

Isolation, Characterization and Genetic Studies on Isolates of Phosphate Solubilizing Bacteria in Egyptian Calcareous Soils

Keywords: Phosphate solubilizing bacteria; Phosphate uptake plant growth promoting traits; 16s rDNA; Phylogenetic and calcareous soils

Abstract

Phosphorous (P) is an essential nutrient element and plays an important role in plant growth and development, it mostly presented in form unavailable for plants. Phosphate Solubilizing Bacteria (PSB) can be successfully used for solubilizing such forms rendering them available for plants. Thirty-two PSB strains were isolated on a Pikovskaya (PKV) agar medium containing Tricalcium Phosphate (TCP) and examined for plant growth promoting effects. A high portion of isolates (68.8%) produced Indole Acetic Acid (IAA) in contents ranging from 5 to 15 $\mu\text{g mL}^{-1}$ and 12.5% produced salicylic acid (SA) in contents < 100 $\mu\text{g mL}^{-1}$ while 50.0% fixed gaseous N_2 nitrogen in medium deprived completely of nitrogen. A portion of 28.1% produced cellulose enzyme and 15.6% produced chitinase enzyme. *In vitro* tests showed that isolates were capable in controlling some fungus plant pathogens and isolates were resistance to some adverse conditions involving pH, temperature and salinity. Use of 16s rDNA analysis and other procedures showed that the most 3 effective isolates were *Bacillus megaterium*-MH142578, *Acinetobacter lwoffii*-MH142579 and *Acinetobacter lwoffii*-MH142580. The results of cluster analysis (Similarity index) showed that were high and low similar values between the bacterial genera under studies.

Introduction

Phosphate (P) is one of the most important elements for plant growth. Although it is abundant in soils in both organic and inorganic forms, its availability is limited to plants as it presents mostly in insoluble forms [1]. Chemical fertilizers are added to the soils, plants can only utilize few amounts of phosphoric fertilizers, which are quickly converted into insoluble forms. Consequently, chemical fertilizers are frequently applied during crop planting, but its regular use is costly and produces undesirable environmental impacts, such as soil and water contamination. Therefore, P is often regarded a limiting nutrient in agricultural soils [2,3]. Soil microorganisms play a great role in availability of phosphate to plants [4].

Biofertilizers are considered to be the alternate source to meet the nutrient requirement of crops and to bridge the future gaps. Soil microorganisms which can solubilize phosphorus, fix atmospheric nitrogen or stimulate plant growth through synthesis of growth promoting substances could be used as safe alternative for the overuse of harmful agrochemicals [5-7]. Soil microorganisms play a great role in availability of phosphate to plants [8]. Microorganisms have the capacity to solubilize Phosphate, These include bacteria, fungi, actinomycetes and algae, and they are collectively called as Phosphate Solubilizing Microorganisms. Bacteria are more successful in P solubilization than others. The Plant Growth Promoting Rhizobacteria (PGPR) include Phosphate solubilizing bacteria [9,10].



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Consequently, the present work aims to study the prevalence of the phosphate solubilizing bacteria in various soils of Fayoum governorate, isolation of some individuals and studying some of their characters and activities (N_2 -fixation, siderophores, Salicylic Acid (SA) and Indole Acetic Acid (IAA) production. The capability of these isolates in controlling some plant pathogenic fungi (*in vitro*) was also determined. The resistibility of isolates to some adverse conditions prevailing in our conditions (pH, temp. and salt content) was also included. The isolates were identified by using 16s rDNA analysis after DNA bacteria isolation and the most 3 effective isolates *Bacillus megaterium*-MH142578, *Acinetobacter lwoffii*-MH142579 and *Acinetobacter lwoffii*-MH142580 were involved on proceeding study. Generally strains of PSB have plant growth-promoting activities and antagonistic potential against phytopathogenic fungi that could be used as alternate source for the overuse of harmful agrochemicals.

Materials and Methods

Collection and characterization of soils samples

Soil samples were collected from two sites distinguished by calcareous soils conditions, site 1 in Youssef El-Sedik and site 2 in Domo in Fayoum Governorate area; surface soil sample (0-30 cm) was collected from the two sites. Collected soil samples were air-dried, gently crushed and passed through a 2 mm sieve and stored in plastic bottles. Some physical and chemical properties of the soil were analyzed. Particle size distribution of the collected soil samples were carried out by the international pipette method [11]. Soil texture class was obtained. The pH values of soil samples were measured in the saturated soil paste using Bekman pH meter also Electrical conductivity values were determined in the saturated soil-water paste extract as dS/m, using CM25 conductivity meter [12]. Total calcium carbonates were determined using Collin's Calcimeter [13]. Soil organic matter contents were determined using the wet combustion method according to Walkly and Black's method [14]. Total nitrogen was determined using micro-kjeldahl method [15]. Available potassium was extracted using 1.0 N ammonium-acetate solution at pH 7.0 [12]. Available P was extracted with sodium bicarbonate

0.5 N (NaHCO₃) solution at pH 8.5 [16]. Available micronutrients (Fe, Mn, Zn and Cu) were extracted by DTPA solution [17]. Then measured with Inductively Coupled Plasma (ICP) atomic emission spectroscopy.

Isolation of phosphate solubilizing bacteria

Ten grams of each soil sample was suspended in 90 ml sterile saline solution and was shaken for 1 h. The serially diluted soil samples were placed on Petri dishes containing Pikovskaya's (PKV) agar medium by pour plate technique and incubated at 28 ± 2 °C for 48-96 h [18]. The bacterial colonies showing clear zone around them were considered as Phosphate Solubilizing Bacteria (PSB) as shown in [19] (Figure 1). Pure culture of the isolates was made by repeated sub culturing for 2-3 times on fresh PKV plate and was maintained on PKV slants at refrigerator temperature.

Assay of solubilization index (SI)

To determine the Solubilization Index (SI), the sterilized Pikovskaya's (PKV) agar medium was poured into sterilized Petri plates, containing insoluble Ca₃(PO₄)₂ at 5 g/L⁻¹. After solidification of the media, the plates were inoculated with the isolated bacteria, incubated at 28 ± 2 °C for two weeks and assayed visually. The solubilization index was determined by measuring the halo (clear zone) diameter and the colony diameter, using the following formula [20]. All assays were replicated three times.

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

Morphological, physiological and biochemical characters of isolates

Different morphological characters of isolates obtained were observed on NB agar media incubated for 48 hours at 30 °C. Cell shape was examined microscopically using gram stain in addition to

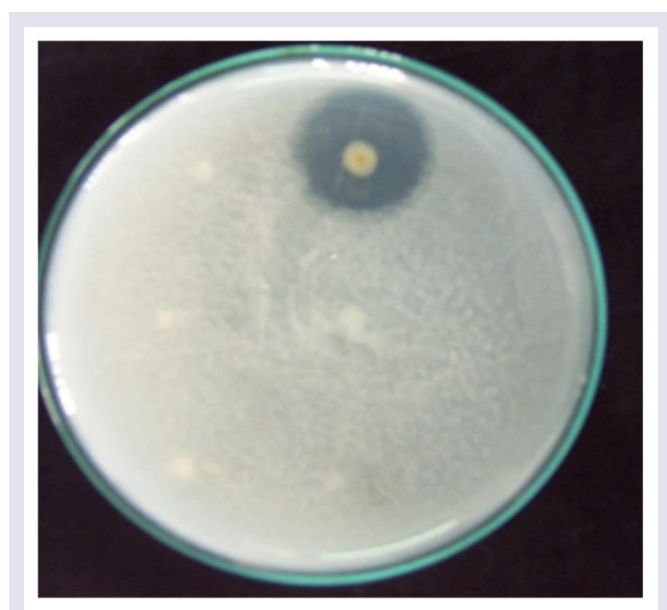


Figure 1: Isolation of Phosphate solubilization bacteria.

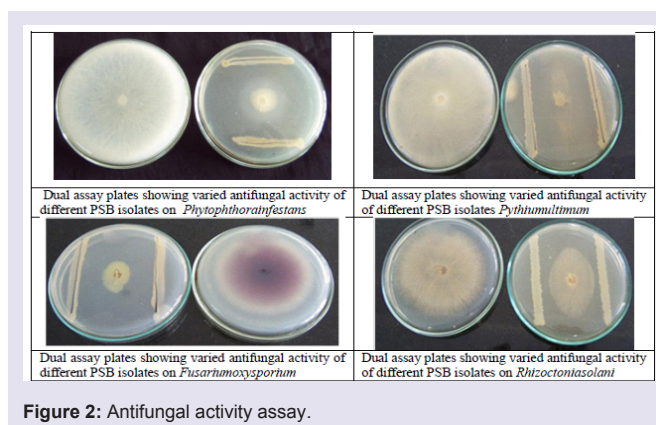


Figure 2: Antifungal activity assay.

motility. Catalase and oxidase activities were measured also.

Characterization of plant growth prompting traits

The capability to produce Plant Growth Promoting substances (PGPs) such as Indoleacetic Acid (IAA) production was measured by the colorimetric method [21], Siderophores production by different isolates was detected using the method described by Schwyn with several modification by Pallai [22, 23]. Salicylic acid (SA), isolates were grown at 28°C for 48h on a rotary shaker at 200 rpm in flasks containing 25 ml of the standard succinate medium, the absorbance of the purple iron-SA complex developed in the aqueous phase was measured at 527 nm, using SA dissolved in the growth medium and treated as described above as a control [24, 25]. Cellulose production was visualized by flooding the cellulose decomposition medium plates with 0.1% (w/v) Congo red for 15 to 30 min followed by bleaching the plates with 1M NaCl, according to the method of [26]. Chitinase production was assessed qualitatively by a microbiological method based on growth of isolates in chitinase medium amended with colloidal chitin [27]. The isolates were screened in vitro for N₂ fixation by growing them on plates of N-free agar medium for 48h at 28°C. The isolates that grow after being sequentially transferred 10 times to the same medium were considered presumptive positive for N₂ fixation [28-30].

Antifungal activity assay

Phosphate Solubilizing Bacteria (PSB) isolates were screened *in vitro* for antagonism towards four soil borne pathogenic fungi: *Pythium ultimum*, *Rhizoctonia solani*, *Fusarium oxysporum* and *Phytophthora infestans* in dual culture test [31]. Five µl of an exponentially growing bacterial culture was streaked along two opposite sides of surface-dried Potato Dextrose Agar (PDA) plates. Plates subsequently incubated at 28 °C for 24 h. Following bacterial growth, mycelial agar disc of 5-mm-diameter from a 7-day-old culture from the target fungi grown on PDA plate was placed in the center of the plate between the two parallel streaks of the test bacteria. Plates inoculated with target fungi alone served as control plates, and two replicate plates were used for each bacterial isolate. Plates were then incubated at 25 °C for 7 days. In vitro antagonistic activity was assessed by relating mycelial diameter on plates inoculated with bacteria to mycelial diameter on control plates and computing percentage inhibition as shown in (Figure 2).

Effect of pH, temperature and salt content on growth of selected strains

The PSB isolates were examined against some adverse environmental conditions to determine their capability to live, proliferate sustain life and perform their activities. They were tested against temperature, increasing pH and salt tolerant characters which prevail in our conditions. They were inoculated at fixed counts on 100 ml complete nutrient broth medium in 250 ml flasks which incubated for 24, 48 and 72 hours at elevated pH, temperature and salt content. Each character was studied separately and in combination with one or two of the other characters. Each treatment was replicated three times.

Genotypic characterization by 16s-rDNA

Bacterial DNA extraction and PCR amplification

The nucleic acid was extracted using protocol of beat beading with zirconia silica beads combined with ABT genomic DNA mini extraction kit spin column (Applied biotechnology, Egypt) according to the manufacture's instruction. A 16s rDNA reference was considered as full-length if it covered the position of the primer (27 F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492 R 5'-GGTTACCTTGTTACGACTT-3'), used in a previous study [32]. The PCR was performed in the thermal cycle 2720 (Applied Biosystem, USA) in a total volume of 25 µl containing 1 µl of each primer, 200 µM from the four ribonucleotide triphosphates (dNTPs), 5 µl of 10X PCR buffer, 1 µl of 25 m mol MgCl₂, 1 µl of template DNA, 1 µl of Taq DNA polymerase and 14.5 µl of water nuclease-free.

Amplification of DNA was carried out under the following conditions: denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1.5 min and final extension at 72 °C for 10 min. PCR products of bacterial isolates were checked by electrophoresis with 1.8% agarose gel and stained by ethidium bromide and then visualized and photographed under UV transilluminator. The PCR have specific bands at size around 1500 bp. The amplified PCR product was purified using Montage PCR Clean up Kit (Millipore), following manufacture instructions to remove unincorporated PCR primers.

Sequencing of 16s rDNA and Phylogenetic analysis

The purified PCR products were subjected to sequencing through Solvent Sequencing Service located in Korea. The sequences of 16s rDNA of the three bacteria isolates were submitted to GenBank.

The data of the nucleotide sequence of the ITS regions of rDNA obtained from the three isolates were compared with 16s rDNA collected from *Bacillus megaterium* and *Acinetobacter lwoffii* sequences available in GenBank database ([http://www.ncbi.nlm.nih.gov/ Blast](http://www.ncbi.nlm.nih.gov/Blast)). NCBI, Bethesda, MD, (USA) [33,34]. The 16s rDNA sequences of all bacterial isolates were aligned with reference sequences showing sequence homology from the NCBI database using Clustal omega multiple sequence alignment. Phylogenetic dendrogram was constructed by the clustal omega multiple sequence alignment programs (successor of Clustal W [35]). Phylogenetic trees were constructed by the Un-weighted Pair Group Method with Arithmetic Mean (UPGMA).

Results

Chemical and physical properties of the studied soils

(Table 1) illustrates the available characters of the two samples collected from various locations distinguished by calcareous soils conditions. The soils Chemical and physical properties studied as calcium carbonates%, pHe (in soil paste) ECe dS/m, organic matter contents%, Total Nitrogen mg/kg soil, available Phosphorus mg/kg soil, available Potassium mg/kg soil, available micronutrients such as (Fe, Mn, Zn, and Cu mg/kg soil). Soil texture classes of all samples were diverse between sand clay and sandy loam.

Isolation of phosphate solubilizing bacteria (PSB) and determination of their characters

Thirty-two bacterial isolates were obtained from two samples in PKV medium. The possible morphological and physiological characters of the isolates are summarized in (Table 2). The screened bacteria were able to solubilize TCP on solid culture state by forming clear halo zone, with different degree of solubilization, depending on the type of organism involved. However, all the selected isolates were found to be potent phosphate solubilizers showing clear halo zone around their colonies. Among of 32 potent isolates, strain NSE₁ showed the maximum phosphate solubilization activity as visualized by the size of clear zone developed around the colony, which showed solubilization index of 5.00 as showed in the same table.

Characterization of plant growth prompting traits

The capability of isolates to produce some plant growth promoting traits was also determined as shown in (Table 3). A total 32 isolates which isolated from different samples tested as plant growth promoters bacteria 21 (68.75%) of them were capable to produce IAA in detectable amounts (5 to 15 µg/ml). Four isolates (12.5%), out of total, produce salicylic acid in appreciable amounts (>100 µg/ml), while 13(40.62%) of them produce less than 100 µg/ml and the rest cannot produce the compound. Concerning Siderophores (Sid), it was found that 20 (62.5%) of the total PSB isolates able to produce Sid. The ability of different isolates to grow on N-free medium (putative N₂ fixers) was also examined in suitable liquid culture medium. The isolate capable of growing in medium free of any N-source for ten successive subculture was considered as putative N₂ fixer, whether the fixation was *via* N₂-ase or by scavenging nitrogen from the surroundings. In this study, out of 32 isolates tested 16 (50%) of isolates were able to grown on in free medium. Regarding lyticenzymes, among the enzymes tested number of cellulose producers were nine isolates (28.12%), whereas chitinase-producers recorded five isolates (15.62%).

Antifungal activity assay

Plant Growth Promoting Rhizobacteria (PGPR) has attracted the attention of many researchers because of the potential for developing these bacteria as inocula for plant disease control. Results presented in that 12 (37.5%) of tested isolates had a wide range of antagonistic activity against *Py. ultimum* (Table 4), while about 17 (53.12%), 20 (62.5%) and 17 (53.12%), against *R. solani*, *F. oxysporum* and *P. infestans* respectively.

Assessment of the PSB isolates according to their in vitro traits

Table 1: Chemical and physical properties of the studied soils.

Soil Properties	Site 1	Site 2
Texture class	Sandy Clay	Sandy Loam
Calcium carbonates,%	32.2	10.8
PHe (in soil paste)	8.11	7.86
ECe, dS/m	10.5	4.70
Organic matter contents, %	0.87	0.90
Total Nitrogen, mg/kg soil	14.1	14.8
Available Phosphorus, mg/kg soil	2.56	4.25
Available Potassium, mg/kg soil	28.0	42.57
DTPA extractable – Fe (mg/kg soil)	2.19	3.45
DTPA extractable – Mn (mg/kg soil)	1.58	2.25
DTPA extractable – Zn (mg/kg soil)	0.68	0.87
DTPA extractable – Cu (mg/kg soil)	0.12	0.15

Table 2: Morphological and Physiological characters of PSB Isolates.

Isolates	Colony ship	Cell ship	G-stain	Motility	Oxidase	Catalase	SI
NSE ₁	Circular	L. rod	+	+	+	+	5.00
NSE ₂	Circular	Short rod	-	-	-	+	4.60
NSE ₃	Circular	Short rod	-	-	-	+	4.50
NSE ₄	Circular	L. rod	+	+	+	+	3.60
NSE ₅	Punctiform	L. rod	+	+	+	+	4.25
NSE ₆	irregular	L. rod	+	+	+	+	3.00
NSE ₇	irregular	L. rod	+	+	+	+	4.20
NSE ₈	irregular	L. rod	+	+	+	+	4.00
NSE ₉	Circular	Short rod	-	+	+	+	4.60
NSE ₁₀	Circular	L. rod	+	+	+	+	2.38
NSE ₁₁	Circular	L. rod	+	+	+	+	3.00
NSE ₁₂	Circular	L. rod	-	+	+	+	2.40
NSE ₁₃	Circular	L. rod	+	+	+	+	3.60
NSE ₁₄	Circular	Short rod	-	-	-	+	2.6
NSE ₁₅	Circular	L. rod	+	+	+	+	2.60
NSE ₁₆	irregular	L. rod	+	+	+	+	2.40
NSE ₁₇	irregular	coccobacilli	-	-	-	+	4.40
NSE ₁₈	irregular	L. rod	+	+	+	+	4.60
NSE ₁₉	irregular	L. rod	+	+	+	+	4.25
NSE ₂₀	irregular	L. rod	+	+	+	+	4.40
NSE ₂₁	Circular	Short rod	-	+	+	+	3.20
NSE ₂₂	irregular	L. rod	-	+	+	+	4.00
NSE ₂₃	Circular	L. rod	+	+	+	+	4.20
NSE ₂₄	Circular	Short rod	-	+	+	+	4.60
NSE ₂₅	Circular	L. rod	+	+	+	+	3.80
NSE ₂₆	Circular	L. rod	+	+	+	+	4.25
NSE ₂₇	Circular	Short rod	-	+	-	+	4.25
NSE ₂₈	irregular	L. rod	+	+	+	+	3.33
NSE ₂₉	irregular	L. rod	+	+	+	+	4.20
NSE ₃₀	irregular	Short rod	-	-	-	+	3.67
NSE ₃₁	Circular	Short rod	-	-	-	+	2.86
NSE ₃₂	Circular	L. rod	+	+	+	+	1.83

SI: Solubilization Index; G-stain: Gram stain

that might be associated with ability to function as PGPR

In an attempt to select better bacterial isolates with high plant growth promotion potential, a bonitur scale similar to that described by Krechel was generated and used for assessment of PSB isolates [36] (Figure 3). In this scale, points were given to each bacterial trait in vitro determined within this study. Up to three points each were given for antagonistic activity toward each of the five indicator fungi, one point for each of the hydrolytic enzymes, and PNF, three points for IAA production, and Siderophores were given two points, one as antifungal trait and one for their use by plants for iron acquisition. This generated of bonitur scale of 21 points. As shown in (Table 5), results of the assessment revealed that out of the 32 isolates screened, NSE₁, NSE₂, NSE₃, NSE₂₀ and NSE₂₆ isolates have the top three ranks according to assessment values.

The five isolates characterized by high efficiency in different activities according to assessment values were tasted effect of pH, temperature and salt content on growth of strains under extreme conditions the results as shown in (Table 6).

The more efficient three strains (NSE₁, NSE₂ and NSE₃) were chosen to study their efficiencies in some important biological processes and activities. The activities studied were limited in N₂-fixation ability, cellulose activity, indole acetic acid, siderophores and salicylic acid production. In addition, their inhibition capacity for the growth of four pathogenic fungi which mentioned before. Worth mentioning that the three strains had the same efficiency whether in extreme or in normal conditions.

Molecular characterization and identification of bacterial isolates

PCR amplification of ribosomal DNA was carried out with universal forward and reverse primers of 16s rDNA and produced a fragment of approximately 1500 bp (Figure 4). This size corresponded to the expected size as compared to other bacteria [37]. The variability within the amplified regions was investigated by phylogenetic analysis. The amplified PCR product was run on 1.8% agarose gel viewed under the UV transilluminator.

Phylogenetic analysis of the rDNA sequences

The amplified PCR product of representative isolates was identified and sequenced. A BLASTN analysis carried out through GenBank (<http://www.ncbi.nlm.nih.gov>) revealed that all the isolates were members of two different genera, *Bacillus* genus and the genus

Table 3: Characterization PSB Isolates of plant growth prompting traits.

Isolates	Chitinase	Cellulase	PNF	Sid.	SA	IAA
NSE ₁	+	+	+	+	++	++
NSE ₂	+	+	+	+	++	++
NSE ₃	+	+	+	+	++	++
NSE ₄	-	-	-	+	+	+
NSE ₅	-	-	+	+	+	++
NSE ₆	-	-	-	+	-	+
NSE ₇	-	-	-	+	-	+
NSE ₈	-	-	-	-	+	+
NSE ₉	+	+	+	+	+	++
NSE ₁₀	-	-	-	+	-	-
NSE ₁₁	-	-	-	-	+	+
NSE ₁₂	-	-	-	+	-	+
NSE ₁₃	-	-	-	+	-	-
NSE ₁₄	-	-	-	-	+	-
NSE ₁₅	-	-	-	+	-	+
NSE ₁₆	-	-	+	-	-	-
NSE ₁₇	-	-	+	-	-	-
NSE ₁₈	-	-	+	-	-	-
NSE ₁₉	-	-	-	-	-	+
NSE ₂₀	+	+	+	+	++	++
NSE ₂₁	-	-	+	+	+	++
NSE ₂₂	-	-	-	-	+	++
NSE ₂₃	-	+	+	+	+	+
NSE ₂₄	-	+	+	+	+	++
NSE ₂₅	-	-	+	-	+	-
NSE ₂₆	-	+	+	+	+	++
NSE ₂₇	-	-	+	-	-	++
NSE ₂₈	-	-	-	+	-	+
NSE ₂₉	-	-	-	+	+	-
NSE ₃₀	-	-	-	-	-	+
NSE ₃₁	-	+	-	+	-	-
NSE ₃₂	-	-	+	-	-	-

IAA = Indole acetic acid, - = Negative, + ≤5, ++ ≤10, +++ ≤15 ug/ml
 Sid = Siderophores, - = Negative, + = Positive
 SA = Salicylic acid, - = Negative, + ≤100 ++ >100, ug/ml
 PNF = Putative N₂ Fixation, - = Negative, + = Positive after 10 sub culture in free nitrogen media

Acinetobacter as shown in (Table 7). Phylogenetic tree divided into two clusters. The first cluster includes 14 isolates of *Acinetobacter lwoffii* and two isolates NSE₂ and NSE₃ with accession numbers (MH142579 and MH142580). The second cluster includes 7 isolates of *Bacillus megaterium* and our isolate NSE₁ with accession number MH142578 as shown in (Figure 5).

Discussion

Phosphorus is commonly deficient in most natural soils, since it is fixed as insoluble iron and aluminum phosphates in acidic soils

Table 4: Screening of PSB isolated for antagonist towards phytopathogenic fungi.

Isolates	Phytopathogenic fungi							
	<i>Py. ultimum</i>		<i>R. solani</i>		<i>F. oxysporum</i>		<i>P. infestans</i>	
	MDC=70mm	MDC=60mm	MDC=55mm	MDC=70mm	MDB ² (mm)	GI ³ (%)	MDB(mm)	GI(%)
NSE ₁	17	76	21	68	05	91	05	92
NSE ₂	13	81	12	80	25	55	05	92
NSE ₃	29	59	15	79	10	82	05	92
NSE ₄	70	NI ⁴	60	NI	55	NI	70	NI
NSE ₅	70	NI	37	47	40	17	49	18
NSE ₆	34	48	60	NI	55	NI	51	15
NSE ₇	70	NI	60	NI	55	NI	70	NI
NSE ₈	70	NI	60	NI	35	46	70	NI
NSE ₉	48	31	39	35	21	62	05	92
NSE ₁₀	70	NI	60	NI	55	NI	70	NI
NSE ₁₁	70	NI	60	NI	55	NI	70	NI
NSE ₁₂	70	NI	60	NI	55	NI	70	NI
NSE ₁₃	70	NI	60	NI	55	NI	70	NI
NSE ₁₄	70	NI	60	NI	55	NI	70	NI
NSE ₁₅	70	NI	60	NI	45	18	48	20
NSE ₁₆	70	NI	60	21	39	39	49	18
NSE ₁₇	70	NI	60	21	39	39	49	18
NSE ₁₈	70	NI	60	NI	55	NI	70	NI
NSE ₁₉	70	NI	60	NI	40	38	70	NI
NSE ₂₀	25	64	25	58	15	73	05	92
NSE ₂₁	15	79	28	60	38	31	47	22
NSE ₂₂	34	48	60	NI	55	NI	51	15
NSE ₂₃	31	56	43	34	23	58	15	79
NSE ₂₄	11	83	15	79	32	42	35	42
NSE ₂₅	70	NI	41	41	39	29	70	NI
NSE ₂₆	17	76	23	62	25	55	31	56
NSE ₂₇	29	59	27	55	35	36	45	36
NSE ₂₈	70	NI	60	NI	35	36	51	15
NSE ₂₉	70	NI	45	25	40	17	70	NI
NSE ₃₀	70	NI	60	NI	55	NI	70	NI
NSE ₃₁	70	NI	33	45	45	18	70	NI
NSE ₃₂	70	NI	25	58	55	NI	70	NI

1. MDC = Mycelial Diameter on Control plates
2. MDB = Mycelial Diameter on Bacteria inoculated plates
3. GI = Growth Inhibition percentage
4. NI = No Inhibition

(pH lower than 5.0) or calcium phosphates in alkaline soils (pH above 7.0), as in the Egyptian soils. Phosphate Solubilizing Bacteria (PSB) play a great role in enhance the availability of phosphorus to plants by converting it into soluble form and increasing the crop yield [38,39]. Phosphate Solubilizing Bacteria (PSB) was found in majority of soils, various PSB have been isolated from different plant roots [3,40]. Hence, PSB can be regarded as one kind of plant growth-promoting rhizobacteria, which are widely considered as alternatives to common biofertilizers, also PSB have been found to alleviate harmful impacts of agrochemical [41-44].

In this study 32 strains were isolated as potential phosphate solubilizers based on their ability to solubilize tricalcium phosphate [Ca₃(PO₄)₂] by formation of a clear zone of solubilization around the colony on Pikovskaya agar medium, Solubilization Index (SI) was determined between 1.83 to 5.00.

The PSB isolates were screened for a wide array of traits that might be associated with ability to function as PGPR. Although many studies have been conducted to identifying the specific traits by which PGPR promote plant growth, usually they were limited to studying just one or two of these traits [45].

Table 5: Assessment of phosphate solubilizing bacteria (PSB) isolates.

Rank	Total Ass* (20)	Metabolites and hydrolytic enzymes				Antagonistic activity				Activities related to plant nutrition and growth promotion			Isolates
		Cellulase (1)	Chitinase (1)	SA (1)	Sid (1)	P (3)	F (3)	R (3)	Py (3)	Sid (1)	IAA (2)	PNF (1)	
1	19	1	1	1	1	3	3	2	3	1	2	1	NSE ₁
1	19	1	1	1	1	3	2	3	3	1	2	1	NSE ₂
1	19	1	1	1	1	3	3	3	2	1	2	1	NSE ₃
9	4	0	0	1	1	0	0	0	0	1	1	0	NSE ₄
7	8	0	0	1	1	1	1	1	0	1	2	1	NSE ₅
9	4	0	0	0	1	0	0	0	1	1	1	0	NSE ₆
10	3	0	0	0	1	0	0	0	0	1	1	0	NSE ₇
10	3	0	0	1	0	0	1	0	0	0	1	0	NSE ₈
4	14	1	1	1	1	3	2	1	1	1	1	1	NSE ₉
11	2	0	0	0	1	0	0	0	0	1	0	0	NSE ₁₀
11	2	0	0	1	0	0	0	0	0	0	1	0	NSE ₁₁
10	3	0	0	0	1	0	0	0	0	1	1	0	NSE ₁₂
11	2	0	0	0	1	0	0	0	0	1	0	0	NSE ₁₃
12	1	0	0	1	0	0	0	0	0	0	0	0	NSE ₁₄
10	3	0	0	0	1	0	0	0	0	1	1	0	NSE ₁₅
11	2	0	0	0	0	0	1	0	0	0	0	1	NSE ₁₆
11	2	0	0	0	0	0	1	0	0	0	0	1	NSE ₁₇
11	2	0	0	0	0	0	0	0	0	0	1	1	NSE ₁₈
11	2	0	0	0	0	0	1	0	0	0	1	0	NSE ₁₉
2	18	1	1	1	1	3	3	2	2	1	2	1	NSE ₂₀
6	12	0	0	1	1	0	1	2	3	1	2	1	NSE ₂₁
9	4	0	0	1	0	0	0	0	1	0	2	0	NSE ₂₂
5	13	1	0	1	1	3	2	1	2	1	1	1	NSE ₂₃
5	13	1	0	1	1	1	1	3	3	1	0	1	NSE ₂₄
8	5	0	0	1	0	0	0	1	0	0	2	1	NSE ₂₅
3	16	1	0	1	1	2	2	2	3	1	2	1	NSE ₂₆
7	8	0	0	0	0	1	1	2	2	0	1	1	NSE ₂₇
9	4	0	0	0	1	0	1	0	0	1	1	0	NSE ₂₈
10	3	0	0	1	1	0	0	0	0	1	0	0	NSE ₂₉
12	1	0	0	0	0	0	0	0	0	0	1	0	NSE ₃₀
9	4	0	0	1	1	0	0	1	0	1	0	0	NSE ₃₁
10	3	0	0	0	0	0	0	2	0	0	0	1	NSE ₃₂

PNE = Putative N₂-fixation, - = 0, + = 1

IAA = Indoleacetic acid, IAA, 1,2,3 according to color intensity

Sid = Siderophores,

SA = Salicylic acid

Py = *Pythium ultimum*, R = *Rhizoctonia solani*, F = *Fusarium oxysporum*, P = *Phytophthora infestans*;

Growth inhibition % , 1= 30 – 49.9%, 2=50 – 69.9%, 3=>70

Ass = Assessment

Table 6: Effect of pH, temperature and salt content on growth of selected PSB strains.

Strain No.	pH 7.0				pH 10.0			
	30 °C		50 °C		30 °C		50 °C	
	-S	+S	-S	+S	-S	+S	-S	+S
NSE ₁	+	+	+	+	+	+	+	+
NSE ₂	+	+	+	+	+	+	+	+
NSE ₃	+	+	+	+	+	+	+	+
NSE ₉	+	+	+	-	+	+	+	-
NSE ₂₄	+	+	+	+	+	+	-	-

• +S +15% salt

• -S no salt

• Incubation period 72h

• Three replicates were used for each treatment.

In the present work, the 32 PSB isolates were screened for a wide array of traits that might be associated with ability to function as

Table 7: Identification of bacterial strains on the basis of 16s rDNA gene sequence similarity.

Strain code	Accession no.	Bacterial name
NSE ₁	MH142578	<i>Bacillus megaterium</i>
NSE ₂	MH142579	<i>Acinetobacter lwoffii</i>
NSE ₃	MH142580	<i>Acinetobacter lwoffii</i>

PGPR several bacterial strains have been reported to fix N₂. Among of 32 PSB isolated in the present study [45-47], 16 (50%) were found Presumptive Nitrogen Fixers (PNF). Indole Acetic Acid (IAA) is one of the most physiologically active auxins. IAA is a common product of L-tryptophane metabolism by several microorganisms including PGPR [48]. It is presumed that PGPR producing plant growth promoting agents play a critical role in plant growth promotion. In the present work, the 32 PSB isolates were screened for their ability to

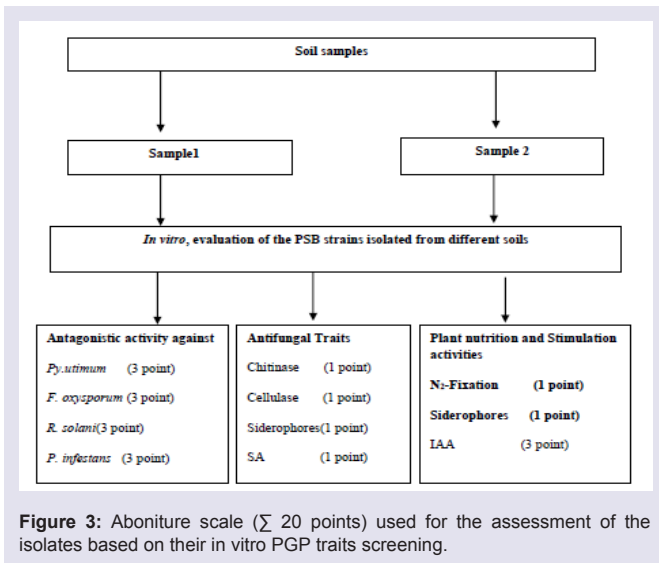


Figure 3: Aboniture scale (Σ 20 points) used for the assessment of the isolates based on their in vitro PGP traits screening.

Table 8: Genetic similarity percentage of 7 isolates of *Bacillus megaterium* and isolate NSE1 with accession number MH142578 based on ITS region of genomic 16s rDNA gene.

	1	2	3	4	5	6	7	8
1	100	98.12	98.12	98.12	98.12	98.12	98.12	98.12
2		100	98.83	99.38	98.77	99.52	99.31	99.72
3			100	99.24	98.83	99.23	99.31	99.31
4				100	99.45	99.65	99.52	99.52
5					100	99.38	99.17	99.31
6						100	99.72	99.58
7							100	99.52
8								100

1: |MH142578|NSE1*, 2: |MG544100| *B.megaterium*, 3: |MG430262| *B.megaterium*, 4: |MG430259| *B.megaterium*, 5: |MG430255| *B.megaterium*, 6: |MG430252| *B.megaterium*, 7: |MG430250| *B.megaterium* and 8: |MG430248| *B.megaterium*

produce IAA. Results presented in this study show that 68.5% of the PSB isolates produced detectable levels of IAA in culture supernatants. These isolates varied greatly in their ability to produce IAA.

It was reported that the ability of bacterial strains to antagonize pathogenic fungi was related to the production of extracellular siderophores which deprive phytopathogenic microflora of iron, thus limiting their growth [49,50]. It was reported that plants use microbial Sid for iron acquisition [51], and Sid is among factors involved in ISR [52,53]. Concerning Siderophores (Sid), it was found that a 25 % of the total PSB isolates able to produce Sid.

With regard to SA, the data showed that ability to produce SA appears to be widespread among the PSB isolates. More than half of isolates (53%) were able to produce SA. Many studies indicated that SA plays an important role in plant defense response against pathogen attack and is essential for development of both SAR and ISR in plants [54,55].

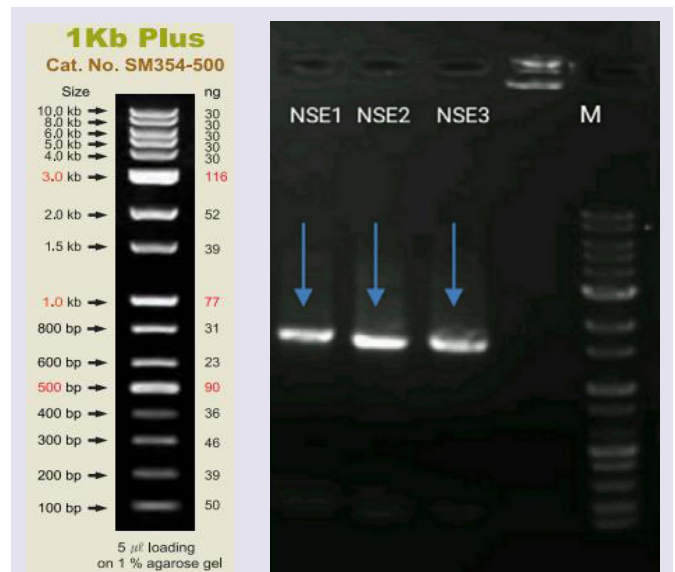


Figure 4: Agarose gel electrophoresis after 16s rDNA-PCR amplification of three bacterial isolates. Lane NSE₁ represents *Bacillus Megaterium* (MH142578), Lane NSE₂ represents *Acinetobacter lwoffii* (MH142579) and Lane NSE₃ represents *Acinetobacter lwoffii* (MH142580) respectively. Lane M represents the molecular size marker (1 kb leader).

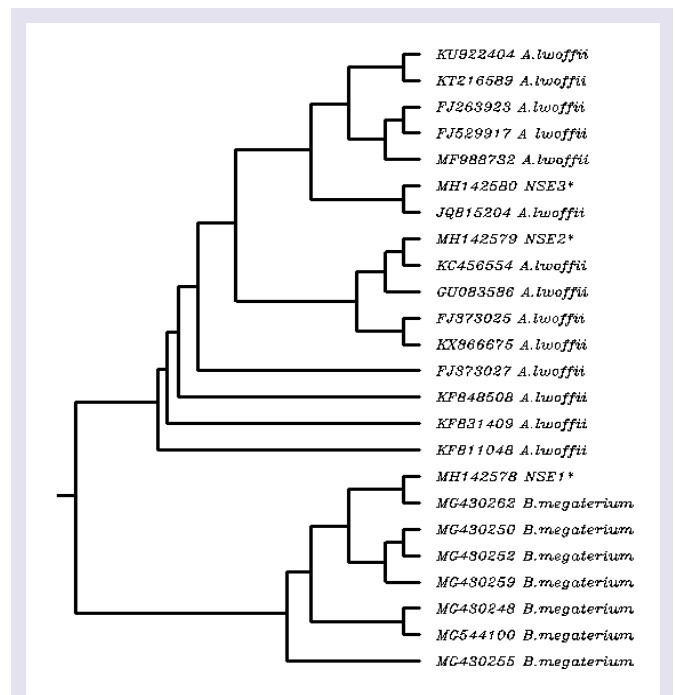


Figure 5: Phylogenetic analysis of bacterial isolates based on ITS region of genomic rDNA gene showing the relationship between our three isolates and 21 representative strains. The tree based on the clustal omega multiple sequence alignment programs. Rooted phylogenetic tree (UPGMA). *MH142578: *Bacillus Megaterium* (NSE₁), MH142579: *Acinetobacter lwoffii* (NSE₂) and MH142580: *Acinetobacter lwoffii* (NSE₃).

Biological control of plant pathogens has been the focus of many studies in plant protection that search for alternative or complementary methods to the use of chemical pesticides. PGPR have captured the attention of many researchers because of the potential for developing

Table 9: Genetic similarity percentage of 14 isolates of *Acinetobacter lwoffii* and two isolates NSE₂ and NSE₃ with accession numbers (MH142579 and MH142580) based on ITS region of genomic 16s rDNA gene.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	100	98.63	98.63	98.72	98.53	98.9	98.99	98.53	96.51	97.71	98.26	98.72	98.53	98.53	98.44	97.8
2		100	99.3	98.53	98.46	97.47	98.46	99.44	97.09	97.45	98.31	98.1	99.3	98.65	98.88	98.37
3			100	97.3	98.81	97.21	98.47	97.92	96.91	96.6	97.49	97.63	98.45	98.8	98.41	98.45
4				100	98.67	97.56	97.29	97.17	97.54	97.15	97.98	97.08	98.95	98.5	97.65	98.52
5					100	98.25	98.6	97.97	97.27	97.2	97.97	98.04	98.1	97.67	98.39	98.73
6						100	97.91	96.44	97.36	97.21	96.86	97	97.89	97.67	97.14	98.23
7							100	97.15	97	96.74	97.28	97.42	98.6	98.42	97.99	98.66
8								100	97.09	96.81	97.7	97.01	99.16	99.85	98.2	97.74
9									100	98.18	97.73	97.27	97.09	97.09	97.09	96.91
10										100	97.28	96.52	97.68	97.9	97.29	98.23
11											100	97.14	98.17	98.27	97.56	98.59
12												100	97.96	98.12	97.56	98.02
13													100	99.02	98.95	98.45
14														100	98.87	97.45
15															100	98.73
16																100

1:|MH142579|NSE2*, 2:|KU922404|A.lwoffii, 3:|FJ373027| A.lwoffii, 4:|KF831409|A.lwoffii, 5: KC456554|A.lwoffii, 6:|GU083586|A.lwoffii, 7:|FJ373025|A.lwoffii, 8:|KT216589|A.lwoffii, 9:|MH142580|NSE3*, 10: KF811048|A.lwoffii, 11: JQ815204|A.lwoffii, 12:|KF848508|A.lwoffii, 13:FJ529917|A.lwoffii, 14:|FJ263923|A.lwoffii, 15: |MF988732|A.lwoffii and 16: |KX866675|A.lwoffii

these bacteria as inocula for plant disease control. The PGPR under most scrutiny for potential use in agriculture are those belonging to the genera *Pseudomonas* and *Bacillus* [56]. In the present work, of the 25 isolates showed antifungal activity 12 (37.5%) 17 (53.12%), 20 (62.5%) and 17 (53.12%), against *Py. ultimum* *R. solani* *F. oxysporum* *P. infestans* respectively.

We can summarize the extremophiles and the extremophilic properties in some abbreviations as mentioned by Satyanarayana in the following: Psychrophiles, Thermophiles, Acidophiles, Alkalophiles, Baraophiles, Halophiles, Oligocarophiles, Oligonitrophiles, Radiation resistant, Methanogenic, Tolerant, Xerophiles and Organic Solvents Tolerant [57].

In the present study Five strains were chosen because they proved to have the higher activities in the different characters studied (IAA, SA, N₂-fixation ...etc) according to assessment values, All isolates were capable of growing at high temperature (50 °C) and pH 7.0 producing observable growth in absence of salt after 24 hours incubation period. This phenomenon was also observed when the pH increased to 10.0 but temperature was at 30 °C in absence of salt but the growth was pronounced after 48 hours, in all strains tested. In presence of 15% salt at pH 10.0 and 50 °C the picture seemed to somewhat different as three strains only were capable of producing observable growth. They were strains No NSE₁, NSE₂ and NSE₃ after 48 hours incubation period. All the three isolates (NSE₁, NSE₂ and NSE₃) showed an inhibitory (*in vitro*) against the test fungi. The three strains had the same efficiency whether in extreme or in normal conditions.

Molecular studies for the three bacterial isolates with its region of genomic rDNA gene showed band with approximately size 1500 bp using 16s rDNA - PCR two different bacterial genera, *Bacillus* and *Acinetobacter* showed closed phylogenetic tree between two isolates of *Acinetobacter lwoffii*, NSE₂ and NSE₃ and other subunit with NSE₁ from *Bacillus megaterium*. It means that there are relationships between the two bacterial isolates (*Acinetobacter lwoffii*, NSE₂ and NSE₃) and 14 representative strains, and there are relationships

between the one bacterial isolate (*Bacillus megaterium* NSE₁) and 7 representative strains.

The results of cluster analysis (Similarity index) showed that the highest similarity value 99.44 observed between 2 (KU922404) *A. lwoffii* and 99.85 between 8 (KT216589) *A. lwoffii*, 99.85 between 8 (KT216589) *A. lwoffii* and 14 (Fj263923) *A.lwoffii*. The Lowest similarity was 96.51 between 1 (MH142579) NSE₂ and 9 (MH142580) as shown in (Tables 8 and 9).

Conclusion

Considerable research efforts are underway globally to exploit the potential of Phosphate Solubilizing Bacteria (PSB) as alternative for chemical pesticides and chemical fertilizers. The main objective of this thesis was to find strains of Phosphate Solubilizing Bacteria (PSB), have broad spectrum of plant growth-promoting abilities and antagonistic potential against phytopathogenic fungi, which could be used as safe alternative for the overuse of harmful agrochemicals. In general, The most three effective isolates were identified as *Bacillus megaterium* MH142578, *Acinetobacter lwoffii* MH142579 and *Acinetobacter lwoffii* MH142580 which have plant growth-promoting abilities and antagonistic potential against phytopathogenic fungi involved on another proceeding study.

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