.3	3.6	3	2.2
pH 6	2.4	1.7	2.4	4.7	4.2	2.1
pH 7	2.7	1.3	3.1	3.9	3.3	2.2
pH 8	2.7	2	2.3	3.6	2.9	2.2

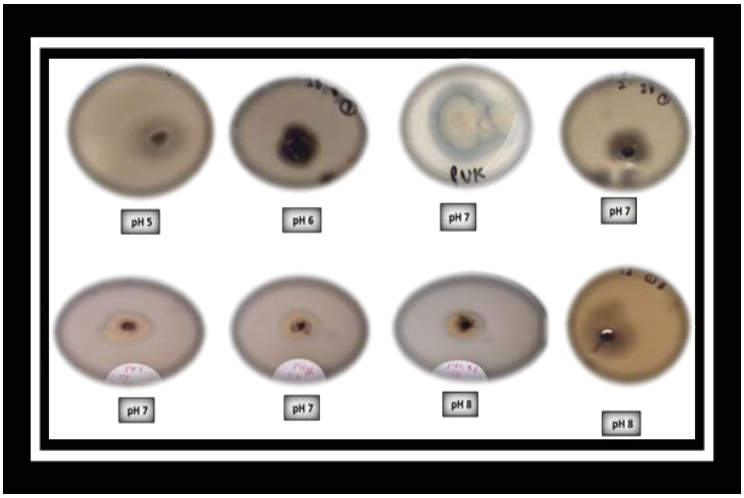

Fig. 10: Clear zone of *A. niger* (LA) at 28 ˚C under pH 5, 6, 7, and 8 on PVK medium.

Table 6: Solubilization index measurement of *A. niger* (LA) at 40 ˚C under pH (5, 6, 7 and 8).

40 ˚C	Zone diameter	Colony diameter	SI 2days	Zone diameter	Colony diameter	SI 3 days
pH 5	2.5	1.9	2.3	4.3	3.9	2.1
pH 6	2.4	1.8	2.3	5.7	4.9	2.2
pH 7	3.5	2.2	2.6	5.7	5.3	2.1
pH 8	3	2.5	2.2	4.7	4.2	2.1

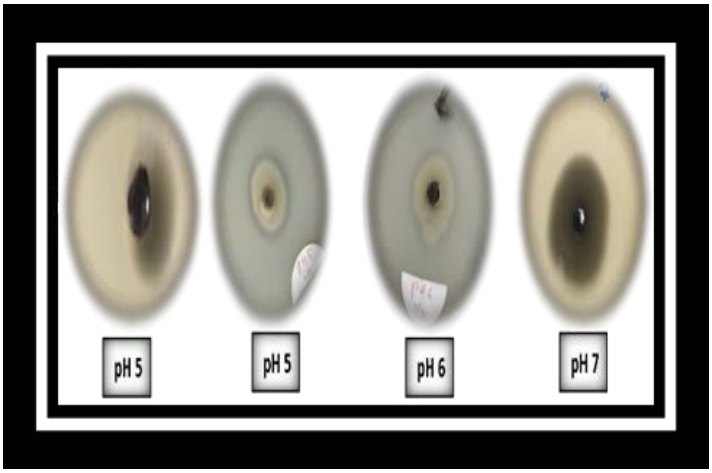

Fig. 11: Clear zone of *A. niger* (LA) at 40 ˚C under pH 5, 6, 7, and 8 on on PVK medium.

Table 7: Solubilization index measurement using spore suspension of *A. niger* (LA) at 28 ˚C under pH 5, 6, 7, and 8.

28°c	Zone diameter	Colony diameter	SI 2days	Zone diameter	Colony diameter	SI 3days
pH 5	1.5	1.1	2.5	3.9	3.3	2.2
pH 6	2.6	1.9	2.4	4.1	3.5	2.1
pH 7	3.4	2.0	2.7	4.4	3.4	2.3
pH 8	2.3	1.8	2.3	3.8	8.2	2.2

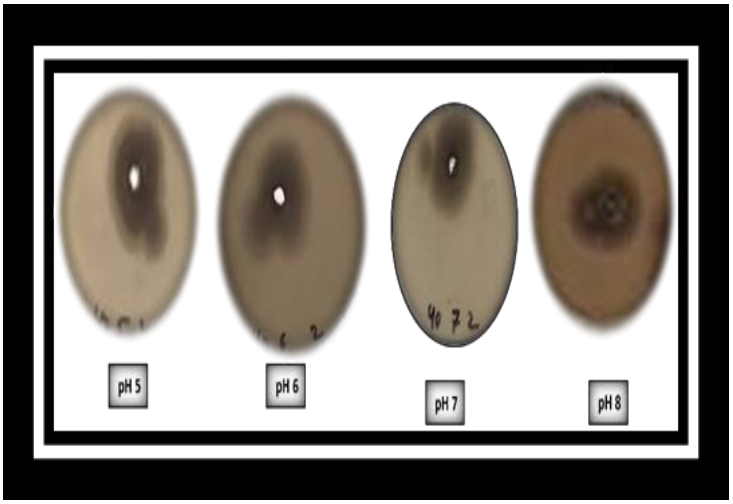

Fig. 12: Clear zone of *A. niger* (LA) at 28 ˚C under pH 5, 6, 7, and 8 on PVK medium.

Table 8: Solubilization index measurement using spore suspension of *A. niger* at 40 ˚C under different PH (5, 6, 7 and 8).

40 ˚C	Zone diameter	Colony diameter	SI 2days	Zone diameter	Colony diameter	SI 3 days
pH 5	2.6	2.3	2.1	3.9	3.5	2.1
pH 6	3	2.5	2.2	3.8	3.4	2.1
pH 7	2.8	2.1	2.3	4.2	3.6	2.1
pH 8	3.2	2.4	2.3	4.3	4	2.1

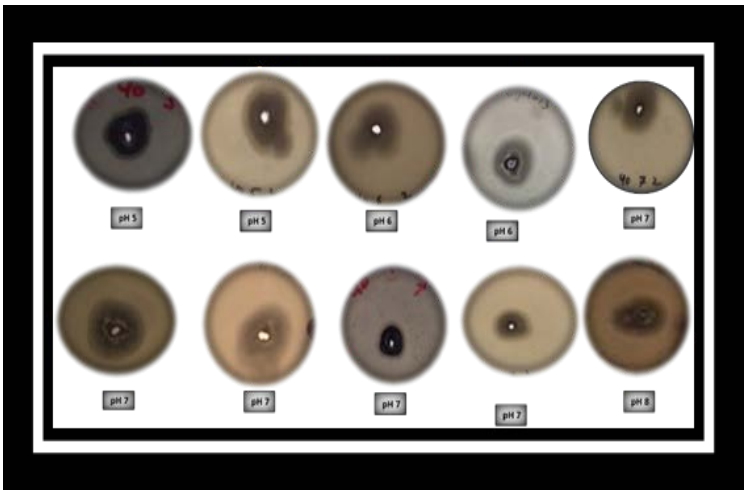

Fig. 13: Clear zone of *A. niger* (LA) at 40 ˚C under pH 5, 6, 7, and 8 on PVK medium

Discussion:

The plant growth promoting bacteria (PGPB) are rhizosphere and soil bacteria which will improve plant development [29]. *S. maltophilia* has been isolated from both *Ocimum* and *Mentha* plants and tested against pathogenic fungi [30]. As reported by Chabot et al. [31], bacteria solubilizing inorganic phosphate could raise phosphate content and profit crops like maize, lettuce, and legumes when inoculated alone. Sundara-Rao and Sinha [32] isolated these bacteria from the rhizosphere of the many terrestrial plants.

Biotechnological approaches could be used instead of chemical fertilizers [33]. PGPB were used to reduce the chemicals in agriculture [34]. P fertilizer in the soil increased the cost of agriculture and environmental problems [35-37]. PSM in the soil, transform P (insoluble) into an available form. The major P-solubilization mechanism in PSM is secretion of organic acids. Crop growth could be promoted by the presence of many PSM in the soil [38]. Nikmehr *et al.* [39] reported that phosphorus was the most essential macro element required for plants growth, and 0.1% of which is available to plants because of low solubility [40]. The most stable organic forms of phosphorus in soil, resemble 50% of the total organic P [41]. Root is the main organ for absorbing minerals and water and participates in material transport [42]. The fresh and dried plant weight and plant height are significantly increased with the improved root system [36, 43]. Previous studies reported that different species of bacteria (*Enterobacter* sp. and *B. cereus)* that solubilize phosphate, were isolated from rhizosphere of various plants such as sunflower, Chinese cabbage, and potato. *Pseudomonas* strains were isolated from lettuce, *Chrysanthemum*, *Eruca sativa* and maize plant rhizospheres [35, 36, 44, 45], and walnut [46]. *Enterobacter cloacae* was isolated from *Portulaca oleracea* [26].

Vazquez *et al*. [47] obtained phosphate-solubilizing bacterial strains *B. licheniformis, B. artrophaeus, Bacillus amyloliquefaciens, Panibacillus macerans, E. aerogenes, Entrobacter taylorae, E. asburiae,* and *P. stutzeri* from mangrove rhizosphere.

Our results were screened on PVK and NBRIP media, and reported that 9 out of 100 isolates had halo zone around the colonies; these isolates included 5 isolates (R1B2, R3B28, R4B31, R8B40, R6B34) from *Ocimum basilicum*, 3 isolates (R7C35, C15, R5C33) from *Cucumis sativus*, and 1 isolate *(*R2W22S) from *Eruca sativa*.

Seven isolates out of 9 showed a clear zone on PVK medium plates. IS ranged from 3 to 2.1 and from 4 to 2.6 on PVK and NBRIP media, respectively. The isolates (C15) and *(*R3B28) showed a clear zone on NBRIP and not on PVK media. Also, Karpagam and Nagalakshmi [48] reported that, six out of 37 isolates collected from the soil and screened on Pikovskayas agar medium containing tricalcium phosphate (TCP) (insoluble) were able to solubilize phosphate, and SI ranged from 1.13 to 2.23. Halo zone was better on NBRIP as reported by [17], who compared NBRIP with PVK medium and concluded a larger and clearer zone on NBRIP; this was because of the inhibitory effect of yeast extract on mineral phosphate solubilization by bacterial strains. Solubilization of phosphate by different genera of bacteria was based on organic acids' production such as gluconate, ketogluconate, acetate, lactate, oxalate, tartrate, succinate, citrate, and glycolate [2, 49, 50]. Carbon source utilized as control substrate of organic acid produced for P solubilization and the highest P solubilization has been observed when glucose, sucrose, or galactose were used as sole source of carbon in the medium [51, 52].

Based on biochemical tests, the isolates were identified as *E. cloacae* (R4B31, R5C33, R6B34, R7C35, R8B40, and R1B2), *K. pneumoniae* (C15*), B. cereus* (R2W22) and *B. megaterium* (R3B28). Identification of selected eight bacterial isolates (R1B2, R2W22, R3B28, R4B31, R5C33, R6B34, R7C35 and R8B40) was confirmed using sequencing of 16S rRNA region. UI Hassan and Bano [53] reported that, strongest soil bacteria in phosphate solubilization were *Enterobacter*, *Bacillus*, *Rhizobium*, and *Pseudomonas*. The best optimum conditions for *E. cloacae* to solubilize phosphate in this study were at 30 ˚C and pH 7, which was in agreement with [26] and [54] for *E. hormaechei.*

SI was different between *Enterobacter* isolates, ranging from 2.5 to 2.1 for *E. cloacae* (R5C33) and *E. cloacae* (R8B40), and from (2.6 to 2.4), (2.4 to 2.2), and (2.3 to 2.7) for *E. cloacae* (R7C35), *E. cloacae* (R6B34), and *E. cloacae* (R4B31), respectively. SI for *E. cloacae (*R1B2) was 3. SI for *B. cereus* (R2W22) ranged from 2.5 to 2.2. on PVK medium. SI ranged from 2.1 to 3 for all strains. SI was ranged from (2.4 to 3.5), (3 to 3.3), (2.8 to 2.5), (2.8 to 2.4), (2.7 to 2.5), and (3.3 to 2.4) for *E. cloacae* (R5C33), (R1B2), (R7C35), (R4B31), (R6B34) and (R8B40), respectively. SI ranged from 2.6 to 2.2 and from 4 to 2.2 for *B. cereus (*R2W22) and *B. megaterium* (R3B28), respectively. SI for *K. pneumoniae* (C15*)* ranged from 3.6 to 2.4 on NBRIP medium agar plates. Comparisons between solubilization indexes using both media reported that all isolates possess phosphate solubilization activity on NBRIP medium. Also, the highest SI was calculated for *B. cereus (*R2W22), on the first day of incubation on NBRIP agar medium. Also, 17 *Bacillus* isolates were isolated from soil samples, and the potential of phosphate-solubilizing ability was observed on the plates of PVK agar medium and UBPSB-07 isolate showed relatively high SI (1.4 in 72 hrs.) [55]. The phosphate solubilization of *B. cereus* [56] and *B. megaterium* [57] has been reported. *B. megaterium* was tested on (NBRIP) medium containing phosphate, pH was gradually declined, and the clear zone (which indicated phosphate solubilization) was significantly increased [58]. Jena and Rath [59] found that, the best day of phosphate solubilization was third day of incubation for the five bacterial isolates. Also, Walpola and Arunakumara [60] reported that *E. ludwigii* and *E. hormaechei* strains on the second and third days of the incubation showed the highest efficiencies. This study also recorded the highest efficiencies in phosphate solubilization for *E. cloacae (*R6B34) and *E. cloacae* (R7C35) isolates after one day of incubation, for *E. cloacae* (R840) isolate at the first and second days, for *E. cloacae* (R5C33) isolate at the first and 7th days, for *E. cloacae* (R4B31) at the 7th and 1st days, and for *E. cloacae (*R1B2) after 7 and 3 days on PVK and NBRIP media. But, *R. leguminosarum* had highest dissolve of phosphates on day 7 of incubation on PVK agar medium [61].

Filamentous fungi were highly important in phosphate solubilization. The samples from soils were collected to isolate fungi and detect phosphate solubilization. One *A. niger* (LA) isolate out of 7 isolates of fungi showed a clear zone on PVK medium, which was in accordance with Nopparat *et al.* [62], who identified 145 phosphate solubilizing fungal isolates, and *A. niger* had high phosphate solubilization. Furthermore, *Aspergillus* sp. isolates were capable of solubilizing all the natural

phosphates. *A. tubingensis* (AT1), *A. niger* and some *Penicillium* species showed solubilization activity [56, 63, 64]. *A. niger* was more dominant than *Penicillium* sp. and *Aspergillus* sp. [65]. *Fusarium* sp. along with *Aspergillus* and *Penicillium* sp. were isolated from tomato rhizosphere with a high proportion of phosphate solubilization [24]. Also, *A. niger* (LA) was identified using sequencing of 18S rRNA gene.

In this study, the colony and zone diameter (cm) was observed on PVK medium for *A. niger* (LA), and SI was calculated after incubation for two days at 28 ˚C as 3.1, 2.4, 2.3, and 2.3 at pH 7, 6, 5, and 8, respectively.SI after 3 days was 2.2 for all pH values. While after two days of incubation at 40 ˚C, SI were 2.6, 2.3, 2.3, and 2.2 at pH 7, 5, 6, and 8, respectively. The maximum clearing zones were at 28 ˚C, and pH 7, and phosphate solubilization was greater at 28 ˚C than 40ºC after two days, declining in three days. Naik *et al.* [66] reported that, SI gradually was increased with the increasing hour of incubation and *A. niger* showed a maximum solubilizing index of 2.0 after incubation for 5 days. In this study, SI was 3.0 after 1 day at 28 ˚C and pH 7. El-Azouni [23] noted that SI of phosphate-solubilizing fungal strains ranged between 2.42 to 3.15, and reported higher SI for *A. niger* strain than the *Penicillium italicum*. Both *A. niger* strains were grown at different pH and temperatures to solubilize TCP, and maximum clearing zones were observed at pH 7.2 and 20 ˚C. The maximum SI was calculated 2.2 and 1.12 by strains 1 and 2, respectively [67]. Two fungi out of 20, isolated from the saline soils, showed a significant zone of P solubilization, increasing up to the 7th day [68]. Phosphate solubilization depends on growth, nutritional, and physiological conditions of the culture [69]. The maximum SI was shown by *A. niger* (LA), ranged from 2.7 to 2.3 after 2 days at 28 ˚C. Elias, *et al.* [24] reported that solubilization index of 3 isolates of phosphate solubilizing fungi ranged from 1.10 to 3.05 at 7th day of incubation at 25–28 ˚C.

Bacteria are more effective than fungi in phosphorus solubilization [70]. Antarikanonda *et al* [71] reported that fungi are more active than bacteria in solubilizing phosphate. *Penicillium* sp. and *Aspergillus sp.* strains were active for phosphate solubilization in both solid and liquid media supplemented with tricalcium phosphate [72]. Inoculation with the soil mineral phosphate solubilizing fungus, *P. bilaii*, could increase phosphate absorption by plants [73]. *P. variable* poorly solubilizes inorganic phosphates in vitro [74]. Several bacterial strains such as *Pseudomonads* and *Bacillus* and fungal strains such as *Aspergilli* and *Penicillium* have been identified as PSM. Their performance under *in situ* conditions is not reliable, and therefore needs to be improved by using either genetically modified strains or co-inoculation techniques [75].

Conclusion

Improved knowledge is the best alternative method to reduce the application of chemical fertilizers, which in turn reduces the pollution of the environment and the cost of production, and increases the yield in terms of quality and quantity, in addition to the search for good ways to reduce the damage of chemical fertilizers such as the use of bacterial and fungal isolates due to good solubility of phosphate. These bacterial strains need to be improved genetically to obtain modified strains.

References

1. Theodorou, M. E. and Plaxton, W. C. (1993). Metabolic adaptations of plant respiration to nutritional phosphate deprivation. Plant Physiology, 101(2), 339-344.
2. Khan, A., Jilani, G., Akhta, M. S., Naqvi, M. S. and Rasheed, M. (2009). Phosphorus solubilizing bacteria: occurrence, mechanisms and their role in crop production. Journal of Agricultural Biological science, 11:48-58.
3. Richardson, A. E. (1994). Soil microorganisms and phosphorus availability. In: Pankhurst CE, Doubeand BM, Gupta VVSR, editors. Soil biota: management in sustainable farming systems. Victoria, Australia: Commonwealth Scientific and Industrial Research Organisation, 1994: 50–62.
4. Mclaughlin, B. R., Russell, P. R. and Carroll D. J. (1988). Cogen plant includes12-acre greenhouse, oyster farm. Power Engineering, 92:40–42.
5. Holford, I. R. (1997). Soil phosphorus: its measurement, and its uptake by plants. Australian Journal of Soil Research, 35:227–239.
6. Hayman, D. S. (1975). In: Soil microbiology (Ed. Walker, N.). Butterworth Scientific Publications London and Boston, 67-92.
7. Kannapiran, E. and Ramkumar, S. V. (2011). Solation of phosphate Solubilizing bacteria from sediments of Thondi coast, Palk Strait, Southeast coast of India. Annals of Biological Research, 25:157-163.
8. Zaidi, A., Khan, M. S., Ahemad, M., Oves, M. and Wani, P. A. (2009). Recent advances in plant growth promotion by phosphate-solubilizing microbes. In Microbial strategies for crop improvement, 23-50.
9. Alexander, M. (1977). Introduction to Soil Microbiology, 2nd edition, John Wiley, New York.
10. Reyes, I., Bernier, L., Baziramakenga, R. and Antoun, H. (2001). Solubilization of phosphate rocks and minerals by a wild-type strain and two UV-induced mutants of Penicillium rugulosum. Soil Biology and Biochemistry, 33:1741–1747.

11. Khan, M. S., Ahmad, E., Zaidi, A. and Oves, M. (2013). Functional aspect of phosphate-solubilizing bacteria: importance in crop production. In Bacteria in Agrobiology: Crop productivity Springer, Berlin, Heidelberg, 237-263.
12. Igual, J. M., Valverde, A., Cervantes, E. and Velázquez, E. (2001). Phosphate solubilizing bacteria as inoculants for agriculture: use updated molecular techniques in their study. Agronomies, 21:561-568.
13. Mikánova O. and Nováková, J. (2002). Evaluation of the P-solubilizing activity of soil microorganisms and its sensitivity to soluble phosphate. RostlinnáVýroba, 48:397-400.
14. Reddy, M. S., Kumar, S., Babita, K. and Reddy, M. S. (2002). Biosolubilization of poorly soluble rock phosphates by Aspergillus tubingensis and Aspergillus niger, 84(2):187-9.
15. Difco L. (1953). Deco manual of dehydrated culture media and reagents for microbiological and clinical laboratory procedures. (9).
16. Pikovskaya R.I. (1948). Mobilization of phosphorus in soil in connection with the vital activity of some microbial species, Mikrobiologiya, 17: 362-370.
17. Nautiyal, C.S. (1999). An efficient microbiological growth medium for screening phosphorus solubilizing microorganisms. FEMS Microbiology, 170:2017-2021.
18. De Freitas, J.R., Banerjee, M. R. and Germida, J. J. (1997). Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (Brassica napus L.). Biology and Fertility of Soils, 24(4):358-364.
19. Edi- Premono, Moawad, A. M. and Vlek, P. L. G. (1996). Effect of phosphate-solubilizing Pseudomonas putida on the growth of maize and its survival in the rhizosphere. Indonesian Journal of Crop Science. 11: 13–23.
20. Salle, A. J. (1992). Fundamental principles of bacteriology. VII Ed. Mc Graw Hill Publishing Co. Ltd. New York.
21. Weisburg, W. G., Barns, S. M., Pelletier, D. A. and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. Journal of bacteriology, 173(2), 697-703.
22. Bokhari, F. M. (1986). Studies on thermophilic and thermotolerant fungi in the soil of the Kingdom of Saudi Arabia (Western Region). MSc thesis, Jeddah, The Girls University.
23. El-Azouni, I. M. (2008). Effect of phosphate solubilizing fungi on growth and nutrient uptake of soybean (Glycine max L.) plants. Journal of Applied Science Research, 4: 592–598.
24. Elias, F., Woyessa, D. and Muleta, D. (2016). Phosphate solubilization potential of rhizosphere fungi isolated from plants in Jimma Zone, Southwest Ethiopia. International Journal of Microbiology, 1-11.
25. Kumari P. D., Nanayakkara J. M. and Bandara J. M. (2010). Development of a fungal inoculum for efficient phosphate utilization in agriculture. Proceedings of the 15th International Forestry and Environment Symposium, 15: 26-27.
26. El-Hamshary, O. I. M., Effat, S. A. M., Al-Muslam, H. J. K. and Al-Hebeshi, A. (2018). Genetic comparison among high phosphate solubilizing mutants of enterobacter cloacae via RAPD-PCR Analysis. Asian Journal of Microbiology, Biotechnology & Environmental Sciences, 20 (2): 643-654.
27. Al-ENZI, R. M. J. and Al-Charrakh, A. H. (2012). Heavy metal resistant bacteria pseudomonas aeruginosa as a model. Lap Lambert academic publishing, 1-93.
28. Timmusk, S., Paalme, V., Pavlicek, T., Bergquist, J., Vangala, A., Danilas, T. and Nevo, E. (2011). Bacterial distribution in the rhizosphere of wild barley under contrasting microclimates, PLOS One, 6 (3):17968
29. Souza, R. D., Ambrosini, A. and Passaglia, L. M. (2015). Plant growth-promoting bacteria as inoculants in agricultural soils. Genetics and Molecular Biology,38 (4):401-19.
30. Al-Sheheri, W. A., El-Hamshary, O. I., Al-Qourashy, N. H. and Ahmad, S. E. (2016). Identification and characterization of chitinolytic strains of stenotrophomonas maltophilia, isolated from mentha and ocimum plants of Saudi Arabia. International Journal of Scientific Research, 5(7):69-117.
31. Chabot, R., Antoun, H. and Cescas, M. P. (1996). Growth promotion of maize and lettuce by phosphate-solubilizing Rhizobium leguminosarum biovar phase oil. Plant Soil, 184: 311–32.
32. Sundara-Rao, W. V. B and Sinha, M. K. (1963). Phosphate dissolving microorganisms in the soil and rhizosphere. Indian Journal of Agriculture Science, 33:272–278.
33. Dobbelaere, S., Vanderleyden, J. and Okon, Y. (2003). Plant growth-promoting effects of diazotrophs in the rhizosphere. Critical reviews in plant sciences, 22(2): 107-149.
34. Welbaum, G. E., Sturz, A. V., Dong, Z. and Nowak, J. (2004). Managing soil microorganisms to improve productivity of agro-ecosystems. Critical Reviews in Plant Sciences, 23(2): 175-193.
35. Shahid, M., Hameed, S., Imran, A., Ali, S. and van Elsas, J. D. (2012). Root colonization and growth promotion of sunflower (Helianthus annuus L.) by phosphate solubilizing Enterobacter sp. Fs-11. World Journal of Microbiology and Biotechnology, 28 (8), 2749-2758.
36. Hanif, K., Hameed, S., Imran, A., Naqqash, T., Shahid, M. and Van Elsas, J. D. (2015). Isolation and characterization of a β-propeller gene containing phosphobacterium Bacillus subtilis strain KPS-11 for growth promotion of potato (Solanum tuberosum L.). Frontiers in Microbiology, 6: 583.
37. Majeed, A., Abbasi, M. K., Hameed, S., Imran, A. and Rahim, N. (2015). Isolation and characterization of plant growth-promoting rhizobacteria from wheat rhizosphere and their effect on plant growth promotion. Frontiers in Microbiology, 6: 198

38. Fernández, L., Agaras, B., Zalba, P., Wall, L. G. and Valverde, C. (2012). Pseudomonas spp. isolates with high phosphate-mobilizing potential and root colonization properties from agricultural bulk soils under no-till management. Biology and fertility of soils, 48(7): 763-773.
39. Nikmehr, S. and Akhgar, A. (2016). Effect of combined application of phosphate solubilizing bacteria and phosphrous fertilizer on growth and yield of Sesame. Majallah-i āb va Khāk, 29(4), 991-1003.
40. Paul, E. A. and Clark, F. E. (1988). Soil microbiology and biochemistry. Academaic Press Inc San Diego. Calif
41. Harley, J. L. and Smith, S. E. (1983). Mycorrhizal symbiosis. London, New York: Academic Press.
42. Heppell, J., Talboys, P., Payvandi, S., Zygalakis, K. C., Fliege, J. and Withers, P. J. A., et al. (2015). How changing root system architecture can help tackle a reduction in soil phosphate (P) levels for better plant P acquisition. Plant Cell Environ, 38: 118–128.
43. Shahid, M., Hameed, S., Tariq, M., Zafar, M., Ali, A. and Ahmad, N. (2014). Characterization of mineral phosphate-solubilizing bacteria for enhanced sunflower growth and yield-attributing traits. Ann. Microbiol, 65, 1525–1536.
44. Wang, Z., Xu, G., Ma, P., Lin, Y., Yang, X. and Cao, C. (2017). Isolation and characterization of a phosphorus-solubilizing bacterium from rhizosphere Soils and Its colonization of chinese cabbage (Brassica campestris sp. Chinensis sp.). Frontiers in Microbiology, 8:1270.
45. Cipriano, M. A., Lupatini, M., Lopes-Santos, L., da Silva, M. J., Roesch, L. F., Destéfano, S. A. and Kuramae, E. E. (2016). Lettuce and rhizosphere microbiome responses to growth promoting Pseudomonas species under field conditions. FEMS microbiology ecology, 92 (12).
46. Xuan, Y.u., Liu, X.u., Tian, H. Z., Guang, H. L. and Cui, M. (2011). Isolation and characterization of phosphate solubilizing bacteria from walnut and their effect on growth and phosphorus mobilization. Biology and Fertility of Soils, 47: 437-46.
47. Vazquez P., Holguin, G., Puente, M.E., Lopez-Cortes, A. and Bashan, Y. (2000). Phosphate-solubilizing microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon. Biol Fertil Soils, 30:460–468.
48. Karpagam, T. and Nagalakshmi, P. K. (2014). Isolation and characterization of phosphate solubilizing microbes from agricultural soil. International Journal Current Microbiology Applied Sciences, 3(3): 601-614.
49. Stella, M. and Halimi, M. (2015). Gluconic acid production by bacteria to liberate phosphorus from insoluble phosphate complexes. Journal of Tropical Agriculture and Food Science, 43 (1): 41–53.
50. Yadav, A.N. (2015). Bacterial diversity of cold deserts and mining of genes for low temperature tolerance. PhD. Thesis, Indian Agricultural Research Institute, New Delhi and Birla Institute of Technology, Ranchi.
51. Vyas, P. and Gulati, A. (2009). Organic acid production in vitro and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent Pseudomonas. BMC Microbiol, 9 (1), 174.
52. Park, K. H., Lee, O. M., Jung, H. I., Jeong, J. H., Jeon, Y. D., Hwang, D. Y., Lee, C. Y. and Son, H. J. (2010). Rapid solubilization of insoluble phosphate by a novel environmental stress-tolerant Burkholderia vietnamiensis M6 isolated from ginseng rhizospheric soil. Applied Microbiology and Biotechnology, 86 (3), 947–955.
53. Hassan, T. and Bano, A., (2015). The stimulatory effects of L-tryptophan and plant growth promoting rhizobacteria (PGPR) on soil health and physiology of wheat. Journal of Soil Science and Plant Nutrition, 15(1): 190-201.
54. Mardad, I., Serrano, A. and Soukri, A. (2014). Effect of carbon, nitrogen sources and abiotic stress on phosphate solubilization by bacterial strains isolated from a Moroccan rock phosphate deposit. Journal of Advanced Chemical Engineering, 1:102.
55. Thomas, S., Mathew, L., and Rishad, K. S. (2018). Isolation and molecular identification of phosphate solubilizing bacteria, Bacillus licheniformis UBPSB-07 capable of enhancing seed germination in Vigna radiata L. St. Teresa's College (Autonomous), Kochi, Kerala, 682 011.
56. Chuang, C.C., Kuo, Y.L., Chao, C.C., and Chao, W.L. (2007). "Solubilization of inorganic phosphates and plant growth promotion by Aspergillus niger," Biology & Fertility of Soils, 43(5): 575–584.
57. Han, H. S. and Supanjani, L. K. D. (2006). Effect of co-inoculation with phosphate and potassium solubilizing bacteria on mineral uptake and growth of pepper and cucumber. Plant soil environment, 52:130–136.
58. Radhakrishnan, R. (2014). Phosphate solubilizing bacillus megaterium mj1212 regulates endogenous plant carbohydrates and amino acids contents to promote mustard plant growth. Indian Journal of Microbiology, 0476-6.
59. Jena, S.K. and Rath, C.C. (2013). Optimization of culture conditions of phosphate solubilizing activity of bacterial sp. isolated from biosphere reserve in solid-state cultivation by response surface methodology. International Journal Current Microbiology Applied Sciences, 2 (5): 47-59.
60. Walpola, B.C. and Arunakumara, K.K.I.U. (2015). Assessment of phosphate solubilization and indole acetic acid production in plant growth promoting bacteria isolated from greenhouse soils of Gonju-gun, South Korea. Tropical Agricultural Research & Extension, 18(1): 31–39.
61. Belal, E.B., Hassan, M.M. and El-Ramady, H.R. (2013). Phylogenetic and characterization of salt-tolerant rhizobial strain nodulatingfaba bean plants. African Journal of Biotechnology, 12 (27): 4324-4337.

62. Nopparat, C., Jatupornpipat, M. and Rittiboon, A. (2013). Isolation of phosphate solubilization fungi in soil from Kanchanaburi, Thailand. KMITL Science and Technology Journal, 7: S2.
63. Richa, G., Khosla, B. and Reddy, M. (2007). "Improvement of maize plant growth by phosphate solubilizing fungi in rock phosphate amended soils," World Journal of Agricultural Sciences, 3. 481–484.
64. Pandey, A., Das, N., Kumar, B., Rinu, K. and Trivedi, P. (2008). Phosphate solubilization by Penicillium spp. isolated from soil samples of Indian Himalayan region. World Journal of Microbiology and Biotechnology, 24(1), 97-102.
65. Mahamuni, S. P., Khose, R. D., Menaa, F., and Badole, S. L. (2012). Therapeutic approaches to drug targets in hyperlipidemia. Bio Medicine, 2(4), 137-146.
66. Naik, S. K., Maurya, S., Kumar, R. I. T. E. S. H., Choudhary, J. S., Das, B. I. K. A. S. H. and Kumar, S. (2013). Evaluation of rhizospheric fungi from acid soils of Jharkhand on phosphate solubilization. The Bioscan, 8(3), 875-880.
67. Mohan Singh, S., Sahab Yadav, L., Kumar Singh, S., Singh, P., Nath Singh, P., and Ravindra, R. (2011). Phosphate solubilizing ability of two Arctic Aspergillus niger strains. Polar Research, 30(1), 7283.
68. Gupta, N., Sabat, J., Parida, R. and Kerkatta, D. (2007). Solubilization of tricalcium phosphate and rock phosphate by microbes isolated from chromite, iron and manganese mines. Acta Botanica Croatica, 66: 197-204.
69. Reyes, I., Bernier, L., Simard, R. R., Tanguay, P. and Antoun, H. (1999). Characteristics of phosphate solubilization by an isolate of a tropical Penicillium rugulosum and two UV-induced mutants. FEMS Microbiology Ecology, 28(3), 291-295.
70. Alam, S., Khalil, S., Ayub, N., and Rashid, M. (2002). In vitro solubilization of inorganic phosphate by phosphate solubilizing microorganism (PSM) from maize rhizosphere. International Journal of Agriculture and Biology, l4:454-458.
71. Antarikanonda, P., Sassanarakit, .S., Amaret, P. and Waiamai, P. (1991). Biological system for improving availability of rock phosphate to plants. In: Kinoshita, S. and A. Bhumiratana (eds.). Polar Research, 612–19.
72. Pradhan, N. and Sukla, L. B. (2006). Solubilization of inorganic phosphates by fungi isolated from agriculture soil. African Journal of Biotechnology, 5(10).
73. Cunningham, J. E. and Kuiack, C. (1992). Production of citric and oxalic acids and solubilization of calcium phosphate by Penicillium bilaii. Applied and Environmental Microbiology, 58(5): 1451-1458.
74. Vassilev, N., Fenice, M. and Federici, F. (1996). Rock phosphate solubilization with gluconic acid produced by immobilized Penicillium variable P16. Biotechnology Techniques, 10 (8) 585-588.
75. Sharma, S. B., Sayyed, R. Z., Trivedi, M. H. and Gobi, T. A. (2013). Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils, International Journal of Agriculture and Biology, 2(1), 587-594.