Solubilization potential of *Bacillus* subtilis and *Pseudomonas aeruginosa* on Phosphorus form in Calcareous Soil

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Abstract

Due to its importance in several physiological and biochemical processes, phosphorus (P) is necessary for all known forms of life. Calcareous soils are one of the major challenges in agriculture due to their high ability to fix phosphorus and make it unavailable to plants. There are rare studies focusing in role of P solubilizing bacteria in calcareous soils in Iraqi Kurdistan Region. This study aims to evaluate the role of *Bacillus subtilis* and *Pseudomonas aeruginosa* bacteria as Phosphate solubilizers in calcareous soils amended with organic (sheep manure) and mineral (DAP) fertilizers. The experiment was conducted at the Faculty of Agricultural Engineering Sciences – Duhok University during the period from December 2024 to June 2025, using 22 agricultural pots for wheat cultivation (Jehan classification 99). Two types of soil with different percentages of calcium carbonate (18% and 35%) were used, and bacteria were isolated and diagnosed using PCR techniques,16S rRNA gene sequencing and VITEC 2. Result revealed, 16S rRNA gene sequencing blast revealed strong similarity to *Bacillus subtilis* (93.65%). The VITIC 2 technique of *Pseudomonas aeruginosa* gives probability (99%). Total phosphor was recorded significantly ($p<0.01$) the highest value (1649.112) in the combination Qesrok with DAP. *Bcillus subtilis*, followed by Qesrok and DAP. *Pseudomonas aeruginosa* (1456.562). Organic phosphor (OP) was recorded highly significant ($p<0.01$) the highest value (370.206) in the combination Qesrok and manure. *Bacillus subtilis*, followed by Korader with DAP (331.913). Available phosphor was recorded significantly ($p<0.01$) the highest value (68.304) in the combination Qesrok with DAP and *Bacillus subtilis*, followed by Korader with DAP and *Bacillus subtilis* (63.013). *Bacillus subtilis* population was significantly achieved by the combination of Qasrok soil with DAP (13.17×10^6 CFU/g) followed by *Pseudomonas aeruginosa* (12.46×10^6 CFU/ g) in Qasrok soil with DAP.As a conclusion, the substantial synergistic interaction was found with the highly soluble DAP fertilizer and the superior enzymatic activity of *Bacillus subtilis*.

Keywords: Total Phosphorus, Available Phosphorus, Organic Phosphorus, $CaCO_3$, *Bacillus subtilis, Pseudomonas aeruginosa*.

Introduction

Phosphorus (P) is an essential element determining plants' growth and productivity. P's availability in soil is rarely adequate for plants to grow and develop to their full potential because of soil fixation**[1]**.Phosphorus which accounts for around 0.2% of a plant's dry weight, is a crucial macronutrient. Because it is a part of essential components like ATP,

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phospholipids, and nucleic acids, plants cannot develop without a steady supply of this nutrient**[2]**. In plants, phosphorus is essential for controlling physiological reactions and improving resistance to abiotic stressors like heat, salinity, drought, waterlogging, elevated CO2, and heavy metal toxicity**[3]**. Calcareous soils are defined by the presence of the mineral calcium carbonate (CaCO3 or lime) in the parent material and a buildup of lime, these soils often have a pH of more than 7, sometimes reaching 8.5. The pH of these soils may be higher than 9 when sodium carbonate is present. CaCO3 can concentrate into very hard layers in some soils called caliche, which are impervious to plant roots and water**[4]**. Soil having a pH higher than neutral, usually between 7.5 and 8.5, is referred to as alkaline soil. Significant amounts of free excess lime (calcium or magnesium carbonate) are present in calcareous soil **[5]**. As the pH rises into the alkaline region, the maximal solubility and plant availability of P at pH 6.5 decrease once again**[6]**. This effect of reduced P availability in alkaline soil is driven by the reaction of P with calcium, with the lowest solubility of these calcium phosphate minerals at about pH 8 The P availability issue is made worse by the presence of lime in alkaline soil**[7]**. These alkaline and calcareous soils are common in arid and semi-arid regions with little rainfall**[8]**. Not all of the fertilizer P added to soils is accessible to plants because of surface adsorption and precipitation processes. Precipitation of insoluble Ca-P is thought to have a significant role in calcareous soils**[4]**. It is well known that a variety of bacterial species may solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate ($CaHPO_4$), hydroxyapatite, or naturally occurring rock phosphate**[9]**. Through the processes of phosphate solubilization and mineralization of complex phosphorus compounds, soil microorganisms have drawn a lot of interest as potentially useful agents in boosting phosphorus availability to the plant from organic and inorganic sources. P exists in three main forms organic P (orthophosphate, phospholipids, and nucleic acids), Al/Fe bound inorganic P (in acidic soils), and Ca bound inorganic P (in alkaline soils). P interacts in both acidic and alkaline soil conditions**[10]**. Compared to the N and C biogeochemical cycles, the P cycle does not produce any stable gaseous species at Earth's temperatures and atmospheric pressures**[11]**. By mineralizing organic P, solubilizing inorganic P minerals, and storing significant quantities of P in biomass, phosphate solubilizing microorganisms (PSMs), a sizable microflora that mediates bioavailable soil P, are essential to the soil P cycle. PSMs can dissolve soil P into soluble and plant-available orthophosphate forms (primarily PO_4^{3-}, HPO_4^{2-}, and H_2PO^{4-}) by releasing phosphatase enzymes and organic acids, lowering soil pH, and boosting chelation activities with more P adsorption sites**[11]**. While PSM addition is a commonly recognized, eco-friendly method for raising soil soluble P concentrations and agricultural output. In order to improve plant P absorption and increase crop yields, the amount of bioavailable P in the soil is crucial. To improve P absorption from soil to plants, soil PSM can use several biogeochemical techniques to use the unavailable P forms**[11]**. Compared to other microorganisms, bacteria are the most common microorganisms in nature that solubilize mineral phosphate**[12]**. PSB-containing microflora, such as Rhizobium, Bacillus, Pseudomonas, and Escherichia species, are commonly found in soil phosphate**[13]**. *Bacillus subtilis* and

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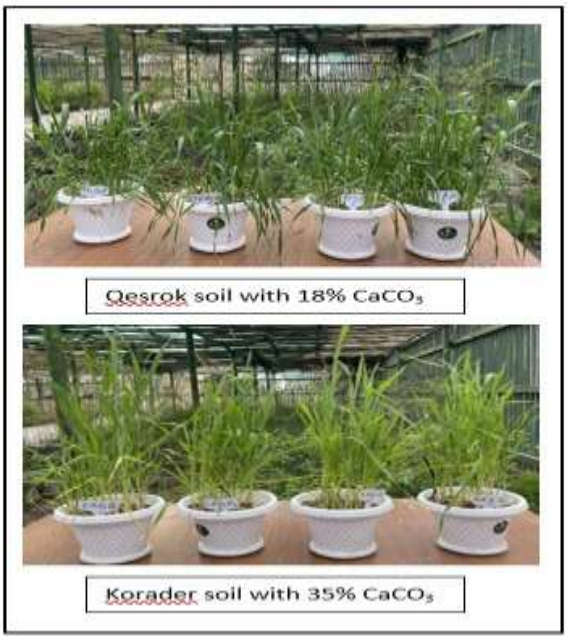
Pseudomonas aeruginosa acts as the highly effective Phosphate Solubilizing Bacteria (PSB) in the soil. *Pseudomonas* is the most significant and preferred bioinoculant among them for encouraging plant development and managing phytopathogenic and plant diseases**[14]**. *Pseudomonas aeruginosa* is a very effective plant-growth-promoting rhizobacteria (PGPR) that lowers soil pH and releases chelating agents by solubilizing insoluble soil phosphorus through organic acid secretion**[15]**. This mechanism greatly increases agricultural crop yields and plant nutrient intake by converting inaccessible minerals (such as tricalcium phosphate) into accessible forms. The bacteria use a number of main mechanisms to mobilize phosphorus, Low-molecular-weight organic acids, including gluconic, citric, lactic, succinic, and malic acids, are secreted by *Pseudomonas aeruginosa***[16]**. These acids produce protons that replace calcium, iron, and aluminum ions linked to phosphates and reduce the pH of the surrounding environment. Another mechanism phosphate is released into the soil solution when the hydroxyl and carboxyl groups of the secreted organic acids chelate cations linked to insoluble phosphates. Additionally, the bacteria produce enzymes such as acid and alkaline phosphatases that mineralize organic phosphorus molecules into inorganic HPO_4^{2-} or $H_2PO_4^-$, which are easily absorbed by plants**[17]**. *Bacillus* is a crucial component of agroecosystems because it enhances plant development and nutrient uptake, also the Bacillus use a number of main mechanisms to mobilize phosphorus, Low-molecular-weight organic acids, including gluconic acid and 2-ketogluconic acid, are secreted by both bacteria. By dissolving insoluble mineral phosphates (such as calcium, iron, and aluminum phosphates) and releasing free phosphate ions, this acidifies the rhizosphere**[18]**. They create phosphatases, like phytases, which mineralize organic phosphorus in the soil so that roots may absorb it, they create phosphatases, like phytases, which mineralize organic phosphorus in the soil so that roots may absorb it**[19]**. In order to support agricultural sustainability, PSB may be a suitable replacement for biofertilizers. Thus, the study's goals are to assess the role of Pseudomonas aeruginosa and Bacillus subtitles as phosphate solubilize bacteria in different pools of phosphorous in calcareous soils amended by organic (Sheep manure) and mineral fertilizers DAP (Diammonium phosphate).

Material and Methods

Study Area: experimental location description

In the December of 2024, the experiment was performed at the college of agricultural engineering science in the University of Duhok, Kurdistan region, Iraq. The college of agriculture engineering science in Duhok has a latitude of approximately 36°51'32.39"N and a longitude of approximately 42°52'58.95"E, (22) pots were prepared under the wooden house in the college of agriculture engineering science, as showed in the figure (fig.1).

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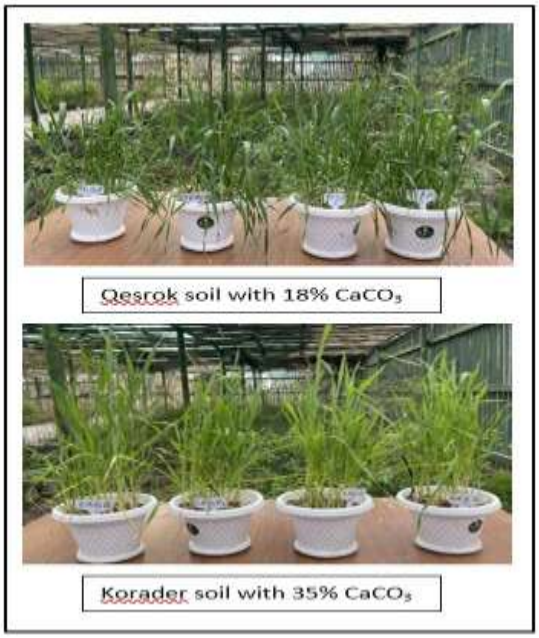


Figure 1: Pots that cultivated with wheat in different soil CaCO3 percentage.

Sampling collection and pots experiment

In this study, two different types of soils with different physical and chemical characteristics used, and two deferent species of PSB were isolated from local soils that are used , in this study 50Kg of the A- horizon soil collected from about 20 subsamples in soil with 18% CaCo3 in Qasrok(36°42'23"N 43°37'03"E) as show in (fig.2) and 35%CaCo3 in Koredare village(36°52'13.65"N 43°10'42.3"E) as show in (fig.3) by the way of randomize methods to represent all of the region, The characteristics of the soils were given in the (Table :1).

Table 1: -characteristics of both soils that were sprayed with PSB

Soil type	Qesrok	Korader
pH	7.4	7.8
EC (Us)	232	186
NO_3 (ppm)	56.04	84.06
NH_4 (ppm)	42.03	56.04
Available p (ppm)	4.3	5.6
Organic P (ppm)	210.095	251.353
Total P (ppm)	712.435	582.498
$CaCo_3$ (%)	18.5	35.01
Active carbonate (%)	20.5	25
Organic matter (%)	0.72	0.76
Texture	55.7% Clay	53.3% Clay
	28.0%	1.7%

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	Sand 16.3% Silt	Sand 45.0% Silt

Figure (2): The location of Qasrok



Figure (3): The location of korader.

The sample immediately sieved at 2mm sieve after sieving the soil, the percentage of CaCO3 were analyzed to estimate the soil CaCO3 and putted in plastic pots. the soils put into plastic planting pots with a surface diameter of (25cm), a height of (30 cm) and 1.70 kg soil for each pot, the bottom drainage holes of the pots were opened to drain excessive water and prevent water logging. In this study, two different types of fertilizer were used Sheep manure and Diammonium phosphate (DAP), 40 g of sieved (Sieve 2 mm) fresh sheep manure in the (Table 2) present the Chemical analysis of sheep manure used in the pot , experiment and according to the amount of DAP fertilizer for one dunam near to (28.125Kg/do) and for one pot with a surface diameter of (25 cm) is (2.13g) so in two doses added 5 g in each dose were added, and 2.5 g of DAP fertilizer. Before planting wheat, were isolated two species of bacteria from the local soil phosphorus solubilizing bacteria (PSB) the first bacteria (*Bacillus subtilis*) the second bacteria (*Pseudomonas aeruginosa*). In this Study soil cultivated mainly by wheat (Jihan 99) the science name (*Triticum aestivum L.),* were cultivated (20) seeds of wheat for each pot, wheat cultivated in winter (12/11/2024) and harvested in summer (13/6/2025).

Parameters	Value
Moisture contents%	15
Organic matter%	27
Nitrogen%	0.65
Phosphorous%	0.37
Potassium%	0.63
C/N	22
pH	7.1

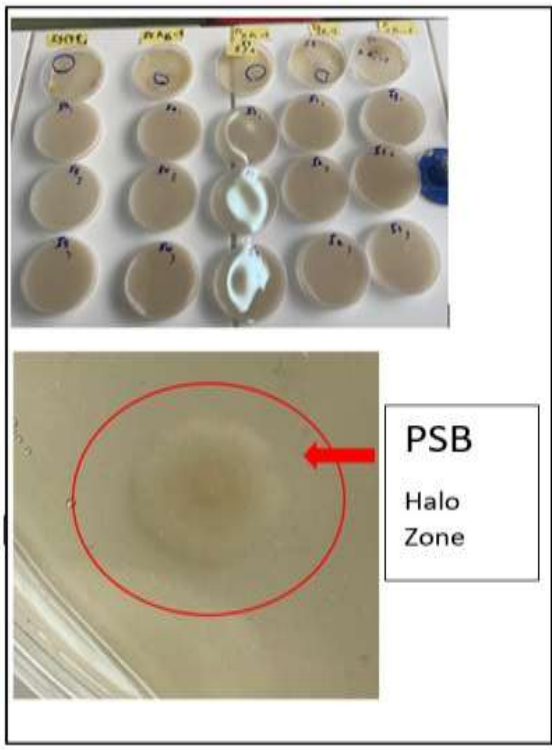
Table (2): Chemical analysis of sheep manure used in the pot experiment

Isolation of phosphate solubilizing bacteria:

After collection of the soil sample, the first step in isolation of bacteria was serial dilution, which suspended 10 g of soil in 90 ml of sterile distilled water. Perform a serial dilution up to (10^{-5} to 10^{-6}) to ensure well-separated colonies. And a serial dilution of (10^{-5}, 10^{-6}) was prepared using sterile distilled water, and 0.1 mL from each dilution to inoculate agar plates. Pikovskaya's Agar media consist from (0.5 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4.7H_2O$, 0.3 g NaCl, 0.3 g KCl, 0.03 g, $FeSO_4.7H_2O$, 0.02 g $MnSO_4.H_2O$, 10.0 g $Ca_3(PO_4)_2$, 10.0 g Glucose and 15.0 g Agar). Incubation at 30 Celsius for 24 hours clear halo diameter was successively measured after 24 h, up to 7 days. Isolation will well-developed and

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separated colonies of pure culture bacteria, the five isolated bacterial on Pikovskaya's Agar media as show in (fig .4). After preparation and isolation of Phosphor solubilizing bacteria were identified two of the best types of Phosphors solubilizing bacteria from Qesrok and korader soils. By Vitic procedure in (VIN private hospital) and Polymerase Chain Reaction (PCR)in Korea.



Figer (4): five isolated bacterial on Pikovskaya's Agar

Molecular identification of bacteria by PCR

-DNA extraction

After overnight incubation of the bacterial isolates from soil samples, the DNA was extracted from harvested 1-2 ml of the inoculated broth. The nucleic acid extraction was performed using AddPrep Bacterial Genomic DNA Extraction Kit (add bio, South Korea). The quality and quantity of the extracted DNA was measured using NanoDrop 2000/2000c (ThermoFisher Scientific, USA). The samples were saved at -20 oC until use.

-Partial 16S rRNA amplification

For bacterial identification, a universal primer set was used targeting a partial 16S rRNA (660 bp) of the bacterial genome (Forward5'GGAACTGAGACACGGTCCA G'3,Revers5'CCAGGTAAGGTTCTTCGC GT'3). A total of 50 µl PCR reaction was performed containing; 25 µl of the 2X AddStart Taq Master Mix (add bio, South Korea), 2 µl of each forward and reverse primers (10 pmol/ µl), 4 µl of genomic DNA (50-100 ng/ µl) and the volume was adjusted to 50 µl with the nuclease free water. The reaction was set up in the Techne Thermal cycler (Barloworld Scientific, UK); one cycle of initial denaturation for 5 minutes at 94 oC, 40 cycles of denaturation for 30 seconds at 94 oC, annealing for 45 seconds at 57 oC, extension for 90 seconds at 72 oC, and one cycle of final extension for 5 minutes at 72 oC. The results of the partial 16S rRNA (660 bp) amplification was confirmed by 1.5% agarose gel electrophoresis. A 30 µl of the confirmed amplicons along with the forward and reverse primers were used for sequencing (Macrogene, South Korea). The sequences were cleaned and consensus files were made from forward and reverse sequences. Therefore, the bacterial species identification was checked with the blast in the NCBI website.

-Confirm the Identification using VITEC 2 compact.

The bacterial isolates that had previously been detected by biochemical testing were

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confirmed using the VITEK 2 Compact system (bioMérieux, France). The *Pseudomonas aeruginosa* bacteria were identified by VITIC 2 technique. By automatically carrying out 64 biochemical processes, the system can identify both Gram-positive and Gram-negative bacteria, aerobes, anaerobes, and yeasts as well as establish their antibiotic resistance profiles, eliminating the need for manual testing.

Addition of bacteria (PSB) to pots

After the incubation were identified, there species by Vitic and PCR procedures. Then, the identified bacteria were added in their nutrient broth (Pikovskaya's broth media) and applied on the both soil with deferent amount of CaCO3 qesrok 18% and korader 35% in order to enhance the availability of available phosphorous. Were this bacteria broth media adding in two dose the first time before cultivating of wheat (17/2/2024)and the second dose (6/3/2024) in each dose 100ml of bacteria broth media were added.

Determination of soil physiochemical properties

a-physical analysis

The soil's texture was examined using either particle size distribution or mechanical analysis (using the hydrometer method).

b-Chemical analysis

A pH meter was used to measure the saturation extract's soil pH**[20]**. An EC meter and glass electrode were used to test the soil EC of the extracts**[20].** Calcimeter was used to determine the percentage of calcium carbonate (%CaCO3), The percentage of calcium carbonate was according to**[21]**. The Walkley-Black technique were used to determine the organic matter , Walkley-Black technique is the name given to this approach (Walkley, 1947; FAO, 1974)**[20]**. The sodium bicarbonate (NaHCO3) method developed by Olsen et al. (1954) is widely recognized as an appropriate measure of P "availability" for alkaline soils, where the precipitation of Ca as CaCO3 increases the solubility of calcium phosphate. The available phosphorous was estimated on the Spectrophotometer at 882-nm wavelength. By ignition the soil at 550 °C, the Ignition Method estimates the amount of organic phosphorus (P) in the soil. Organic Phosphorus was estimated on the Spectrophotometer at 882-nm wavelength**[20].** In order to determine total P, a soil sample must be digested with a strong acid (Perchloric Acid) (HClO4) concentrated (60%) to dissolve all insoluble inorganic and organic P forms of minerals. Typically, only soil genesis or mineralogical investigations use this measurement (Olsen and Sommers, 1982). Total Phosphorus was estimated on the Spectrophotometer at 410-nm wavelength. Kjeldahl methods were used to calculate the quantities of accessible NO3-N and NH4-N. Steam distillation and titration methods were used after the air-dried soil samples were extracted using 2M Potassium Chloride (KCL) (1:5), (W/V) when 25 ml of 2M KCL were applied to 5g of air-dried soil according to (Peters et al., 2003).

Statistical Analysis

statistically. Descriptive Statistics, Two-way ANOVA, Repeated Measure and correlation analysis were applied, after ensure the normality of data (Shapiro test $p>0.05$). However, the mean's differences were separated using Duncan Multiple range test (Duncan, 1955) **[22]**for two-way ANOVA and Bonferroni test for Repeated measure.

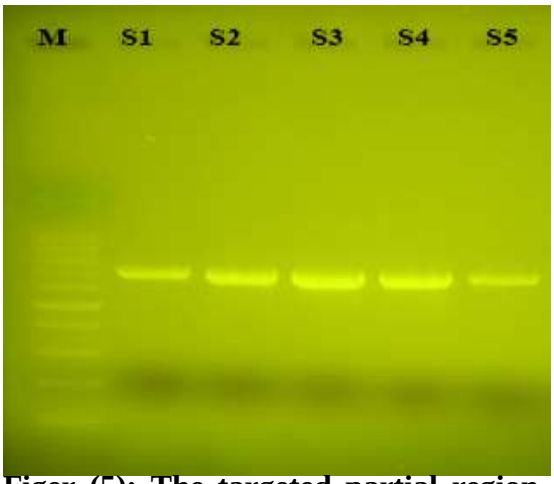
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Results and Discussion

Results

Plotted on the USDA Soil Texture Triangle, the Korader soil sample with 28.0% sand, 16.3% silt, and 55.7% clay is classified as (clay), and also plotted on the USDA Soil Texture Triangle, the Qesrok soil sample with 1.7% sand, 45.0% silt, and 53.3% clay is classified as (Silty Clay).

The results of Molecular identification of bacteria, The DNA of the five inoculated bacterial samples was successfully extracted. The targeted partial region of the 16S rRNA was amplified with the universal primer (660 bp) (Fig. 5).



Figer (5): The targeted partial region of the 16S rRNA was amplified with the universal primer (660 bp).

From S1to S5, each land shows single, distinct band. This demonstrates that the targeted partial portion of the 16s rRNA gene was successfully amplified in each of five bacterial samples. About high purity and specificity there are no genomic DNA stains close to the wells, primer dimer artefacts at the bottom, or secondary nonspecific bands. This demonstrates the great specificity of the universal primers.

After PCR amplification confirmation, 30 µl of the PCR products and 20 µl (10 pmol/µl) of both primers (Forward and Reverse) were sent for sequencing in Macrogen, South Korea. The sequencing was performed in both directions. After sequence cleaning and alignment, they were used in blast search in NCBI and the bacteria were confirmed based on the most nucleotide identity percentage. Based on a 16S rRNA gene sequencing blast search, these percentages reflect the genetic similarity between bacterial samples and known strains .the result of five samples are (S1) *Paenibacillus polymyxa strain* (72.76%)lowest similarity,(S2) *paenibacillus polymyxa strain* (73.03%)low similarity, (S3)*Bacillus tianshenii spp.* (90.40%) moderate similarity to another species in the Bacillus genus,(S4) *Bacillus subtilis* (92.02%) Strong similarity to a different strain of the same species,(S5) *Bacillus subtilis* (93.65%) this strain is genetically closest to the sample with the greatest similarity. But in this experiment were used the (S5) *Bacillus subtilis.* Also, the result of the unfirm the Identification using VITEC 2 compact shown in Table (2), The *Pseudomonas aeruginosa* bacteria were identified by VITIC 2 technique the probability (99%) this is indicating a highly definitive identification.

Table (3): The result of the unfirm the Identification using VITEC 2 compact

99%Probability (Pseudomonas aeruginosa)

Biochemical Details																			
2	APPA	-	3	ADO	-	4	PyrA	-	5	1ARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	-	18	Dmal	-	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BALap	+
23	ProA	+	26	LIP	+	27	PLE	-	29	TyrA	-	31	URE	-	32	Dsor	-
33	ASAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	1HISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	1MLTa	+	62	ELLM	-	64	1LATa	+			

It could be noticed from Table 3, that total phosphor was recorded significantly ($p<0.01$) the highest value (1649.112) in the combination Qesrok.DAP.*Bcillus subtilis*, followed by Qesrok.DAP.*Pseudomonas aeruginosa* (1456.562), while the lowest value was recorded by the combination Control Korader (549.126), and the rest combinations were intermediate. DAP is a highly efficient weak alkali-soluble P fertilizer. According to other research, after being applied to neutral soil, DAP changed into accessible P in the forms of CaHPO4 and Ca8H2(PO4)5 · 5H2O such that plants could readily absorb it **[23]** . The total phosphorus (P) pool in the soil is immediately increased by applying diammonium phosphate (DAP). The use of DAP immediately increases the absolute mass of phosphorus in the soil system due to its high phosphorus content (46% $P_2 O_4$)**[24].** The Qesrok's have highest total and available phosphorus accumulation. The substantial synergistic interaction between the highly soluble DAP fertilizer and the superior enzymatic activity of *Bacillus subtilis* is responsible for the DAP. *Bacillus subtilis* combination. Although DAP offered a quick and plentiful supply of phosphorus, its use usually results in quick soil fixing**[25]**. Nevertheless, this restriction was successfully lessened by the *Bacillus subtilis* inoculation. This particular strain of bacteria probably grew well in Qesrok soil, releasing phosphatase enzymes and low-molecular-weight organic acids. By chelating the binding cations, these microbial exudates kept the concentration of both total and easily accessible phosphorus fractions in the soil matrix high and prevented phosphorus precipitation. Regarding to time effect, the highest value of total phosphor was achieved significantly ($p<0.01$) at week 1, followed by week 2 (1004.072), and the lowest value was recorded at time (week8), however, before and week 4 were intermediate. Soil chemical and biological activities can account for the temporal dynamics of total

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phosphorus (TP)**[26]**, which show a strong rise in Week 1 and a gradual drop until Week 8. The fast disintegration of the highly soluble DAP fertilizer and the instantaneous metabolic burst of the implanted bacteria are the main causes of the increasing at Week 1. However, phosphorus fixation or precipitation a frequent occurrence in calcareous soils where soluble phosphorus combines with calcium ions to produce insoluble calcium phosphate complexes can be blamed for the steady decline in TP levels over time. As the experiment moved into the week8, the measured phosphorus pool in the soil was further reduced by biological immobilization caused by the growing microbial biomass and subsequent plant absorption**[27]**. It could be noticed from Table 3, that organic phosphor (OP) was recorded highly significant ($p<0.01$) the highest value (370.206) in the combination Qesrok.Manure. *Bacillus subtilis*, followed by Korader.DAP (331.913), while the lowest value of OP was recorded by the combination Control Qesrok (208.429), and the rest combinations were intermediate. the highbies accumulation of organic phosphorus in the Qesrok.Manure. *Bacillus subtilis* combination can be effected to the combined effect of the organic fertilizer (sheep manure)and microbial inoculation, Manure is inherently a rich source of complex organic phosphorus compounds, phospholipids, including phytin, and nucleic acids**[28]**. When introduced into the Qesrok soil, the addition of *Bacillus subtilis* likely facilitated a temporary microbial immobilization process. n this process, the microbial biomass to maintain development assimilates available phosphorus to their cellular structures, trapping it into organic forms**[29]**. However, the microbial priming effect might account for the high OP value shown in the Korader.DAP treatment, ever though DAP is a completely inorganic chemical fertilizer. Available nitrogen and phosphorus were instantly added to the Korader soil because to the high solubility of DAP fertilizer. The native soil phosphorus solubilizing bacteria were greatly boosted by this nutrient's influx, which led to fast comultiplication. Also, these native microbes took up the free inorganic phosphorus and converted into microbial biomass phosphorus in order to build their biomass**[30]**. Regarding to time effect as shown in (Table 4), the highest value of organic phosphor was achieved significantly ($p<0.01$) at before bacteria (319.418) and statistically equal with week 1(315.101), followed by week 2 (288.543), and the lowest value was recorded at time week4(217.638) and statistically equal with week 8(240.082).The fluctuating equilibrium between microbial immobilization and biological mineralization is reflected in the dynamic variation in OP level throughout the experimental periods as show in (Table 4). The initial build-up of organic inputs from the manure and the early stage of microbial immobilization, when free phosphorus is bound with in microbial cells, are responsible for the noticeably higher OP values sustained throughout the before bacteria stage (319.418) and week1 (315.101). On the other hand, a phase of intense biological mineralization is clearly indicated by the quick and large reduction in OP by week 4 (217.638). In order to hydrolyze organic phosphorus molecules in to inorganic forms that plants may use, both native and inoculated soil bacteria actively produce extra cellular phosphatase enzymes during this time. It's interesting to note that slight recovery in OP by week 8 (240.082) is a typical cyclic phenomenon in soil biology, it represents the decrease in the active bacterial population as a result of nutrient depletion, which causes cell lysis and the

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subsequent return of cellular phosphorus as microbial debris to the soil's organic phosphorus pool. It could be noticed from (Table 4), that Available phosphor was recorded significantly ($p<0.01$) the highest value (68.304) in the combination Qesrok.DAP. *Bacillus subtilis*, followed by Korader.DAP. *Bacillus subtilis* (63.013), while the lowest value was recorded by the combination Control Qesrok (4.002), and the rest combinations were intermediate. In the Qesrok. DAP. *Bacillus subtilis* the available phosphorus has the highest value because of diammonium phosphate (DAP) highly soluble source of orthophosphates, when bacterial was applied, it produced phosphatase enzymes and organic acid. In order to stop the quick chemical fixation of soluble phosphorus in to soluble solid phases, this microbial activity successfully lowered localized soil PH and chelated competing cations such(calcium) .The obvious advantage of (*Bacillus subtilis*)over (*Pseudomonas aeruginosa*) suggests that this particular stain has greater metabolic ability to preserve the fluidity of control treatments low phosphorus availability emphasizes how important it is to combine chemical nutrient in puts with specific biofertilizers in order to maintain phosphorus availability . Regarding to time effect, the highest value of Available phosphor was achieved significantly ($p<0.01$) at week 2 (53.478), followed by week 1 (44.289), and the lowest value was recorded at time week8(27.442), however before bacteria (statistically equal with week 2) and week 4 were intermediate. In soil sciences, the particular variation in available phosphorus over time is a well-known bio-geochemical phenomenon. It is driven by the dynamic interplay of chemical fixation, microbial control, and fertilizer dissolution**[31]**. The maximum value of available phosphorus represents the microbial release phase. The added phosphorus solubilizing bacteria (PSB) finish their lag phase, develop quickly, and reach highest metabolic activity. They actively release phosphatase enzymes and low molecular weight organic acids. This is explicitly by data, where organic phosphorus significantly decreases and from 315.101(week 1) to 288.543(week 2), indicating the organic P was successfully mineralized in to available P. Also, the week 1 (44.289) is lower than week 2 and before bacteria because of biological immobilization, bacteria go through a brief "shock" and adaption period when they are first added to the soil (week1). The accessible soil pool temporarily decreases before the bacteria start aggressively dissolving it because they actively take and lock up free available phosphorus in to their cells (a process known as microbial immobilization)in order to develop their own biomass and live**[32]**.The lowest volume was in week 8 (27.442), the significantly decreases in week 4 (29.587)to week 8(27.442)because of the soil chemical fixation (precipitation) and the exhaustion of carbon sources .The chemical fixation in soil ,free phosphorus in highly unstable, soluble orthophosphate ions rapidly react with soil minerals over aperid of week4 and week 8.phosphorus and calcium precipitation to produce insoluble calcium phosphate in alkaline/calcareous soil, which are present in areas like (Qesrok,Korader) **[33]**.Exhaustion of carbon sources ,the readily decomposable organic matter (from manure or fertilizer transporters)becomes through week 4 and 8.The added bacteria's proportion and metabolic activity therefore decrease ,which significantly lower the amounts of organic acid they produce of active bacterial breakdown, the phosphorus is locked up by the soil's natural chemical fixing

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Table (4). Effect of the combination (Area, Fertilizer and Bacteria) and time on the soil's Phosphorus

Combination	**Effect of Combination**		
	Total Phosphor(ppm)	**Organic Phosphor(ppm)**	**Available Phosphor((ppm)**
Control Qesrok	720.401 fg	208.429g	4.002g
Qesrok.DAP. *Bacillus subtilis*	1649.112 a	217.274 fg	68.304 a
Qesrok.DAP. *Pseudomonas aeruginosa*	1456.562 b	242.745 defg	59.119 bc
Qesrok.Manure. *Bacillus subtilis*	902.265 def	370.206 a	34.746 e
Qesrok.Manure. *Pseudomonas aeruginosa*	921.137 de	324.916 b	36.496 e
Control Korader	549.126 g	257.852 def	6.637 g
Korader.DAP. *Bacillus subtilis*	1442.804 b	314.104 bc	63.013 b
Korader.DAP. *Pseudomonas aeruginosa*	1196.860 c	280.720 cd	53.745 d
Korader.Manure. *Bacillus subtilis*	741.470 efg	230.495 efg	32.271 e
Korader.Manure. *Pseudomonas aeruginosa*	844.115 def	230.858 efg	34.356 e
Korader.DAP	953.030 d	331.913 b	56.593 cd
Korader.Manure	567.457 g	267.040 de	34.879 e
Qesrok.DAP	1214.087 c	267.308 de	57.915 cd
Qesrok.Manure	739.997 efg	322.334 b	25.035 f
SEM	67.87	13.10	1.39
P value	<0.001 **	<0.001 **	<0.001 **
Effect of Time			
Before Bacteria	955.143 C	319.418 A	47.745 A
Week 1	1326.195 A	315.101 A	44.289 B
Week 2	1004.072 B	288.543 B	53.478 A
Week 4	890.228 D	217.638 C	29.587 C

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Week 8	788.085 CD	240.082C	27.442 D
SEM	26.54	5.26	0.73
P value	<0.001 **	<0.001 **	<0.001 **

Means having different letters (small for combination and capital for time effect) are differed significantly (p<0.05).

About the bacterial population it could be noticed from Table 5, revealed that the treatments significantly influenced the population of total heterotrophic bacteria (p<0.01). The highest bacterial *Bacillus subtilis* population was significantly achieved by the combination of QDAP_ *Bacillus subtilis* (13.17×10^6 CFU/g) followed by QDAP_ *Pseudomonas aeruginosa* Bac2 (12.46×10^6 CFU/ g), while the lowest microbial counts were statistically shared between Qesrok Control (28.2×10^5 CFU/ g) and korader Control (29.8×10^5 CFU/g). however, the rest combinations were intermediate. The strong combinatorial interaction between the chemical fertilizer and the bio-inoculant is responsible for the notable expansion of Total Heterotrophic Bacteria shown in the QDAP_ Bacillus subtilis treatment. Nitrogen (N) and phosphorus (P), two vital macronutrients that are easily accessible in diammonium phosphate (DAP), serve as rapid substrates for microbial cellular synthesis and metabolic pathways, causing an exponential increase in the bacterial population. Furthermore, when using additional nutrients, Bacillus species have an advantage over native microflora due to their spore-forming capabilities. Due to a lower baseline of available phosphorus than Korader soil, where organic manure demonstrated a comparatively higher compatibility, the clear variation between locations indicates that Qesrok soil was more responsive to inorganic fertilization combined with microbial inoculation. The experimental results presented in Table 6, revealed that the treatments significantly influenced the population of Phosphorus solubilizing bacteria (p<0.01). The treatment Qesrok.DAP. *Pseudomonas aeruginosa* reached the highest counts (10.45×10^6 CFU/g), Here are the specific scientific reasons why these treatments showed a uniform and statistically homogenous result, followed by the QDAP_ *Bacillus subtilis* (75.60×10^5 CFU/g), all other combined treatment is statistically homogenous. Here are the specific scientific reasons why these treatments showed a uniform and statistically homogenous result, the Manure and Biological Incompatibility (QM/KM). Slow - rate nutrient release, As compared to instant chemical availability, manure provides nutrients through Slow decomposition**[34]**,And slow bacteria(*Pseudomonas aeruginosa, Bacillus subtilis)* growth were unable to grow quickly enough to distinguish themselves from control in the absence of a rapid food supply such as DAP. Another reason the competition of Native microbes the native soil bacteria readily outcompeted the injected starting strains in treatments using manure or single fertilizers**[35]** .Another reason specifically for korader soil , unfavorable baseline conditions: High soil pH, heavy clay texture, or high phosphorus-fixation capacity in Korader likely neutralized the treatments. Overall, the results showed a highly significant positive association between the mobilization of phosphorus fractions in the soil and the population density of Phosphate Solubilizing Bacteria (PSB). The greatest bacterial population (10.45×10^6 CFU/g) was found in the treatment QDAP_ Pseudomonas aeruginosa, which is very consistent with

the significant amounts of Total Phosphorus (1456.562) and Available Phosphorus (59.119) found in the same treatment. This suggests that P. aeruginosa and DAP fertilizer produced an ideal microenvironment that sped up the conversion of bound phosphorus into forms that were accessible. bacterial count (75.60×10^5 CFU/g), graded as ab). This particular treatment had the greatest absolute values for both Total Phosphorus (1649.112) and Available Phosphorus (68.304) during the whole investigation, which may be directly explained by this peak in microbial density. In contrast, the application of organic manure (Qesrok.Manure. Bacillus subtilis) resulted in the largest accumulation of Organic Phosphorus (370.206), although the bacterial counts decreased to (44.91×10^5CFU/g). This implies that inorganic starters, such as DAP, are more efficient in quickly multiplying the inoculated PSB populations to release available phosphorus, whereas manure increases organic phosphorus stores. The influence of time further validates this microbial-chemical interplay. While available phosphorus peaked in Week 2, total phosphorus peaked in Week 1. The biological "incubation window" needed by the growing PSB populations to metabolize, proliferate, and produce the organic acids required to solubilize the phosphorus is shown by this obvious 1-week shift.

Table 5. Effect of treatment (Combination) on No. (Total Heterotrophic Bacteria)

No. (Total Heterotrophic Bacteria) CFU/g			
Treat	Mean	± SE	p value
Qesrok Control	28.20×10^5 e	971287.8	<0.001 **
QDAP_ *Bacillus subtilis*	13.71×10^6 a	1485519	
QDAP_ *Pseudomonas aeruginosa*	12.46×10^6 ab	1233892	
QM_ *Bacillus subtilis*	10.63×10^6 abc	3242463	
QM_ *Pseudomonas aeruginosa*	84.80×10^5 abcde	1278871	
Korader Control	29.80×10^5 e	852877.5	
KDAP_ *Bacillus subtilis*	80.50×10^5 abcde	2272016	
KDAP_ *Pseudomonas aeruginosa*	64.80×10^5 bcde	1477821	
KM_ *Bacillus subtilis*	58.10×10^5 cde	1460856	
KM_ *Pseudomonas aeruginosa*	88.09×10^5 abcde	1387955	
Korader.DAP	10.02×10^6 abcd	630396.7	
Korader.Manure	11.44×10^6 abc	1952076	

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Qesrok.DAP	36.60×10^6 de	404475	
Qesrok.Manure	65.40×10^5 bcde	430813.2	
Overall mean	7992071	1362951	

*Means with different letters are differed significantly. **= significant (p<0.01)*

Table 6. Effect of treatment (Combination) on No. Phosphorus Solubilizing Bacteria (PSB)

No. Phosphorus Solubilizing Bacteria (PSB) CFU/g			
Treat	Mean	± SE	p value
Qesrok Control	27.60×10^5 b	193907.2	<0.001 **
QDAP_ *Bacillus subtilis*	75.60×10^5 ab	989635.2	
QDAP_ *Pseudomonas aeruginosa*	10.45×10^6 a	3520267	
QM_ *Bacillus subtilis*	44.91×10^5 b	1447266	
QM_ *Pseudomonas aeruginosa*	43.20×10^5 b	420792.9	
Korader Control	23.20×10^5 b	780640.8	
KDAP_ *Bacillus subtilis*	50.40×10^5 b	370944.7	
KDAP_ *Pseudomonas aeruginosa*	51.50×10^5 b	821673.6	
KM_ *Bacillus subtilis*	31.10×10^5 b	752543.8	
KM_ *Pseudomonas aeruginosa*	38.70×10^5 b	673803.5	
Korader.DAP	33.0×10^5 b	557673.7	
Korader.Manure	41.80×10^5 b	886792	
Qesrok.DAP	28.0×10^5 b	622093.2	
Qesrok.Manure	49.40×10^5 b	651613.4	
Overall mean	45.92×10^5	906403.3	

*Means with different letters are differed significantly. **= significant (p<0.01)*

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Conclusions:

Bacillus subtilis showed higher efficiency in solubilizing phosphorus compared to Pseudomonas aeruginosa when combined with DAP fertilizer. Time period had a morale effect, with total phosphorus peaking in the first week, while available phosphorus peaked in the second week. The growing PSB incubation period for the populations to metabolize, proliferate, and produce the organic acids to solubilize the phosphorus is one week. However, phosphorus fixation or precipitation a frequent occurrence in calcareous soils where soluble phosphorus combines with calcium ions to produce insoluble calcium phosphate complexes can be blamed for the steady decline in TP levels over time. The study recommended the use of *Bacillus subtilis* in combination with DAP to improve phosphorus availability in calcareous soils, reducing the need for chemical fertilizers and supporting sustainable agriculture.

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