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Studying the Ability of some Bacteria Isolated from Egyptian Soils to Fix Nitrogen and Solubilize Phosphate, *In Vitro*

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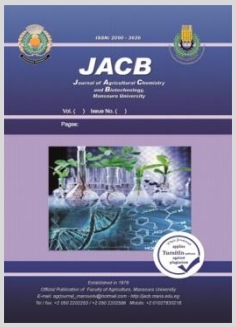

ABSTRACT

Nitrogen and phosphorous are critical determinants for plant growth and productivity. Some soil bacteria can provide them in available forms to plants. These bacteria can be used as nitrogen fixers biofertilizers. In the current study, eighteen isolates of *Azotobacter* spp. were separated. The efficacy concentrations of the N_2-fixation were examined using acetylene reduction technique, the isolates reduced acetylene at rates of 31.01 to 861.01 nmoles C_2H_4/ml/day. The highest lively isolate was No. AZ 14; recognized as *Azotobacter beijerinckii*. 8 phosphate-solubilizing bacteria (PSB) were insulated. These 8 P-solubilizing bacteria were analyzed for the tendency to solubilize P from tri-calcium phosphate in liquid Pikovskaya's medium. The uppermost amount of free phosphorus in vigorously developing culture was 0.3652g g/100 ml after 28 days of cultivation and 0.1515g/100 ml after 7 days of cultivation on tri-calcium phosphate, correspondingly by isolate PSB 5 which was recognized as *Bacillus paramycoides*. Both nitrogen fixing bacteria and P-solubilizing bacteria isolates were genetically identified according to www. ncbi.nlm.nih.gov/blast data bank. The two isolates were defined according to standard morphological and biochemical tests as well as molecular tests.

Keywords: Biofertilizers, N_2-fixating and P-solubilizing bacterial isolates and Blast Gene bank.

INTRODUCTION

A biofertilizer is a ingredient comprises living microorganisms which, when added to seed, plant surfaces, or soil, colonizes the rhizosphere or the inner of the plant and stimulates development by enhancing the resource or obtainability of prime nutrients to the host plant (Vessey, 2003). The expression plant growth promoting rhizobacteria (PGPR) was primary used by (Kloepper and Schroth, 1978). Some PGPR can be deliberated as biofertilizers, though others that encourage plant development by regulating harmful creatures, are biopesticides. Biofertilizers encourage plant growth by nitrogen fixation, phytohormone, phosphate and potassium solubilization (Bashan and de- Bashan, 2005).

Azotobacter spp. are Gram negative, free–living nitrogen fixation, aerobic soil bacteria, oval or spherical bacteria that form thick–walled cysts (way of asexual reproduction below satisfactory circumstance). There are about 6 species in the genus *Azotobacter* part are motile by peritrichous flagella, others are not, *A. chroococcum* is the prime aerobic free–living nitrogen fixer, *Azotobacter* spp. is sensible for acidic pH, elevated salts, and temperature. *Azotobacter* has advantageous influences on crop development and produce through, production of biologically energetic materials, encouragement of rhizospheric microbes (Jnawali *et al.*,2015).

PSB can change the insoluble inorganic phosphate mixtures, such as tri-calcium phosphate, di-calcium phosphate, hydroxyapatite, and rock phosphate, to plant obtainable forms. Numerous bacterial genera were documented to have P-solubilization action as *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Erwinia* and *Klebsiella* (Rodríguez and Fraga, 1999; Yao *et al.*, 2006 & Walpola *et al.*, 2014).

This research aimed to emphasis on isolation, effectiveness assessment, and identification of effective bacterial strains, which can fix nitrogen and solubilize phosphorus.

MATERIALS AND METHODS

Soil samples

The rhizosphere soil samples were gathered from ground cultivated with moringa; a rhizosphere soil sample was assembled for microbiological investigation.

Isolation of *Azotobacter* spp.

Azotobacter spp. were isolated from Most Probable Number (MPN) tubes on modified Ashby's medium as a selective medium for *Azotobacter* growth (AbdEl-Malek and Ishac, 1968). For refinement a loopful of the culture was relocated to 20 ml sterile water in a bottle having glass beads, routinely shaken for 30 min to break the mucous round the cells, which conveys contaminations used for streaking the agarized plates.

Growen colonies were selected and re-purified at least 5 periods, untill demonstrating no contamination. Cultures were considered pure when microscopical

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checkup shown only typical *Azotobacter* and without contaminating organisms. A single colony was transported and saved in Ashby's agar slants.

Isolation of phosphate-solubilizing bacteria:

PSB were isolated by pouring plate technique (Pikovskaya's, 1948). Plates were kept at 30°C for 7 days. PSB were identified by clear zones round the grown colonies. A single colony was taken, purified and kept on Pikovskaya's agar slants.

Estimation of Nitrogenase activity:

Nitrogenase activity was assayed by evaluating examined reduction technique (Hardy *et al*., 1973).

Estimation of water-soluble phosphorus:

The picked effective bacteria were developed in 250 ml conical flasks each containing 100 ml sterile Pikovskaya's medium, tri-calcium (as basis of insoluble phosphorus at rate of 50 mg P/100 ml culture). The estimation of water-soluble phosphorus and pH values were done at day 0, 7, 14, 21 & 28 of incubation. Water soluble phosphorus in culture media was assessed using the method of Boltz and Mellon (1948) modified by Hemalatha *et al*. (2013).

Identification of the bacterial isolates:

Some morphological observations and biochemical characteristics were accomplished. The genetic identification were assessed (Sigma Scientific Services Co). Nucleotide sequences were entered to National Center for Biotechnology Information database (NCBI), website: www.ncbi.nlm.nih.gov/blast, to clear identification with known taxonomic data obtainable at the databank of NCBI (Altschul *et al*., 1997).

RESULTS AND DISCUSSION

Isolation and efficiency of *Azotobacter* spp.:

18 isolates of *Azotobacter* spp. were seprated from MPN tubes. Table (1) shows that efficiency levels of the N_2-fixation varied among the isolates.

Table 1. Nitrogenase activity of *Azotobacter* spp.

Isolate No.	Nitrogenase activity (nmoles C_2H_4/ml/day)
AZ 1	233.56
AZ 2	134.65
AZ 3	339.01
AZ 4	860.01
AZ 5	132.01
AZ 6	31.03
AZ 7	650.71
AZ 8	341.38
AZ 9	31.01
AZ 10	550.94
AZ 11	232.03
AZ 12	651.94
AZ 13	760.03
AZ 14	861.01
AZ 15	113.02
AZ 16	350.71
AZ 17	139.19
AZ 18	234.55

The isolates weaken acetylene actively in the range from 31.01 to 861.01 nmoles C_2H_4/ml/day for AZ 6 and AZ 14 bacterial isolates, respectively. The most active isolate namely after identification *Azotobacter beijerinckii* that isolated from the soil rhizosphere area of moringa plant, was elected for extra experimentations.

Isolation and efficiency of Phosphate-solubilizing bacteria:

8 bacterial colonies display clear zones were separted from Pikovskaya's agar plates. The isolates were then inoculated in Pikovskaya's liquid media. Table (2) shows the variations in pH values as prompted by the activity of PSB on tri-calcium phosphate. Data revealed that there was a speedy reduction in pH values in the first 3 days of the experimentation demonstrating the activity of isolates that led to degrade the organic carbon source of the culture media discharging organic acids. On tri-calcium phosphate, the diminution in pH was followed by a minor lessening and minor variations until 21 days of the experimentation. For instance, isolate PSB4, PSB5, PSB6, PSB 7, PSB8 the pH inclined to increase once more. This may because of a consequent oxidation of organic acids created in culture media and/or to the development of natural materials at the expense of organic acids formerly made. The gotten outcomes are in agreement with those of Hauka *et al*. (2017) and Walpola *et al*. (2014).

Table 2. Changes in pH values as influenced by the activity of phosphate-solubilizing bacteria on tri-calcium phosphate

Isolate No.	Incubation period (days)			
	7	14	21	28
PSB 1	4.93	4.97	4.96	5.53
PSB 2	4.74	5.5	4.73	4.7
PSB 3	4.81	4.83	5.3	7.3
PSB 4	5.01	4.7	5.8	8.54
PSB 5	4.72	7.2	5.4	6.63
PSB6	4.5	6.57	7.4	7.5
PSB 7	5.1	4.7	5.2	6.3
PSB 8	4.7	7.46	7.1	7.3

Initial pH value = 7.0

Table (3) showed the variations in water-soluble phosphorus values as affected by the activity of PSB on tri-calcium phosphate.

Table 3. Changes in water soluble phosphorus (WSP) (g/100 ml culture) as influenced by the activity of phosphate-solubilizing bacteria on tri-calcium phosphate

Isolate No.	Incubation period (days)			
	7	14	21	28
PSB 1	0.1486	0.1553	0.2211	0.2680
PSB 2	0.0623	0.1015	0.1729	0.2641
PSB 3	0.1350	0.1735	0.1870	0.3080
PSB 4	0.1350	0.1431	0.1531	0.1501
PSB 5	0.1515	0.1729	0.2130	0.3652
PSB 6	0.0632	0.0752	0.0823	0.1386
PSB 7	0.1331	0.1501	0.1671	0.2752
PSB 8	0.0352	0.1530	0.1730	0.2750

Initial soluble P = 0.0315 g/100 ml.

The highest liberation of soluble phosphorus from tri-calcium phosphate amounting 0.3652 g p /100 ml culture media for the isolate of PSB 5 after 7,14, 21 and 28 days, respectively. The diminution in P content with the advance of incubation period could be due to the consumption of P causing the changeable levels of P discharge, accessibility of soluble phosphorus in the culture medium might also has an inhibitory effect on further

phosphate solubilization, excretory toxic yields may also responsible for such weakening in P-solubilization. The existing outcomes are matched with that of Hemalatha *et al*. (2013), Walpola *et al*. (2014) and Hauka *et al*. (2017). The maximum amount of liberated phosphorus in actively developing culture was verified by isolate PSB 5 which was used as PSB inoculant in further experiments.

Identification of the bacterial isolates:

Records in Table (4) revealed that the morphological and biochemical features of isolates AZ 14 and PSB 5 according to Bergy's Manual of Systematic Bacteriology (2005).

For isolate AZ 14, cells were rods to ellipsoid shaped, make a cinnamon colored pigment in aged cultures, Gram-negative, capsulated, non-motile, non-spore forming, catalase positive. Hydrolyzing starch, not hydrolyzing casein, can integrate mannitol, glucose, galactose, fructose, mannose, maltose, cellulose, sucrose, sorbitol, xylose and starch as a sole carbon sources and can live up to 7% Na CL containing media. While, isolate PSB 5 cells were rod shaped Gram-positive, non-motile, spore forming, catalase positive, hydrolyzing starch, casein and lipids, negative for Indole and positive for V. P. test, can assimilate glucose, mannose, mannitol, xylose, sorbitol and starch as a sole carbon sources. Agreeing with the morphological, biochemical characteristics and the phylogenetic trees (Figs.1&2) isolates accession numbers in NCBI Gene Bank showed at Table (5),the isolates displayed nearby proximity with *Azotobacter beijerinckii* and *Bacillus paramycoides* fitting with the GeneBank database were attained in BLASTN searches at the NCBI (http://www.ncbi.nlm.nih.gov).

Table 4. Morphological and biochemical properties of bacterial isolates

Isolate No. / Characteristics	Isolate AZ 14	Isolate PSB 5
Morphological		
Shape	Rods to ellipsoid	Rods
Spore forming	–	+
Capsule formation	+	ND
Motility	+	–
Pigment	Cinnamon	ND
Gram stain	–	+
Biochemical		
Catalase production	+	+
Starch hydrolysis	+	+
Casein hydrolysis	-	+
Lipase production	-	+
Indole produced	-	-
Voges-Proskauer test	-	+
Growth at		
1 % NaCL	+	+
2 % NaCL	+	+
3 %NaCL	+	+
4 %NaCL	+	+
5 %NaCL	+	-
6 %NaCL	+	-
7 %NaCL	-	-
8 %NaCL	-	-
9 %NaCL	-	-
10 %NaCL	-	-
Assimilation of sugars		
Glucose	+	+
Galactose	+	-
Fructose	+	-
Mannose	+	+
Maltose	+	-
Cellulose	+	-
Mannitol	+	+
Sorbitol	+	+
Xylose	+	+
Sucrose	+	-
Starch	+	+

+ = positive - = negative ND = not determined.

Fig. 1. Phylogenetic tree of isolate AZ14 *Azotobacter beijerinckii* ICMP8673.

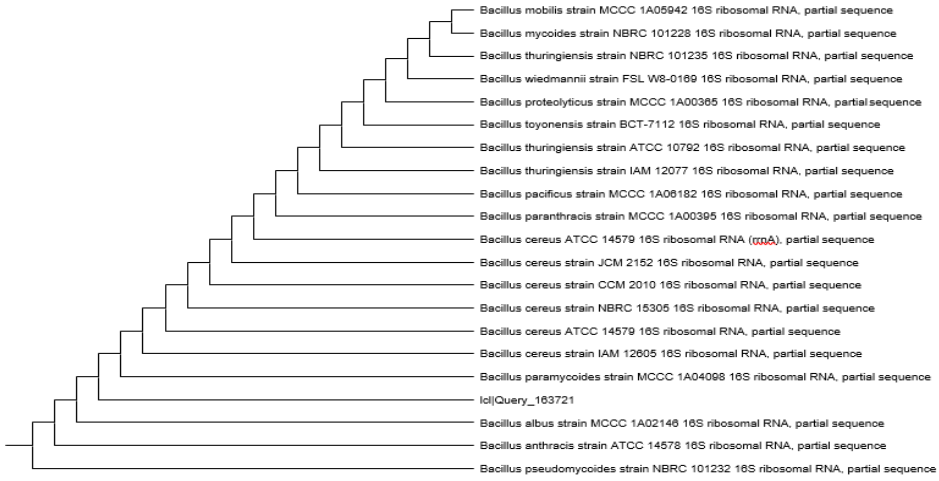

Fig. 2. Phylogenetic tree of isolate PSB5 *Bacillus paramycoides* MCCCIAQ93.

Table 5. Isolates accession numbers in NCBI Gene Bank

Isolate cod No.	Bacterial Species	Sequence length(bp)	Similarity (%)	Accession number
PSB 5	*Bacillus paramycoides*	800	100	NR157734
AZ 14	*Azotobacter beijernckii*	800	97	NR042071

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دراسة قدرة بعض البكتيريات المعزولة من الأراضى المصرية على تثبيت النيتروجين وإذابة الفوسفات معملياً

آية أحمد نادر ، محمد عبد الله العوضى سليم ،عايدة حافظ عفيفى و فتحى اسماعيل على حوقة

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يعتبر النيتروجين والفوسفور من العوامل الضرورية لنمو النبات وإنتاجيته ، فبعض البكتيريا الموجودة في التربة يمكن أن توفرها في صورة ميسرة للنبات وبالتالى يمكن استخدام هذه البكتيريا كأسمدة حيوية. وفي هذه الدراسة ، تم عزل ثمانية عشر عزلة من جنس الأزوتوباكتر، تم اختبار مستويات كفاءتها فى تثبيت نيتروجين الهواء الجوى عن طريق اختبار اختزال الاسيتلين بمعدلات ٣١.٠١ - ٨٦١.٠١ نانومول /مل /يوم وكان أكثرها قدرة على تثبيت النيتروجين العزلة رقم ١٤ التي عرفت باسم ازوتوباكتر بيرجينكيايي. كما تم الحصول على ثمانية عزلات من البكتيريا المذيبة للفوسفات. تم اختبار كفاءة العزلات الثمانية فى قدرتها على إذابة الفوسفات فى بيئة سائلة تحتوى علي ثلاثي فوسفات الكالسيوم وذلك فى بيئة بيكوفسكايا السائلة. وكانت أعلى كمية من الفوسفور الذائب هى ٠.٣٦٥٢ جم / ١٠٠ مل بعد ٢٨ يومًا من التحضين و ٠.١٥١٥ جم / ١٠٠ مل بعد ٧ أيام من التحضين ، على التوالي وذلك باستخدام العزلة رقم ٥ الذي تم تعريفها على أنها بكتريا باسيلس بارامايكويدس. وقد تم تعريف العزلتين طبقا للإختبارات المورفولوجيه و البيوكيميائيه القياسيه بالإضافه إلى الإختبارات الجزيئيه .