



ISSN: 0067-2904

## Isolation and identification of phosphate solubilizing bacteria and evaluate its effect on of Mung bean (*Vigna radita* [L.] R.Wilczek) growth

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### Abstract

Sixteen soil samples were collected from wheat, barley and yellow corn rhizosphere in Abu-Ghraib, Aqraqof, Latifieh, Tarmiah, Jadriya and of Agriculture in Baghdad university/ Baghdad city. The results found nine phosphate solubilizing bacteria (PSB) isolates (Y1, Y2, Y3, Y4, Y5, Y6, Y7, Y8, Y9), formed clear zones on National Botanical Research Institute's (NBRIP) agar. The solubility index (SI) of PSB isolates ranged from 2.00 to 3.66. Y4 have the highest SI (3.66) followed by Y3 and Y6 (3.33). Phosphate solubilization abilities varying from (20.10-39.00  $\mu\text{g.ml}^{-1}$ ), Y4 was the highest (39.00  $\mu\text{g.ml}^{-1}$ ) followed by Y3 (37.00  $\mu\text{g.ml}^{-1}$ ). The results of hydrolytic enzymes production showed that almost all nine isolates are able to produce protease and pectinase, while Y1 and Y2 showed negative results in cellulase production. Maximum ability for hydrogen cyanide (HCN) and indole acetic acid (IAA) production were showed by Y3 and Y4 isolates. The isolate Y4 was found to be the most efficient isolate, so it was selected identified as *Bacillus cereus* using biochemical tests confirmed by VITEC 2 compact system. The results of High performance liquid chromatography (HPLC) revealed that *Bacillus cereus* produce oxalic acid (2.996), citric acid (9.117) and malic acid (3.734). *Bacillus cereus* (Y4) enhanced the growth of mung bean plants. A significant increase in branches number (12.33), plant length (83.0cm), fresh weight (27.25 g) and dry weight (1.427g) were obtained compared with control treatments. The main objective of this study is to isolate PSB and evaluate their roles in plant growth promotion. The results showed the high phosphate solubilization efficiency of PSB isolates and the identified isolates was found to be good enough for plant growth promoting.

**Keywords:** *Bacillus cereus*, hydrolytic enzyme, organic acid production, phosphate solubilizing bacteria, plant growth promoter.

### عزل وتشخيص البكتريا المذيبة للفوسفات وتقييم تأثيرها في نمو نبات الماش (*Vigna radita* [L.] R.Wilczek)

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### الخلاصة

تم جمع ستة عشر عينة من التربة الجذرية لنبات القمح والشعير والذرة الحمراء الصفراء في أبو غرب ، اللطيفية ، الطارمية ، وكلية الزراعة في جامعة بغداد / مدينة بغداد. وظهرت النتائج تسع عزلات من البكتريا المذيبة للفوسفات ( Y1 ، Y2 ، Y3 ، Y4 ، Y5 ، Y6 ، Y7 ، Y8 ، Y9 ) ، كونت مناطق واضحة على وسط المعهد الوطني للأبحاث النباتية (NBRIP). تراوح مؤشر الذوبانية (SI) للعزلات المذيبة للفوسفات

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(PSB) من 2.00 إلى 3.66. تمتلك العزلة Y4 أعلى مؤشر ذوبانية (3.66) يليها Y3 و (3.33) Y6. القدرة على إذابة الفوسفات تراوحت من (20.10-30.00 مايكروغرام.مل<sup>-1</sup>)، العزلة Y4 كانت الأعلى (30.00 مايكروغرام.مل<sup>-1</sup>) يليها Y3 (30.00 مايكروغرام.مل<sup>-1</sup>). أظهرت نتائج إنتاج الإنزيمات المحللة أن جميع العزلات التسع تقريبا قادرة على إنتاج البروتيز والبكتينيز ، في حين أظهرت Y1 و Y2 نتائج سلبية في إنتاج السيلوليز. أظهرت العزلات Y3 و Y4 القدرة القصوى على إنتاج سيانيد الهيدروجين والاندول استك اسد. وقد وجد أن العزلة Y4 هي العزلة الأكثر كفاءة ، ولذلك تم تحديدها على أنها *Bacillus cereus* باستخدام الاختبارات الكيموحيوية المؤكدة بواسطة نظام VITEC 2 المضغوط. كشفت نتائج كروموتوكرافيا السائل عالية الاداء (HPLC) أن *Bacillus cereus* تنتج حمض الأكساليك (2.996) ، وحمض الستريك (9.117) وحمض المالك (3.734). عززت عزلة *Bacillus cereus* نمو نبات الماش. وتم الحصول على زيادات معنوية في عدد الفروع (12.33) وطول النبات (83.0 سم) والوزن الطازج (27.25 جم) والوزن الجاف (1.427 جم) بالمقارنة مع المعاملات القياسية. الغرض من هذه الدراسة هو عزل البكتيريا المذيبة للفوسفات وتقييم دورها في تعزيز نمو النبات. أظهرت نتائج هذه الدراسة كفاءة ذوبانية الفوسفات العالية للعزلات المذيبة للفوسفات وان العزلة المشخصة كانت جيدة بدرجة كافية في تعزيز نمو النبات.

## Introduction

Phosphor (P) availability to plant is limited despite its higher content in soil, particularly in tropical soils. The rapid conversion of supplied chemical phosphate fertilizers into insoluble phosphate sources leads to regularly applying P fertilizers, which are costly and environmentally undesirable. Therefore, some innovative technologies tend to develop and focus on the use of soil microbes, particularly PSB as a biofertilizers. phosphate solubilizing bacteria (PSB) which belongs to genera *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Rhizobium*, *Erwinia*, *Enterobacter*, *Pseudomonas*, and *Bacillus* have the ability to solubilize insoluble P and supply plants with available phosphorus by producing various organic acids, mineral acids, siderophores, protons, humic substances, CO<sub>2</sub> and H<sub>2</sub>S [1].

Biofertilizers have various mechanisms for enhancing plant growth, such as solubilizing insoluble phosphate, biological nitrogen fixation, and inhibit the growth of phytopathogens by the production of hydrocyanic acid (HCN), protease, cellulose and pectinase, when inoculated to seeds, root or into the soil [2, 3].

Various strategies have been demonstrated for solubilization of phosphates in liquid medium [4, 5]. One of the most accepted theory is secretion of low molecular mass organic acids (OA) by microorganisms. In various studies, the presence of organic acids (i.e. oxalic acid, citric acid, malic acid, succinic acid etc.) in filtrated liquid culture were determined by modern techniques such as High-Performance Liquid Chromatography (HPLC), Isotachopheresis and enzymatic methods have been used by others to allow more accurate identification of unknown organic acids [6].

This study aimed to isolate PSB from rhizospheric soil, screening them for their solubilization efficiency, HCN, protease, pectinase and cellulase production, characterization and identification of the most efficient isolate which then detected for organic acids production (citric acid, oxalic acid, malice acid, fumaric acid, succinic acid and gibberellic acid) using high performance liquid chromatography (HPLC), then test its ability to stimulate the growth of mung bean (*Vigna radita* [L.] R. Wilczek) plant.

## Material and methods

### Isolation of phosphate solubilizing bacteria

samples were collected from rhizospheric soils of , wheat, barley, and corn plants from fields in Abu-Ghraib, Araqof , Latifieh, Tarmiah, Jadriya, and College of Agriculture / Baghdad university. The soil suspension was prepared by suspending 10g of each soil sample in 100 ml normal saline (NaCl 85%) and shaken at 120 rpm for 2 h. The soil suspension was serially diluted followed by spreading of 1 ml of 10<sup>-3</sup> and 10<sup>-4</sup> on National Botanical Research Institute Phosphorus (NBRIP) agar medium (MgCl<sub>2</sub>.6H<sub>2</sub>O 5 g, MgSO<sub>4</sub>.H<sub>2</sub>O 0.25 g, KCl 0.2 g, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.1g, Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> 5 g, glucose 10 g, and agar 20.0 g, diluted at 1 L distilled water (D.W) at pH 7) [7]. Plates were incubated at 30 °C for 14 days. Colonies with clear halos were considered as PSB [8].

### Determination of phosphor solubilizing activity

Bacterial isolates were screened for their activity to solubilize inorganic phosphate by adding 1 µl of 24 h. age cultures on the top of NBRIP media plates. The plates were incubated at 30°C for 2 weeks. Solubilization index (SI) was estimated by using the following formula [9]:

$$\text{Solubilization Index (SI)} = \frac{\text{Diameter of colony(cm)} + \text{halo zone diameter(cm)}}{\text{Diameter of colony(cm)}}$$

Quantitative estimation of soluble phosphate was done by inoculating 100 ml of NBRIP broth with 24 h, cultured bacteria and incubated in shaker (180 rpm) at 30°C for 14 days, control treatment was inoculated with D.W, then centrifuged at 10,000 rpm for 10 min. The supernatant was separated from the bacterial cells by filtration through Whatman No.1 filter paper and used to estimate the released phosphate, from tri-calcium phosphate (TCP), spectrophotometrically (880 nm) [10].

### Hydrolytic enzymes, Hydrogen cyanide (HCN), and indole acetic acid (IAA) production

PSB isolates were activated in NB medium at 30 °C for 24 h, in order to detect their ability to produce hydrolytic enzymes and HCN production.

Cellulase production was determined in plates containing Minimal agar (M9) (dextrose 1.0 g, KH<sub>2</sub>PO<sub>4</sub> 2.0 g, K<sub>2</sub>HPO<sub>4</sub> 7.0 g, MgSO<sub>4</sub> 0.1g, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 1.0 g, sodium citrate 0.5 g, and agar 20.0 g, dissolved in 1L D.W at pH 7.0) supplemented with yeast extract (1.2 g.l<sup>-1</sup>) and carboxy-methyl cellulose (10 g.l<sup>-1</sup>). The plates were incubated at 30 °C for 6 days. Appearance of halo zone indicates a positive result.

Pectinase production was detected in M9 medium containing plates. After inoculation and 3 days of incubation at 30 °C, clear zones formation in the medium represent a positive result [11].

Protease enzyme production was detected in skim milk agar (SM powder 28.0 g, tryptone 5.0 g, yeast extract 2.5 g, dextrose 1.0 g. and agar 15.0 g) containing plates, which incubated at 30°C for 96 h. Colonies with transparent zones were recorded as protease producing bacteria [12].

HCN production of the isolates was determined by streaking for 24 h. bacterial culture on plates containing King's B medium (peptone 20.0 g, K<sub>2</sub>HPO<sub>4</sub> 1.5 g, MgSO<sub>4</sub> (H<sub>2</sub>O) 1.50 g and agar 20.0 g, dissolved in 1L D.W at pH 7) supplemented with 4.4 g.l<sup>-1</sup> glycine. Filter paper strips were soaked in solution containing 0.5% picric acid and 0.2% sodium carbonate, and then placed in between the plates. The plates incubated at 28°C for 48 h. Un inoculated plate were served as control. The change of color of filter paper to orange brown or dark brown indicates a positive result [13].

IAA production was determined by growing PSB isolates on Luria Bertani (LB) broth medium (peptone 10 g, yeast extract 5 g, NaCl 5g, L-tryptophan 1.2 g, agar 15 g, dissolved in 1 L D.W at pH 7) for 3 days at 30 °C. Then 4 ml of Salkowski reagent (2 ml from 0.5 M FeCl<sub>3</sub>, 49 ml from 70% perchloric acid and 49 ml DW) was added and re-incubated in the dark for 30 min at 25°C. Developing of a pink to red color indicates a positive result [14].

### Morphological and biochemical identification

The morphology of colony, margins, shape, raised, pigmentation, and Gram staining were recorded after 24 h growth at 30 °C of the most efficient phosphate solubilizing isolate on nutrient agar plates. The isolate was also tested for motility, oxidase, catalase tests, and methyl red production [9, 15 and 16].

### Identification by VITEK2

Bacterial isolate was identified using VITEK2 compact system. The isolate was cultured in NA medium and incubated at 37 °C for 24 h. The suspensions were prepared in 3 ml of sterile saline and adjusted to a McFarland standard of 1.80- 2.20. BCL cards were filled automatically in the instrument vacuum chamber and incubated at 35 °C. Data then analyzed automatically using VITEK2 database.

### Determination of organic acid production

Bacterial isolate was cultured in NBRIP broth (50 ml) and incubated for 5 days at 37 °C. Then the broth culture was centrifuged at 9000 rpm for 15 min and filtrated through a 0.22µm filter (Millipore, Millex- GP Syringe Filter, SLGP033NB) to obtain a cell-free culture supernatant, non-inoculated broth served as a control [17]. Organic acids were detected with high performance liquid chromatography (HPLC), by injection five µl of filtered supernatant into HPLC (Agilent model 1260 infinity) with UV detection at 210 nm. The organic acids separation were carried out on a column (Agilent ZORBAX SB C18, 4.6 mm × 250 mm, 5 µm) with 0.1 M monopotassium phosphate as mobile phase at a constant flow rate of 0.8 ml/min (200 bar), pH of buffer was adjusted to 2.7 at 25 °C

oven temperature. Then retention time of organic acids was recorded and compared with the organic acids standard solutions.

### Biological experiment

#### Inoculum preparation

Bacterial isolate was grown in 50 ml nutrient broth and incubated at 30 °C in incubator shaker for 48 h, then centrifuged at 10000 rpm at 4 °C for 15 min, to collect the bacterial cell. The bacterial suspension was diluted in sterile D.W to a final concentration of  $84 \times 10^8$  CFU. ml<sup>-1</sup> [18].

#### Seeds preparation

The seeds of mung bean (*Vigna radiate* [L.] R. Wilczek), category - local green; procured from General Authority for testing and certification seeds / Abu- Ghraib, were surface sterilized for 10 min with 5% sodium hypochlorite solution and then washed thrice with D.W and left for air drying under laminar air flow for 2 h then, soaked in bacterial cell suspension. The non-soaked seeds were served as control. Furthermore, soaked seeds were air dried inside the laminar air flow for 2 h. and left overnight in the moist chamber [19].

#### Pots experiment

Pot experiment was carried out in Agricultural Research office / Abu Ghraib. Plastic pots (25 cm diameter) were filled with 8 Kg soil, obtained from Abu Ghraib field. Table-1 showed some of the soil properties, which was silty clay loam. The experiment was conducted with two treatments and three replicates: Control, includes soil and non-inoculated seeds (C) and inoculated treatment, includes soil and seeds inoculated with *Bacillus cereus*(T). All the treatments were supplied with 50 mg N.kg<sup>-1</sup>, 160 mgP. kg<sup>-1</sup>and 120 mg K. kg<sup>-1</sup>soil that was applied as urea, Tri-calcium phosphate (TCP),and potassium chlorite respectively, based on the nutrient requirements of mung bean plant. Plant seeds were cultured and covered with 15 mm thick layer of soil, pots were irrigated during the study period. The plants were harvested after 2 months, and plant growth parameters were analyzed, total plant length, number of branches, fresh weight, and dry weight.

**Table 1**-Texture and physiological properties of the soil used in the study

Property	Unite	Value
Soil PH 1:1		7.3
Electric conductivity EC 1: 1	ds.m <sup>-1</sup>	3.0
Cation exchange capacity (CEC)	Cmol. kg <sup>-1</sup> Soil	23.0
Calcium Ca <sup>+2</sup>		7.0
MagnesiumMg <sup>+2</sup>		40.0
Sodium Na <sup>+</sup>		16.0
Nitrogen N	g. kg <sup>-1</sup>	56.0
Phosphor P		26.0
Potassium K		46.0
Organic mater		138
Sand	g .kg <sup>-1</sup>	176
Silt		540
Clay		284
Texture: Silty Clay Loam		

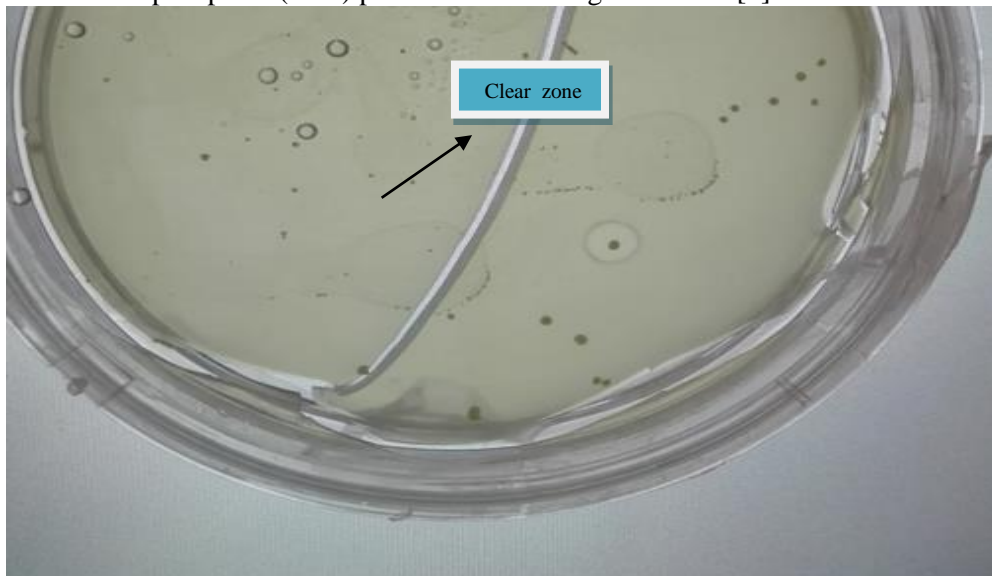
#### Statistical analysis

Pot experiment was performed with a complete random design (CRD). The data were statistically analyzed using ANOVA. The means of triplicate samples were compared with least significant difference (LSD) values at the level of 0.05 [20].

### Results and discussion

#### Isolation of PSB

Out of 16 soil samples obtained, 9 bacterial isolates formed a halo zone around the bacterial colony during growth in NBRIP agar medium (Figure-1), 6 of them were from wheat rhizosphere (Tab. 2). The 9 bacterial isolates were considered as PSB on the basis of their capability to solubilize the insoluble tri-calcium phosphate (TCP) present in NBRIP agar medium [9].



**Figure 1-**Clear halo zone formation by PSB isolates on NBRIB medium.

**Table 2-**The sampling area of PSB isolates

No .of sample	Cultured plant	Site	Isolate
1	Wheat	Tarmiya	Y1
2	Wheat	Abu-Ghraib	Y2, Y3
3	Barley	Latifieh	Y4
4	Wheat	Aqraqof	Y5, Y6
5	Barley	Aqraqof	Y7
6	yellow corn	Jadriya	Y8
7	yellow corn	College of Agriculture	Y9

The nine PSB isolates showed different solubilization abilities (Table-3). The halo zone formed after 2 weeks of incubation in NBRIP agar ranged from 0.3 cm to 0.8 cm, Y4 and Y5 isolates have the widest zone (0.8 cm) followed by Y2, Y3, and Y6 (0.7 cm). SI index ranged from 2.00 to 3.66, Y4 isolate reflex the highest value (3.66) followed by Y3 and Y6 (3.33), then Y5 (3.00). The isolates ability to release phosphate (P), from tri-calcium phosphate (TCP), in NBRIP broth ranged from 39.00 to 20.10  $\mu\text{g.ml}^{-1}$ , the isolate Y4 showed the highest ability (39.00  $\mu\text{g.ml}^{-1}$ ) followed by Y3 (37.00  $\mu\text{g.ml}^{-1}$ ) and Y6 (35.00  $\mu\text{g.ml}^{-1}$ ). The decline in P-solubilization activity may be due to the nutrients deficiency in the culture medium which needed by phosphate solubilizing bacteria, as a substrates to convert it to organic acid [21]. Some researchers reported a wide variation in the P-solubilization capacities for eight PSB strains which, CBP 2 isolate showed maximum P-solubilization reached 45.96 ppm [22], While others found P-solubilization activity ranged from 0.88 to 24.92  $\mu\text{gml}^{-1}$  [23]. A widely accepted hypothesis for phosphate solubilization mechanism is that different types of organic acids, including maleic acid, lactic acid, isovaleric acid, isobutyric acid, acetic acid, oxalic acid and citric acid, are being produced which in turn facilitates the solubilization of insoluble phosphates by chelating the cation bound to phosphate, thereby converting it to a soluble form [24].

**Table 3-**Phosphate solubilizing activity for PSB isolates on NBRIP medium

The isolate	Halo-zone diameter (cm)	Colony diameter (cm)	SI	Soluble P (TCP) ( $\mu\text{g.ml}^{-1}$ )
Y1	0.6	1.50	2.50	26.00
Y2	0.7	1.75	2.75	25.00
Y3	0.7	2.33	3.33	37.00
Y4	0.8	2.66	3.66	39.00
Y5	0.8	2.00	3.00	28.00
Y6	0.7	2.33	3.33	35.00
Y7	0.4	1.33	2.33	21.00
Y8	0.4	1.33	2.33	21.00
Y9	0.3	1.00	2.00	20.10

### Production of hydrolytic enzymes, HCN and IAA

Various PSB features were examined for possible association with the effectiveness of these isolates in the phosphorus solubilization and enhance plant growth, as well as effectiveness against some pathogens. The results showed that, out of 9 isolates, only 7 PSB isolates were able to produce cellulase (Tab. 4), by formation of the clear zone on M9 medium. While all the isolates were able to produce pectinase and protease production through formation of the transparent zone on M9 and skim milk agar plate. Hydrolytic enzymes production is important assistance to the antimicrobial potential of antagonists and also have vital roles in the degradation of phytopathogenic micromycetes' cell wall, therefore the synthesis of the hydrolytic enzymes may associate with the antifungal potential. Moreover, some hydrolytic enzymes, such as pectinases, protease, and cellulose, play a substantial role in the stimulation of plant growth, and also contribute to the plant roots colonization by rhizospheric bacteria, the rhizospheric bacteria, such as *Pseudomonas* and *Bacillus* genera, exhibited high enzymatic activity and both strains secreted proteinase, amylases, pectinase and cellulases in the suitable conditions and medium [25].

Totally nine isolates showed production of HCN on King's B medium which could contribute to their antifungal activity. The Maximum color change of filter paper strip which soaked in picric acid was shown by Y3 isolate followed by Y4 and Y6, while minimum color change was shown by strain Y7, Y8 and Y9. Production of HCN by PSB isolates can inhibit fungal growth and it was reported that *Bacillus* spp. isolate can inhibit fungal growth by the production of HCN [25].

The ability of IAA production in LB broth medium was found in all PSB isolates, but in different chromatic concentrations, which ranged between pink to red or bulbous color. The maximum color change, compared to control, was recognized in Y4 and Y3 isolates, while the minimum color change was recognized in Y7 and Y8 isolates. IAA secreted by some rhizospheric bacteria may directly be involved with the increase of the surface area of the roots which stimulate cell elongation and division, which increases plant uptake of nutrients from the soil. Previous study showed that PSB isolates (JY17 and JY22) improved grape growth by the production of some metabolites facilitating such as IAA, ACC deaminase, and siderophores [26].

**Table 4-**Production of hydrolytic enzymes, HCN and IAA

The isolates	Cellulase	Pectinase	Protease	HCN	IAA
Y1	-	+	+	++	++
Y2	-	+	+	++	++
Y3	+	+	+	++++	+++
Y4	+	+	+	++++	+++
Y5	+	+	+	++	++
Y6	+	+	+	++++	++
Y7	+	+	+	+	+
Y8	+	+	+	+	+
Y9	+	+	+	+	+

**Identification of PSB isolate**

The isolate Y4 was selected from the nine PSB isolates, depending on the previous tests, because it showed the widest solubilizing halo zone (0.8 cm), highest SI values (3.66) and the highest ability to release phosphate (39.00 µg.ml<sup>-1</sup>) (Table. 3). The isolate Y4 was positive for cellulase, pectinase and protease production tests. Moreover, it was able to produce HCN and IAA with high efficiency (Table-5).

Morphological and biochemical tests were performed for Y4 isolate. The obtained isolate was found to be Gram positive, small colony, rod shaped bacteria and also, Showed off-white to creamish colony color when it grown on NA medium. The isolate shows negative results for methyl red and oxidase, and methyl red tests, and positive results for catalase and voges-proskaur. (Table-5). On the basis of these tests, the isolates were closely related to *Bacillus* genera. Furthermore, *Bacillus* species isolate was identified, to detect its species, by using VITEK2.

**Table 5-**Morphological and biochemical properties for selected PSB isolate.

Biochemical test	Result
Pigmentation	off-white to creamish
Colony morphology	Smooth margin
Cell shape	Rod
Motility	Motile
Methyl red	-ve
Gram stain	+ve
Voges-proskauer	+ve
Oxidase	-ve
Catalase	+ve

**Identified of PSB isolate by VITEC 2 compact system**

The Vitek 2 compact system was used to confirm the diagnosis of the PSB isolate under study, the identified isolate was related to *Bacillus cereus* with a probability of 91%, as illustrated in (Table-6). Vitek 2 compact system, a development of - API -20, records the chromatic changes as a result of bacterial growth.

**Table 6-**Identification of *Bacillus sereus* using Vitek 2 compact system

<b>Identification Information</b>	<b>Analysis Time:</b> 13.87 hours	<b>Status:</b> Final
<b>Selected Organism</b>	91% Probability <b>Bacillus cereus</b>	
	<b>Bionumber:</b> 0727101240456661	
<b>ID Analysis Messages</b>		

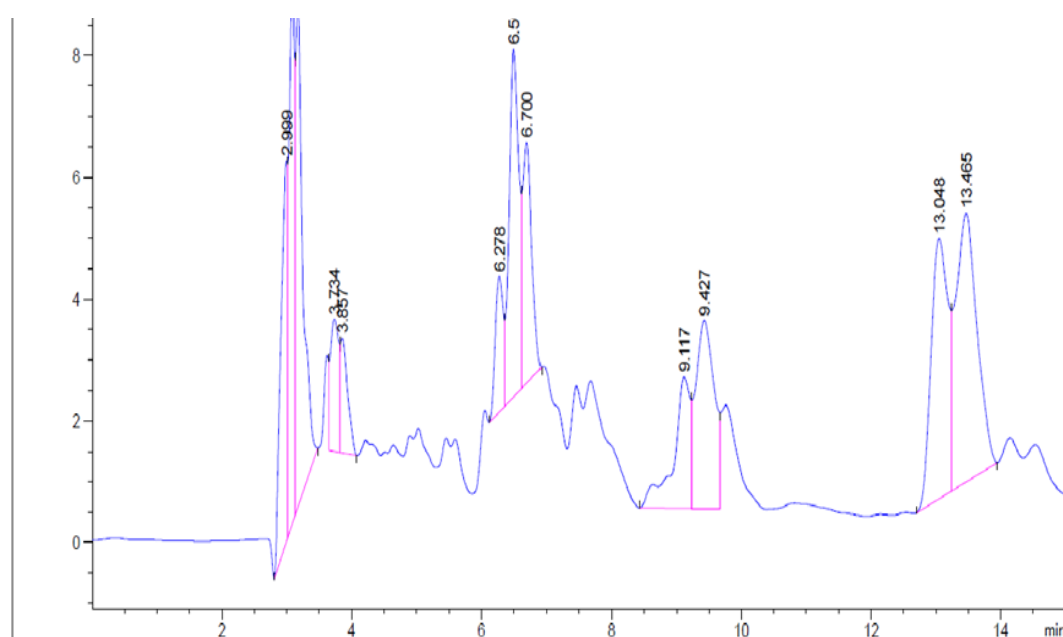
Biochemical Details																	
1	BXYL	-	3	LysA	-	4	AspA	(-)	5	LeuA	+	7	PheA	+	8	ProA	+
9	BGAL	-	10	PyrA	+	11	AGAL	-	12	AlaA	+	13	TyrA	+	14	BNAG	+
15	APPA	+	18	CDEX	-	19	dGAL	-	21	GLYG	-	22	INO	-	24	MdG	-
25	ELLM	+	26	MdX	-	27	AMAN	-	29	MTE	(-)	30	GlyA	+	31	dMAN	-
32	dMNE	-	34	dMLZ	-	36	NAG	+	37	PLE	-	39	IRHA	-	41	BGLU	-
43	BMAN	-	44	PHC	-	45	PVATE	+	46	AGLU	+	47	dTAG	-	48	dTRE	+
50	INU	-	53	dGLU	+	54	dRIB	+	56	PSCNa	-	58	NaCl 6.5%	+	59	KAN	+
60	OLD	-	61	ESC	+	62	TTZ	+	63	POLYB_R	+						

### Detection of organic acid

*Bacillus cereus* ability to produce organic acids in the NBRIP broth medium was determined by HPLC with wavelength 210 nm. The results in Figure-2 illustrated 12 peaks for different organic acids (Appendix 1 and 2).

Three major peaks (Table-7) were detected for oxalic acid (2.996), citric acid (9.117), and malic acid (3.734) by comparing their retention times with those of the authentic standards (Appendix 3,4,and 5).

These results demonstrated that the production of low-molecular organic acids such as oxalic acid, malic acid, succinic acid, tartaric acid and citric acid by acid-producing *Bacillus* strain could significantly increase the mobility and bioavailability of phosphorus by bonding such heavy metals as Fe, Mn, Zn, Cu, Cd, Pb through acidification, chelating, sedimentation and oxidation reduction [27]. Furthermore, secretion of citric acid by PSB isolates can improve their P-solubilizing efficiency [26]. Researchers incorporated artificial citrate operon that contained 2 genes; NADH insensitive citrate synthases (gltA1) and citrate transporter (citC) genes, into the genomes of *Pseudomonas fluorescense* [28] and *Enterobacter hormaechei* strains [29], founding that the hybrids strains have more efficacy to secrete more citric acid than the native strains, therefore they released more soluble P in the mediums.



**Figure 2-**The organic acids produced by *Bacillus cereus*, HPLC chromatogram

**Table 7-**Production of organic acids in NBRIP medium by *Bacillus cereus*

Sr. No	Organic acid	Retention time (min.)
1	Oxalic acid	2.996
2	Citric acid	9.117
3	Malic acid	3.734

This results demonstrated that the production of low-molecular organic acids such as oxalic acid, malic acid, succinic acid, tartaric acid and citric acid by acid-producing *Bacillus* strain could significantly increase the mobility and bioavailability of phosphorus by bonding such heavy metals as Fe, Mn, Zn, Cu, Cd, Pb through acidification, chelating, sedimentation and oxidation reduction [27].

### Pot experiments

The effect of *Bacillus cereus* isolate on mung bean plant as inoculum, was detected in biological experiment. Inoculated plants illustrated a significant increase in branches number (12.33), plant length (83.0cm), fresh weight (27.25g) and dray weight (1.427g) compared with control treatments, after 2 months of growth (Table- 8). These increases in inoculated plants may be due to enhancing the



amount of soluble P presence in the soil which in turn increase the uptake of P by mung bean plants or suppress many diseases caused by different phytopathogens [26, 30].

*Aloe barbadensis* treated with PSB isolates were increased in P content, especially in leaves, and consequently all the growth parameters, including leaf length, root length, drying weight, were elevated [31]. The previous study found that PSB isolates increased the height, stem thickness and root and shoot dry weight of Cabernet Sauvignon(*Vitis vinifera*) [26].

**Table 8-**The effects of *Bacillus cereus* inoculum on mung bean growth

The treatments Growth parameter	C	T	LSD P < 0.05
Number of branches	7.33±1.000	12.33 ±1.000	4.3030
Plant length (cm)	43.3± 2.91	83.0± 2.91	12.50
Fresh weight(g)	23.08±3.132	27.25 ±3.132	3.1320
Dry weight( Mg/ plant)	0.867±0.0651	1.427±0.0651	0.2799

Values are given as means of triplicate samples ± S.E

### Conclusion

The ability of PSB isolates to convert insoluble phosphorus to a soluble form, is an important trait of phosphate solubilizing bacteria to overcome the problem of deficiency of available phosphorus as a result of chemical fertilizers addition to soil. Phosphorus is a master key for plants nutrition, but it is often not available due to its fixation in soil. PSB are beneficial rhizobacteria with all their mechanisms as plant growth promoting bacteria, such as phosphate solubilization, antifungal and production of HCN, plant growth regulators (auxins, cytokinins and gibberellins) and lytic enzymes. Nine PSB isolates were able to solubilize tri-calcium phosphate presence in NBRIP media, most of them were also able to produce cellulase, pectinase, protease, IAA and HCN. *Bacillus cereus* was the most efficient phosphate solubilizing bacteria, by producing high levels of P- solubilization and other plant growth promoting activities. The organic acids producing by this isolate have the most important role in the mechanism of phosphate solubilization which in turn improves mung bean (*Vigna radiata* [L.] R. Wilczek) plant growth parameters.

### Acknowledgment

The authors acknowledge with thanks the Agricultural Research Office/Abu Graib, Baghdad, and Ministry of Science and Technology /Department of researches/ Baghdad, for their contribution and help during the research period.

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