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Efficiency of Phosphate solubilizing bacteria isolated from different regions in dissolving of the insoluble phosphate and the activity of phosphatase enzyme

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Abstract
Ten isolates of Phosphate solubilizing bacteria were isolated from the soil of the rhizosphere for the plants cultivated in different locations of southern Iraq, the study of the culturing, microscopic and biochemical traits of these isolates, as well as testing the efficiency of these isolates in dissolving the phosphates in the liquid culture media, the activity of phosphatase enzyme, solubility index, their production of total acidity and reduce the reaction degree of the liquid media. The results showed that the isolates belonged to the Bacillus genus. B6 isolates were the most efficient isolates in dissolving the phosphate amounted to (36.6 mg P. L^{-1}) and solubility index amounted to (3.428), as well as their superiority in reducing the pH for the growth media from 7 to 5.6, their production to total acidity of (1.24 Meq.L^{-1}). The efficacy of the acid and alkaline phosphatase enzyme was (2.48 and 1.13 IU.L^{-1}), respectively.

Keywords: respectively, enzyme, production, phosphatase, superiority

1. Introduction
Phosphorus is considered the second most important mineral nutrient after nitrogen, which is needed by the plant and is specific for agricultural production because of its important role in the formation of cells and their division, the formation of cellular membranes, the transfer of genetic properties and other, where the Phosphors availability is dependent on the change of the soil pH. In acid soils, phosphorus is deposited in it the form of insoluble iron phosphate and aluminum phosphate (Xaio, *et al.*, 2011) (Al-Taey *et al.*, 2017) [7]. Phosphorus is also less available in the alkaline media as a result of its deposition with calcium carbonate, thus most of the alkaline soil becomes containing on large deposits of accumulated phosphorus resulting from the successive annual additions of phosphate fertilizer (FNCA, 2006) [14]. In order to improve phosphorus availability in the soil, phosphorus solvents are tend to using the Phosphate solubilizing bacteria which have the ability to dissolve insoluble phosphorus compounds through acidizing, chelating and ion exchange (NIIR, 2007) [21]. It produces different types of organic and mineral acids and growth hormones such as Auxins, Gibberellins GA3 and Cytokinins, are considered the most important endogenous substances for modulating physiological and molecular responses, a critical requirement for plant survival as sessile organisms (Al-Taey, 2017, Al-Taey, 2018) [6-7, 5], Phytohormones act either at
their site of synthesis or elsewhere in plants following their transport thus reducing the harmful effects of nutrition imbalance resulting from environmental stresses (AL-Taey and AL-Musawi, 2019) [4], As well as their ability to produce antibiotics that effectively contribute to plant protection from pathogens (Mohamed & Gomaa, 2012: Siddikee, *et al.*, 2011) [20, 26]. Phosphate solubilizing bacteria are considered one of the types of plant growth-promoting rhizobacteria (PGPR) which have different methods of promoting plant growth, such as fixing atmospheric nitrogen, dissolving insoluble phosphorus, controlling some plant diseases, releasing some growth regulators, producing vitamins, enzymes, excretion of Osmosis resistance materials such as polysaccharides, amino acid, proline, acidic acid and ACC denminase, which improve the growth of growing plants under stress conditions (Omer, *et al.*, 2016) [22]. Inoculation with these bacteria reduces the use of mineral fertilizers and keeps the ecosystem from pollution (Vivekanandan *et al.*, 2015) [32]. Therefore, the study aims to isolate the Phosphate solubilizing bacteria from the rhizosphere soil samples of some crops and test their efficiency in dissolving the insoluble phosphates and the activity of phosphatase enzyme.

2. Materials and Methods
Rhizosphere soil samples for Alfalfa, tomato, wheat and barley plants were collected from the fields in Basra and Dhi Qar provinces. The plant was randomly taken off from each field at the flowering stage and obtained the soil of the Rhizosphere according to (Thaher, 2001) [30]. Phosphate solubilizing bacteria was isolated from different Rhizosphere soil samples by plate coumt dillution, using the modified solid Pikovskaya media (Sundara-Rao and Singh, 1963) [28]. After incubation of the plates at 28 °C for 2-3 days, the colonies of Phosphate solubilizing bacteria were identified through forming it a clear zone around the colonies as shown in Figure (1).
The solubility coefficient was estimated according method of (El-Komy, 2005) [13].

SI= D/C
Whereas
SI = solubility coefficient (without unit)
D = total diameter for the colony + transparent halo (cm)
C = diameter of the colony only (cm)
The culture traits of bacterial colonies were also taken and also microscopic and biochemical traits for isolates

(Bergey`s and Manual, 2004) [9].

The efficiency of the Phosphate solubilizing bacteria isolates was determined by using the free phosphate liquid Pikovskaya media, which was placed in 150 mL in a 250 mL flask and 1 g of tri-calcium phosphate was added as a source of insoluble phosphate. After sterilization, the flasks were inoculated with 1mL pure culture of Phosphate solubilizing bacteria, with age of 48 hr at a lighting density of 0.85 (Standard turbidity equivalent containing 1.5 x 108 bacteria. ml^{-1}) according to (Baron and Finegold, 1990) [8], The flasks were incubated in the shaking incubator (100 cycles. min^{-1}) at 28 °C for 3 weeks and 10 ml was then taken from the liquid culture and filtered with centrifugal (6000 cycles / min). The pH for the media was estimated by the pH-meter as indicated by (Black, 1965a) [11] and the total acidity by the Titration was also estimated according to (Sberber, 1957) [27] by the following equation:

Percentage of total acidity =

$$\frac{\text{Normalty of base} \times \text{volume} \times 0.064 \times \text{Final volume of the solution}}{\text{The volume of the Titrated solution} \times \text{Sample volume}} \times 100\%$$

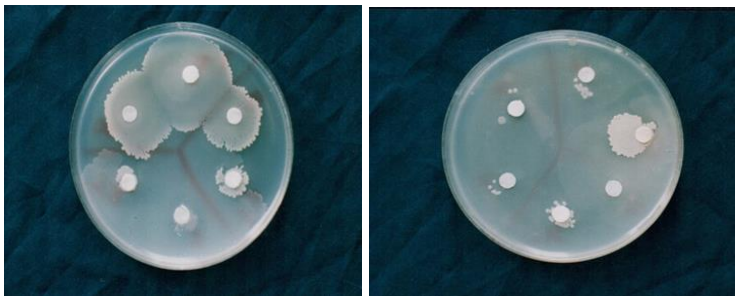

Fig 1: clear zone around colonies of Phosphate solubilizing bacteria isolated from rhizosphere soils for different plants

Dissolved phosphorus was estimated by spectrophotometer along a wavelength of 470 nanometers (Richards, 1954). The efficacy of acid and alkaline phosphatase was estimated by taking 0.5ml from the Pikovskaya media containing phosphate soluble isolates and 0.5 mL of (PNPP 0.115 M) P-nitrophenyl phosphate disodium substrate Tabatabai & Bremmer, 1965) [29]. The activity of phosphatase was estimated according to the equation: (Al-Khan *et al.*, 2006) [3]

The activity of phosphatase enzyme = the intensity of light absorption for the model × 101

A 10 isolates of the Phosphate solubilizing bacteria were obtained for the rhizosphere samples of the roots obtained for the Alfalfa, tomato, wheat and barley plants cultivated in the soils of Basra, Dhi Qar, and Maysan provinces, which their cultivation, microscopic and biochemical traits listed in Table (1, 2), which showed that the isolates belong to the Bacillus genus (Holt *et al.*, 1984; Bergeys manul, 1984). Several studies have indicated that Iraqi soil is rich with Phosphate solubilizing bacteria isolates which belong to the Bacillus genus (Zahir, 1981; Hassan, 2013) [15].

Table 1: the cultivation and microscopic traits for Phosphate solubilizing bacteria isolates

Isolate code	Source of isolate	Cultivation description for bacterial isolates				Microscopic description for bacterial isolates			
		Color	The edge of the colony	Height of the colony	Size	Shape	Gram stain	movement	Aggregation
B1	Al-dawaya/ Alfalfa	Creamy	Regular	High	Middle	Short rod	+	motility	Pairs
B2	Al-bade / barley	White	irregular	High	Big	Short rod	+	motility	Chains
B3	Um Onaij/ Tomato	Creamy	Regular	High	Big	Short rod	+	motility	Chains
B4	Amarah / Alfalfa	White	Serrated	High	Big	Short rod	+	motility	Chains
B5	Al-dawaya/ Tomato	Yellow	Regular	High	Middle	Short rod	+	motility	Pairs
B6	Al-bade/ Alfalfa	White	Regular	High	Middle	Short rod	+	motility	Single
B7	Al Hartha/ Alfalfa	Creamy	Regular	High	Big	Short rod	+	motility	Chains
B8	Al-Zubair / Tomatoes	brouwn	irregular	High	Big	Short rod	+	motility	Chains
B9	Al-Rifai/ Alfalfa	White	Regular	High	Middle	Short rod	+	motility	Pairs
B10	Amarah / wheat	Creamy	Serrated	High	Big	Short rod	+	motility	Single

Table 2: Some biochemical traits for Phosphate solubilizing bacteria isolates

Isolate code	Catalase test	Oxidase test	Decomposition of starch	3% NaCl	37^0	Methyl red	Nitrate reduction	Gelatin decomposition
B1	+	+	+	++	++	+	+	-
B2	+	+	+	++	+	+	+	-
B3	+	+	+	++	+	+	+	-
B4	+	+	+	+	+	+	+	-
B5	+	+	+	++	++	-	+	-
B6	+	+	+	++	++	-	+	-
B7	+	+	+	+	+	+	+	-
B8	+	+	+	+	+	+	+	-
B9	+	+	+	++	++	-	+	-
B10	+	+	+	++	++	+	+	-

Table 3: solubility Index of phosphate solubilizing isolates

Isolate code	Colonial diameter (cm)	Diameter of the transparent halo + colony (cm)	Phosphate Enzyme Index (SI)
B1	0.6	1.7	2.834
B2	1.0	2.1	2.100
B3	0.9	1.7	1.889
B4	0.9	1.8	2.000
B5	0.9	2.2	2.444
B6	0.7	2.4	3.428
B7	0.9	1.7	1.889
B8	0.9	1.6	1.778
B9	0.5	1.5	3.000
B10	0.8	1.8	2.250

Where all of the studied bacterial isolates varied in their ability to dissolve phosphate in the liquid and solid Pikovskaya. This is illustrated by the variation of the dissolving region diameters around the colonies as shown in Figure (1) and Table (3). The isolate B6 that isolated from the rhizosphere of the Alfalfa plant for the Al-bada region was the most efficient isolates in dissolving phosphate. The solubility index for it was 3.428, while the lowest average for dissolving phosphate was (1.778) by isolate B8 that isolated from the tomato rhizosphere in Zubair region as shown in Table (3). This is due to the difference in the Phosphate solubilizing bacteria isolates in the quantity and quality of the metabolic compounds produced by organic acids such as Succinic acid, Glutamic acid, Malic acid, Fumaric acid and phosphatase enzyme activity, all of which work on dissolve phosphate compounds (Mardad *et al.*, 2013; Lin *et al.*, 2006) [19, 18].

Table 4: Activity of alkaline and Acidic Phosphatase Enzyme ($IU.L^{-1}$) for Phosphate solubilizing isolates.

Isolate code	Acidic Phosphatase Enzyme activity ($IU.L^{-1}$)	Alkaline Phosphatase Enzyme activity ($IU.L^{-1}$)
B1	2.10	0.91
B2	1.87	0.59
B3	1.83	0.68
B4	1.80	0.77
B5	2.28	1.01
B6	2.48	1.13
B7	1.75	0.65
B8	1.69	0.73
B9	2.36	1.18
B10	2.00	0.18
Average	2.016	0.846

It is noted from Table (4) that Phosphate solubilizing isolate B6 showed significant efficiency in the activity of acid phosphatase enzyme by recording it (2.48 $IU.L^{-1}$), while isolate B8 was less effective for acid phosphatase enzyme was (1.69 $IU.L^{-1}$), whereas bacterial isolate B9 recorded significant excelling on the rest of the bacterial isolates in the activity of basal phosphatase enzyme amounted to (1.18 IU. L^{-1}), While B2 isolate recorded the lowest activity for basal phosphatase enzyme (0.59 IU. L^{-1}). These results agree with (Poumurugan and Gopi, 2006; Al-Jabouri 2016) [23, 2] found that bacterial isolates differ in their efficacy to produce basal and acid phosphatase enzyme due to the genetic and environmental factors of the bacterial isolates themselves and found a positive correlation between the efficacy of the phosphatase enzyme and the amount of dissolved phosphorus. He *et al.*, (2004) [16] indicated that Phosphate solubilizing bacteria possess phosphatase enzyme, which increases the concentration of metal phosphorus in the media from its low solubility sources, as well as that these bacteria conduct the process of organic phosphorus mineralization. The Phosphate solubilizing bacteria isolate (B6) was excelled on all isolates in the reduction of pH to 5.60 during three weeks of incubation compared to the control treatment (non-inoculated) which gave pH 7.07 as shown in Table (5). This result agrees with (Alam *et al.*, 2004) [1] that the low pH of the liquid media is due to the ability of isolates to produce organic acids such as citric acid, oxalic acid and 2-ketoGlucose acid, and that the quality and quantity of the acid produced is of great importance in dissolving the insoluble phosphate, These results agree with (Al-Jubouri, 2016; El-Komy, 2005) [2, 13]. The bi-interaction between the different isolates and incubation period had a significant effect on the reduction of the degree of reaction for the liquid culture media, The highest decrease (5.22) was found in the interaction between isolate B6 and incubation period (21 days) while the lowest average (7.11) in the treatment of non-inoculation and incubation period 21 days. These results agree with (Salimpour *et al.*, 2010) [25] that one of the most important indicators of Bacillus isolates activity is the low pH values of the culture media in which isolates grow over time.

Table 5: pH values in the liquid culture media inoculated with Phosphate solubilizing isolates treating with Tricalcium phosphate during various incubation periods

Isolate code	Incubation 3 days	Incubation 1 week	Incubation 2 weeks	Incubation 21 days	General average
Control	7.02	7.05	7.10	7.11	7.07
B1	6.56	6.38	5.82	5.89	6.17
B2	6.61	6.50	6.31	6.34	6.44
B3	6.70	6.62	6053	6.55	6.60
B4	6.72	6.63	6.56	6.57	6.62
B5	6.10	5097	5.56	5.53	5.79
B6	6.07	5082	5.29	5.22	5.60
B7	6.75	6071	6.64	6.62	6.68
B8	6.76	6072	6.65	6.71	6.71
B9	6.12	5091	5.50	5.43	5.74
B10	6.66	6052	6.51	6.55	6.56
mean 6.56 6.44 6.23 6.23					
$L.S.D_{0.05}$	Isolate × Incubation period		Isolate		Incubation period
	0.824		0.412		0.275

The isolates also differed in their ability to produce total acidity in the liquid Pikovskaya media, where isolate B6 produced total acidity amounted to (1.33 meq.L^{-1}) after 21 days of incubation compared to the control treatment (non-inoculation) which amounted to (0.35 meq.L^{-1}) as shown in Table (6). This result agree with (Al-Jabouri, 2016) [2] increase in total acidity in the latter stages of incubation. This is due to the use of Phosphate solubilizing bacteria in the metabolism, the construction of its cells and the formation of nucleic acids. It is also noted that the production of total acidity by bacterial isolates was highest during the first periods of incubation and began to decrease after two weeks of incubation and these results agree with (Zahir, 1981; Hassan (2013) [15].

Table 6: Total acidity values in the liquid culture media inoculated with Phosphate solubilizing isolates treating with Tricalcium phosphate during various incubation periods

Isolate code	Incubation 3 days	Incubation 1 week	Incubation 2 weeks	Incubation 21 days	General average
Control	0.47	0.43	0.39	0.35	0.41
B1	0.88	1.02	1.10	1.04	1.01
B2	0.78	0.88	0.93	0.89	0.87
B3	0.73	0.83	0.86	0.82	0.81
B4	0.72	0.85	0.86	0.85	0.82
B5	0.97	1.20	1.28	1.27	1.18
B6	1010	1.22	1.31	1.33	1.24
B7	0.70	0.78	0.80	0.80	0.77
B8	0.67	0.74	0.78	0.77	0.74
B9	1.02	1.19	1.29	1.30	1.20
B10	0.79	0.86	0.90	0.85	0.85
mean 0.80 0.91 0.96 0.93					
$L.S.D_{0.05}$	Isolate × Incubation period		Isolate		Incubation period
	0.216		0.108		0.072

Bacterial isolates differed significantly in the dissolve of tricalcium phosphate in the liquid culture media as shown in Table (7). The B6 isolate was excelled in their solubility for phosphate on the other isolates amounted to (38.7 mg $P.L^{-1}$) after three weeks of incubation, while the lowest average was (4.8 mg $P.L^{-1}$) when untreated at the same time period. This is due to the ability of these isolates to secrete the various organic acids that dissolve the insoluble phosphate (EI-Komy, 2005) [13]. The greatest effect is due to the type of acid produced by bacteria such as lactic acid (Didiek *et al.*, 2000) [12].

Table 7: The amount of dissolved phosphorus (mg $P.L^{-1}$) in the liquid culture media inoculated with Phosphate solubilizing isolates treating with Tricalcium phosphate during various incubation periods

Isolate code	Incubation 3 days	Incubation 1 week	Incubation 2 weeks	Incubation 21 days	General average
Control	6.2	5.8	5.1	4.8	5.48
B1	21.3	25.1	31.6	29.0	26.75
B2	17.1	21.0	22.7	21.2	20.50
B3	11.3	18.2	19.0	17.5	16.50
B4	11.1	19.0	19.2	17.7	16.75
B5	22.3	34.0	37.1	35.6	32.25
B6	32.0	35.3	38.0	38.7	36.00
B7	10.1	17.0	18.2	16.7	15.50
B8	11.2	13.1	17.7	17.0	14.75

B9	25.4	34.3	38.0	37.3	33.75
B10	17.1	18.6	21.1	18.2	18.75
Mean 16.83 21.95 24.34 23.06					
$L.S.D_{0.05}$	Isolate × Incubation period		Isolate		Incubation period
	7.652		3.826		2.381

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