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Research Article

Sidrophore Production and Phosphate Solubilization by *Bacillus cereus* and *Pseudomonas fluorescens* Isolated from Iraqi Soils and Soil Characterization

Zaid Raad Abbas, Aqeel Mohammed Majeed Al-Ezee, Sawsan H Authman

College of science –Dept. Biology -Mustansiriyah University/Iraq

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ABSTRACT
This study was conducted to explore the ability of *Pseudomonas fluorescens & Bacillus cereus* to solubilizing a phosphate in soil for enhancing the planting growth &, its relation with soill characterization. The isolates were identified as *P.fluorescens* and *B. cereus* using convential analysis and, its phosphate solubilization ability and sidrophore was shown by the clear zone formation on National Botanical Research Institute's Phosphate medium. Moreover, *Pseudomonas fluorescens* isolates (n = 9) and three of *B. cereus* isolated from agricultural area in Baghdad university, Mustansiriyah university and Diyala bridge. Results displayed that bacterial count were varied in soil samples according to their region,,and ranging from 30 to 60 *10 2 CFU/g in Baghdad university soil to 10—20 *10 2 CFU/g in Mustansiriyah university soil ,.,the Baghdad soil macronutrient which included: NH4 , NO3 ,P,and K were ., 8.42 , 20.53 , 19.09 218.73 respectively, While the physio analysis revealed that the mean of pH was 7.3 and EC was 8.63. on the other hand the micronutrient analysis indicated that the soil samples were included Ca ,Fe ,Mn ,Zn and Cu which gave their mean 5025.9 , 8.9, 4.9, 0.5 and 1.5 respectevily. Results revealed that all isolated bacteria (9 isolates of *P.fluorescens* and three isolates of *B. cereus* gave ahalo zone which mean their ability to be phosphate solubilizing bacteria at 100%. Results revealed that all isolated bacteria were detected a ability to produce high levels from chelating agents((siderophores)) by *P.fluorescens* &. *B cereus* at 100%,when appeared ahalo clear zone. Furthermore, the high levels of phosphate solubilization and siderophore production were grouped in bacterial species isolated from Iraqi soils. might be attributed to many soil factors such as soil nutrient status, soil acidity, water content, organic matter and soil enzyme activities.

Keywrods:

INTRODUCTION
Soil microorganisms play important role in soil Phosphate movement and subse quent ava ilability of phosphate to growth of plants[1]. Inorganic forms of Phosphate are, solubilized by a type of organisms called hetero trophic microo rgamisms release some of organic acids that have ability to dissolve phosphatic minerals and chelate cationic partners of the ,Phosphore ions directly, and discharge Phosphorse into soil solution[2]. Phosphate solubilizing -bacteria (PSB) are actuality used as biofertilizer from*1950s[3]. Release of Phosphate by PSB, from insoluble and stable or adsorbed shapes is an importance feature regarding Phosphorse obtainability in soils., There are great evidences that bacteria,in soil are able of convert soil Phosphate to the shapes or forms & and stops it from adsorption or fixation[4]. Microbial community effects soil fertility through soil M.O increase the Phosphate avail'ability to root plants by mineralizing organic Phosphorse in soil and by solubilizing speeded the phosphates[5,6]. These type of bacteria in the being of labile(C)serve as a sink for Phosphate by fast immobilizing it even in little Phosphate soils[7]. Sub,sequently, PSB become a basis of Phosphate to plants upon its excreting from their cells. The PSB and plant growth promoting rhizobacteria (PGPR) together could decrease Phosphate fertilizer application even 50% without any important reduction of crop yield[8]. It infers that PSB inoculants bio fertilizers hold great prospects for sustaining harvest production with optimized Phosphate fertilization[9]. Mineralization of earth organic Phosphate (Po) plays an vital role in phosphorus movement of a farming system. Organic Phosphate may form 4,-90 % of the total soil Phosphate., Almost partial of the M.O in soil & plant roots have Phosphate mineralization potential under the act of phosphatases[10] Alkaline and acid phosphatases utilization organic phosphate as a substrate to change it into inorganic form[11]. Main mechanism for mineralization of soil root organic Phosphate is the production of enzymes (acid phosphatases). Secreting of organic anions, and creation of siderophores and acid phosphatase by plant roots bacteria or alkalinee phosphatase enzymes hydrolyz the soil organic Phosphate or fragmented Phosphate from organic residues. The major portion of extracellular

**Author for Correspondence: Zaidraad91@uomustansiriyah.edu.iq*

Table 1: *Pseudomonas fluorescens* and *Bacillus cereus* isolated from Baghdad soil.

Soil samples	*Pseudomonas fluorescens*	*Bacillus cereus*
	No .of isolates	No .of isolates
Baghdad university	3	2
Mustansiriyah university	3	1
Diyala bridge	3	0
Total	9	3

Table 2: The biochemical test for *Bacillus cereus* isolated from Iraqi soil.

Test	Results
Spore formation	+
motility	_
Oxidase	+
Catalase	+

soil phosphatases is resulting from the microbial population[12]. *Pantoea agglomerans* solubilizes hydroxyapatite and hydrolyz the organic Phosphate[13]. Combined cultures of PSMs (*Bacillus, Streptomyces, Pseudomonas* etc.) are maximum effective in mineralizing organic phosphate[14]. Present investigation was designed to study the population density of phosphate solubilizing bacteria especially *Pseudomonas* sp. and *Bacillus* sp. and find out the potential isolate for future inoculants on the basis of phosphate solubilization capacity and its relationship with soil characters of different ecologies .Also would loop out further roads for researchers interested to commercially create the Phosphate solubilized bacteria based bio fertilizers to be effective over awide collection of crops .

MATERIALS AND METHODS

Samples collection

The samples were collected from Baghdad areas (Baghdad University, Mustansiriyah University, Diyala bridage by using a sterile metalic tool at a depth of 10 to 20 centimeter below the surface of studied soils. This soil amples were put in a sterile bags and delivered to the laboratory within -30 minute from collecting. The samples were homogenized and sieved through asterile 2mm mesh. The sieved samples (2 grams) was dissolved in 18 milliliter of physiological normal saline and serially diluted from 5-10 dilution. According to[15] One tenth of amilliliter (0.1 milliliter) of the 2~10 to5~10 dilution was inoculated on to N agar plates. This plates were incubated for 24 hrs at 37^{o} C . The populations of target bacteria in the soil samples (CFU per gram from soil) were determined by enumerating the number of colony that formed on agar plates.

Preparation of soil samples for soil analysis

The collected soil samples were sieved from 2mm mesh size to remove the stones, plant residue and small organisms (earthworms etc.). Then soil samples were air dried grinded, thoroughly mixed and stored below 4o C until further analysis[15].

Physio-Chemical analysis of collected soil samples

Soil texture analysis was done by[16] method. All soil samples were analyzed for their pH and ECe by using 1:1 (w/v) with the helpof method described by[17,18] respectively. The organic matter of all the soil samples was determined by[19].

AB,-DTPA, extraction

AB-DTPA method was used for the determine of macro,nutrients and micro, nutrients[20]. (10grams) of soil samp le was weighed into (125 milliliter,) conical-, flask. Then 20ml from extract (0.005M DT-PA+1.0M NH_4HCO_3) and shaken on reciprocating shaker for (fifteen minute\), ((180)) cycles per minute. Extract was obtained by filtering through filter papers type whatman,, No.1. This process was used to determine of

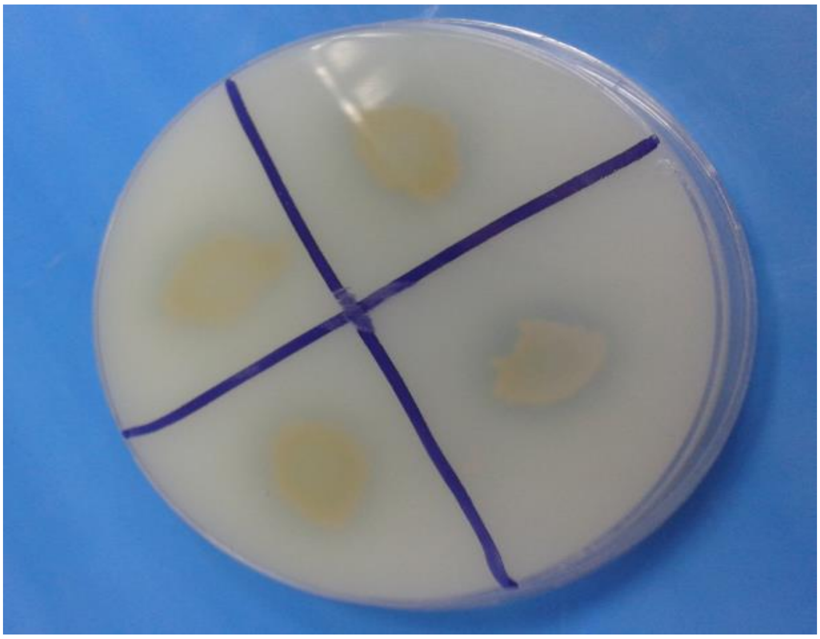

Figure 1:Phosphate solubilizing bacteria *(Pseudomonas fluorescens*)on Pikovskaya agar appere halo zone.

Table 3: API20-E system used for diagnosis of Pseudomonas fluorescens isolated from badhdad soil samples

Test	Substrate	Reaction/enzymes	Result
ONPG	Ortho-nitrophenyl-galactopyranoide	Eta-galactosidase β	Negative(-)
ADH	Arginine	Arginine dihydrolase	Red/Orange(+)
LCD	Lysine	Lysine decarboxylase	Yellow(-)
ODC	Ornithine	Ornithine decarboxylase	Yellow(-)
CIT	Sodium citrate	Citrate utilization	Negative(green-)
H_2S	Sodium thiosulphate	H_2S production	Colorless(-)
URE	Urea	Urease	Yellow(-)
TDA	Tryptophan	Tryptophan deaminase	Yellow(-)
IND	Tryptophan	Indol production	Colorless(-)
VP	Sodium pyruvate	Acetone production	Pink/red(-)
GEL	Kuhn's gelatin	Gelatinase	Black pigment (+)
GLU	Glucose	Fermentation/Oxidation	Yellow(+)
MAN	Manitol	Fermentation/Oxidation	Yellow(+)
INO	Inositol	Fermentation/Oxidation	Yellow(+)
SOR	Sorbitol	Fermentation/Oxidation	Yellow(+)
RHA	Rhamnose	Fermentation/Oxidation	Yellow(+)
SAC	Sucrose	Fermentation/Oxidation	Yellow(+)
MEL	Melibiose	Fermentation/Oxidation	Blue(-)
AMY	Amygdalin	Fermentation/Oxidation	Yellow(+)
ARA	Arabinose	Fermentation/Oxidation	Yellow(+)

Table 4 : Bacterial population in Baghdad soil samples.

Soil region	Total count {CFU/g}*102	Mean
Baghdad university	30-6	45
Mustansiriyah university	10-20	15
Diyala bridge	17-25	21

various nutrients ((zinc ,Fe ,N etc)),.

Identification of bacterial species

After incubation period, the plates of nutrient agar and Blood agar were examined for typical colonies of *Pseudomonas fluorensces* and *Bacillus cereus*.The colonies bearing typical Morphology were purified and subcultured on nutrient agar plates and stored for further assay. Pseudomonas agar in order to isolate *P. fluorescences*. Then plates were incubated at 37°C for 24 hrs.Taxonomic properties (morphology ,cultural ,physiological and biochemical) characteristic of the isolate was determined according to the method and media of the[21] and by API-20E system according to[22].

Colony morphology

Suspension in sterile water was prepared from each of the purified culture and grown on solid media by spread plate method. The inoculated plates were incubated at 37C until colonies appeared Colony morphological characters recorded were color, margins, Colony shape, and elevation as[23].

Microscopic characters

The bacteria purified from selective media were prepared for reactions with grams stain . The shape and color of colony were observed under light microscope#. Bacteria appear Pink consider Gram negative (-ve), while Bacteria appear), purple consider Gram Gram positive (+ ve)[24].

Biochemical tests of bacterial isolates

Biochemical characters were recorded using API 20E kit {biomerieux} the isolates incubated Liquid, cultures were added to the wells of kit following the instructions supplied by supply company. Any results were verified as prescribed in Bergey's Manual[12].

Isolation of phosphate solubilizing bacteria

The bacteria that have ability to Phosphate solubilizing were isolated from every testing sample by using method, called (dilution ,,plate counting method). Ten folds- serial from dilutions were prepared by using suspension from tested soil. From each dilution take(1mililiter) & spread on Pikovskayas medium by using the method of[25], containing insoluble tricalcium phosphate and incubated at 28 C for 48 hrs. Colonies showing halo zones were picked and purified on Pikovsk-ayas agar for studding the characters of isolates.

Detection the siderophore production by the isolates

Table 5: Physio-Chemical analysis of collected Baghdad soil samples.

Soil region	Chemical component (PPm)				Physio-analysis	
	NH4	NO3	P	K	pH	EC
Baghdad university	7.84	20.44	18.50	221.27	7.2	8.60
Mustansiriyah university	8.12	21.0	20.18	227.66	7.5	8.58
Diyala bridge	9.30	20.16	18.58	207.26	7.3	8.70
Mean	8.42	20.53	19.09	218.73	7.3	8.63

The chrome-azurol sulphate agar (CAS) was described by[26]. In brief, (60.5 milligrams) of CAS reagent was liquefied in(50 milliliter) of deionized water, & mixed briefly, with(10 milliliter) of a ((Fe^{3+})) solution (1 mmol L-1 Fe Cl3.6H2O, 10 mmol L-1 HCl) ,,. While stirring, this solution was briefly &slowly, mixed with ((72.9 milligrams) from powder hexa-decyltrimethyl-ammonium- bromide (HDTMA) previously dissolved in (40 milliliter) of distillate water.
The resulting solution appear dark-blue. This solution autoclaved, &,cooled to (45/60°C)and mix with (900 milliliter) sterile MM9 medium containing ((15 gram, /1 agar)),, (kept at 55/60°C). The resulting medium was allowed to gel on Petri dishes, was subsequently inoculated# with target isolate & incubated in the darkling place (in temperature 28°C for 120 hour). The formation of a clear- halo region around the bacterial growth were indicated (+) result ,, showing a visable change in color from dark blue to pink,,. Every assay was performed in triplicate.

RESULTS AND DISCUSSION

Microorganisms in the Soil Rhizospher, influence on crop production in agricultural field by root- growth stimulation and improving the food availability in soil, and many rhizo-bacteria were recognize their responsible on growth of plant[14]. In some, current study, was designed to study the population density of PSB and find out the potential isolates for future inoculants on the basis of phosphate solubilization capacity and its relationship with soil characters of different ecologies. Current study would loop out further avenues for researchers interested to commercially produce the Phosphate Solubilizing Bacteria depend bio-fertilizers to be active over a, wide ranges of agriculture crops.

1-Identification of bacteria

The study involved soil samples of Baghdad areas of isolating and diagnosing nine isolates of *Pseudomonas fluorescens* and three of *Bacillus cereus* where the samples were planted on the optional medium Pseudomonas Agar medium and Nutrient agar. these media promotes the growth of *Pseudomonas spp*. and some other soil bacteria[3]. The Pseudomonas isolates were shown on the Pseudomonas medium after being growth in aerobic conditions at 37° c for 24 h. Small, high, regular colonies were attached to each other. In under microscopy show gram negative, rod shape of a single or pairs. on the other hand *Bacillus cereus* isolates were appeared small, high, irregular colonies and. In under microscopy show gram positive, rod shape of a single or pairs{table-1}.
The results of the conventional biochemical test (Table-2)

Table 7: phosphate solubilizing bacteria in Baghdad area soil samples.

Bacterial species No.of isolate	phosphate solubilize	percentage %
Pseudomonas fluorescens (9)	+	100
Bacillus cereus (3)	+	100

Table 8: Siderophore producing bacteria in Baghdad area soil samples.

Bacterial species No.of isolate	Siderophore production	percentage %
Pseudomonas fluorescens (9)	+	100
Bacillus cereus (3)	+	100

and (Table-3) compared with the characteristics of *P.fluorescens* and *Bacillus cereus* documented by[21,24], The bacterium was sufficient for identification of *P.fluorescens* and *Bacillus cereus*. Biochemical characteristics confirmed by the API-20E system shows that *P.fluorescens* isolates was mobile and catalase positive ,oxidase positive, Lysine decarboxylase negative, ornithin decarboxylase negative B-galactosidase negative, ferment glucose without gas production, urease negative,citrate utilization positive and indol production negative as mention in table-3.

Bacterial population in soil samples

Results in table –(4) shows that bacterial count were varied in soil samples according to their region,,and ranging from 30 to 60 *10 2 CFU/g in Baghdad university soil to 10—20 *10 2 CFU/g in Mustansiriyah university soil.

Physio-Chemical analysis of collected soil samples

Soil texture analysis was done by[16] method. All soil samples were analyzed for their pH and ECe by using 1:1 (w/v) with the help of method described by[17,18] respectively.The organic matter of all the soil samples was determined by[19],Table –(5) and Table –(6) showed that the Baghdad soil macronutrient which included: NH4 , NO3 ,P,and K were, (8.42, 20.53, 19.09, 218.73) PPm respectively, While the physio analysis revealed that the mean of pH was 7.3 and EC was 8.63. on the other hand the micronutrient analysis indicated that the soil samples were included Ca, Fe, Mn, Zn and Cu which gave their mean (5025.9, 8.9, 4.9, 0.5 and 1.5) PPm respectively.

phosphate solubilizing bacteria

Solubilizing of Phosphate- by some type of bacteria can be screened routinely on media containing tricalcium phosphate called Pikovs.kaya –agar medium[25], To test the relative efficiency of these isolate, select microorganisms

Table 6: Micronutrient component of Baghdad soil samples.

Soil region	Ca	Fe	Mn	Zn	Cu
Baghdad university	5237.9	8.9	4.9	0.5	1.5
Mustansiriyah university	5321.9	8.9	5.1	0.5	1.5
Diyala bridge	4517.9	8.8	4.8	0.6	1.5
Mean	5025.9	8.9	4.9	0.5	1.5

that can produc a halo/clear- zone on agar plate refer to produce of organic acids. However, the reliability of organic acids. However, these technique based on appear of halo- is often questioned, as some of bacterial isolates cannot produce clear zones on plates but could be solubilize different kind of inorganic phosphates
insoluble in broth medium[7]. Results revealed that all isolated bacteria (9 isolates *of P .fluorescens* and three isolates *of B. cereus* gave a halo zone which mean their ability to be phosphate solubilizing bacteria as shows in figure (1) and table (7).

Phosphate solubilizing bacteria were isolated from three vegetables rhizospheric soil of Baghdad, Mustansiriyah university and Diyala bridge. Estimation of population was made from various rhizospheric region and the microorganism studied for their characters of agricultural significance with maiming focus on ability of solubilizing tricalcium phosphate., all of these isolates *Pseudomonas* and *Bacillus* were gave positive results at 100%. But with different halo zone, This variation with in halo zone may be related with variation in the population of phosphobacteria in different ecologies might be attributed to many soil elements such as soil nutrient status, soil acidity, moisture content, organic matter and activity of soil enzyme, as correlated with the results in table (5) and table (6).

Siderophore producing bacteria

Some of M.O using systems for siderophore-mediated Fe transport. For bacteria, this systems differ according to type of this bacteria ((gram-positive and gram-negative)). Gram-negative (example: *Escherichia* sp.) have type of receptors called. ((Ton B-dependent outer membrane receptors)) this receptors have a ability to recognitions of Fe(III)–siderophore complexes in the surface of cells[12]. the Fe(III)–siderophore binding to receptor in the part of outer membrane, the complex crosses this part of cell through an energy-dependent- system consisting of many receptor proteins, periplasmic- binding- proteins and inner- membrane –transpor, tproteins. Thereafter, the Fe(III)–siderophore- complex bound to periplasmic binding protein according to a high-affinity between them, which accompanied this complex to membrane of cell, is movement of complexs to the peri-plasmic space[14]. In contrast, in gram+ Ve (example. *Bacillus* species0, which haven't the outer -membrane, therefore this receptors are completely absent. That mean, the Fe(III)–siderophore complexes are bind by proteins ((periplasmic- siderophore binding proteins)) that are anchored to membrane of cell because of the absent of a peri-plasmic space[11].

Results in table (8) revealed that all isolated bacteria were can produce a high levels of chelating factors (siderophores) *by P. fuorescens & B. cereus* at 100%,when appeared a halo clear zone. Further-more, the raise produce of chelating agent ((siderophore)) were done by species of bacteria isolate from Iraqi soils. might be attributed to some factors in soil for example: soil nutrient status, soil acidity, water content, organic material and activities of enzyme in soils,. as correlated with the results in table (5) and table (6).

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