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Quantitative estimation of complex phosphate - solubilization by immobilized or free *Azospirillum Lipoferum* (H3) as vital vaccine in Pikovskaya broth (PVK) medium

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Abstract

Azospirillum species were collected from the rhizosphere and free soil of different plants in Al-Jabal El-Akhdar region. The isolates were identified according to biochemical activities. Isolate (H3) which showed the higher solubilization efficiency (SE) on Pikovskaya medium (PVK) containing insoluble phosphate (inorganic phosphate) $Ca_3(po_4)_2$ Isolate (H3) was identified as *Azospirillum lipoferum* (H3), which used as an inoculum as free cell suspension or as alginate formulation. Phosphate solubilization was measured by *A.lipoferum* (H3) as free or alginate immobilized cells in (PVK) liquid medium and recording the pH of the medium at the same time. The results showed in the phosphorus content by immobilized bacteria in liquid (PVK) broth medium from 2nd day of incubation (2.48 µg/ ml) to 10th day (3.70 µg/ ml) and the free bacteria from 2nd day (8.81 µg/ ml) to 10th day (4.49 µg/ ml). pH of the liquid (PVK) broth medium was recorded from 2nd day to 10th day after incubation which decreased by immobilized *A.lipoferum* (H3) from 7.00 pH to 4.47 on the 10th day and by the free *A.lipoferum* (H3) to 6.00, which improved the production of organic acids from sugars which was response of decreasing the pH of the medium. This present study contribute to make agriculture more productive with less harm to the environment and for developing countries where the use of fertilizers is costly, and to encourage of using the biofertilizers 'plant growth promoting rhizobacteria' (PGPR) instead of the chemical fertilizers to enhance the plant growth which is the main goal to increase the food production in a healthy way.

Key words: *Azospirillum lipoferum*, Phosphate solubilization**,** Pikovskaya broth (PVK) medium, (inorganic phosphate) $Ca_3(po_4)_2$.

Introduction

It is well known that a considerable number of bacterial species, mostly those associated with the plant rhizosphere, are able to exert a beneficial effect upon plant growth. Therefore, their use as biofertilizers or control agents for agriculture improvement has been a focus of numerous researchers for a number of years (Suslov, 1982; Glick, 1995). This group of bacteria has been termed 'plant growth promoting rhizobacteria' (PGPR)

(Kloepper and Schroth, 1978). Solubilization of precipitated Tricalcium phosphate (TCP) in unbuffered solid agar medium plates has been used widely as the initial criterion for the isolation of phosphate solubilizing microorganisms (Pikovskaya,1948). Tricalcium phosphate (TCP) is regarded as a model compound for measuring the potential or relative rates of microbial solubilization of insoluble inorganic phosphate compounds. In addition, the insoluble calcium phosphate forms a major portion of insoluble phosphate in soil (Devi and Narasimhan, 1978).

Materials and methods

1. Isolation of *Azospirillum* spp.

Soil and plant root samples were collected from different regions at EL- Jabal EL-Akhdar, Roots were cut into about 0.5-1 cm long segments. 0.1g of the root pieces were introduced into a sterile test-tubes containing 4 ml of the semisolid (DÖbereiner medium -DN) (DÖbereiner and pedrosa, 1987).

2. Identification of the bacterial isolates

Five isolates had a high (solubilization efficiency) on Pikovskaya (PVK) medium plate from different plants were recognized as belonging to the genus *Azospirillum* spp., according to morphological, cultural and some biochemical characteristics described by Tarrand *et al*., (1978), and the schemes described in the 9th edition of Bergy's Manual of Systematic Bacteriology (Krieg and DÖbereiner, 1984).

3. Biochemical tests

Different biochemical tests were carried out **:** Nitrate reductase activity, catalase activity, growth in the presence of 3% $Nacl_2$, starch and gelatin hydrolysis were also tested, growth on succinate and pyruvate as sole carbon - source . For determination of the different species, utilization of different carbon-sources was performed in aerobic conditions. The organisms were grown in semisolid medium containing the carbohydrate together with a pH indicator (bromothymol blue) according to Hugh and Leifson, (1953). The development of a yellow color during 96 hour incubation at 30°C indicates acidification.

4. Immobilization of *Azospirillum lipoferum* isolate (H3) in alginate pellets

A.lipoferum isolate (H3) which had high (solubilization efficiency) on Pikovskaya (PVK) medium on plate was immobilized by entrapment in 2% Ca-alginate. Cells encapsulated in alginate pellets were prepared by using the method applied at laboratory (Shaban and El-Komy, 2000; El- Komy, 2001; 2005).

5. Quantitative estimation of phosphate- solubilization by bacterial isolates in liquid Pikovskaya (PVK) medium containing insoluble phosphate (inorganic phosphate) $Ca_3(po_4)_2$:

Experiments were carried out in flasks (100 ml) each flask containing 20 ml of PVK medium, pH=7.0, before autoclaving. Flasks were inoculated with either 1 ml of bacterial suspension, 3.0g or 2.7g of fresh alginate agar beads containing 10^9 CFU/flask. The flasks were incubated at 30°C as still-surface culture. Cultures were harvested by centrifugation at 7000 x *g* for 10 min, 2, 4, 6, 8 and 10 days after incubation, and the phosphorus in culture was estimated by the paramolybdate blue method using spectrophotometric at (410 nm) (Olsen and Sommers,1982). Phosphorus content was expressed as (µg/ml) and pH of the medium was recorded at the same time.

6. Bacterial cell – free inoculum:

For preparing the bacterial cell free inoculum, one ml of bacterial suspension was transferred into flask containing 50ml of Nutrient broth (NB) medium, incubated at 30 °C for 24 hours.

Results and discussion

After several transfers for purifications, isolates were identified as bacteria belong to the genus *Azospirillum* according to the following common cultural and cell-morphological characteristic in semisolid (DN) medium, strains were Gram- negative motile , ovoid to curved-rods, and showed the presence of characteristic white sub-surface pellicles (often 10 mm) below the surface of semisolid media. In the present study, *Azospirillum* spp. were isolated and enriched from the rhizosphere or free soil using the nitrogen-deficient medium (DN) which L-malic acid was the sole carbon-source (El-Komy, 1992).
Moreover, the cultural and morphological tests and some biochemical tests were proceeded, for example the acidification of different carbon sources by the tested bacteria according to the level of acidification of the pH indicator, (bromothymol-blue) to colour the reaction. The preference of the organic acids by different *Azospirillum* species was reported by Reinhold *et al*., (1985), and can be explained on the basis that organic acids were the major source of nutrients for microflora in the rhizosphere (Curl and Truelove, 1986). All strains were positive in nitrate reductase, catalse and motility activities and the isolates were negative in gelatin and starch hydrolysis.

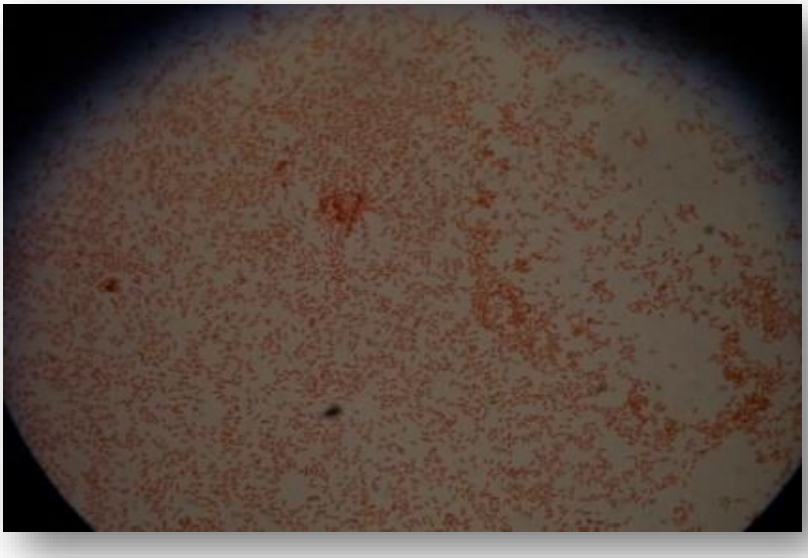

Figure (1) Gram staining of *Azospirillum* spp.

The results showed that *Azospirillum lipoferum* (H3) as free or immobilized bacteria which were further tested for their ability to solubilize tricalcium phosphate in (PVK) broth medium (Table.1). (Figure.2.3) to indirect measurement of phosphate solubilization by plate assay, the direct measurement of phosphate solubilization in (PVK) broth, which is more accurate results, especially for *Azospirillum lipoferum* strains (El-komy, 2005). As a result show on (PVK) broth inoculated with free bacterial formulation of *Azospirillum lipoferum* (H3) and the count of the bacteria recorded 19.5× 10^9 CFU which increased on the first 2nd and 4th day as 8.8 (µg/ml) and 6.9 (µg/ml) of free phosphorus (Figure 2) which determined spectrophotometric and declined slowly after words on the 8,9 and 10th day after incubation, pH values were determined which decreased gradually in (PVK) broth during early days of incubation and no increase was observed in latter days, maximum pH recorded 6.00 on 10th day (Table.2). Data presented in (Figure. 3) showed that phosphate solubilization by the immobilized bacteria in (PVK) broth medium and the count of the bacteria recorded 16× 10^9 CFU which increased slowly from 2nd day 2.4 (µg/ml)to the 10th day 3.7 (µg/ml) of phosphorus without any increase latter compared with free bacterial cell- suspension in (PVK) broth. Earlier reduction in pH values was also observed in (PVK) broth when bacterial strains used in the immobilized forms. pH was recorded 5.62 on the 2nd day an decreased gradually to 4.47 on the 10th day after incubation (Table.2. Figure 4,5).

In general, Ca-phosphate solubilization seems to be linked with pH decrease of the medium but this pH decrease was not strictly proportional to the amount of the phosphate solubilized. These findings were supported by other reports (Illmer and Schinner, 1992).

Table (1) Quantitative estimation of phosphate - solubilization by immobilized or free bacterial isolates in liquid PVK medium.

Isolate	Incubation day	Immobilized bacteria P (µg/ml)	free bacteria P (µg/ml)
A.lipoferum H3	**2**	2.48	8.81
	4	3.20	6.94
	6	3.27	4.93
	8	3.48	4.21
	10	3.70	4.49

Table (2) pH values of *A.lipoferum* (H3) in (PVK) broth and (SE) of immobilized *A.lipoferum* (H3) on (PVK) solid media.

Isolate	Incubation day	pH values in (PVK) broth	
		Free isolate	Immobilized isolate
A.lipoferum H3	2	6.85	5.62
	4	6.21	5.23
	6	6.14	4.75
	8	6.06	4.71
	10	6.00	4.47

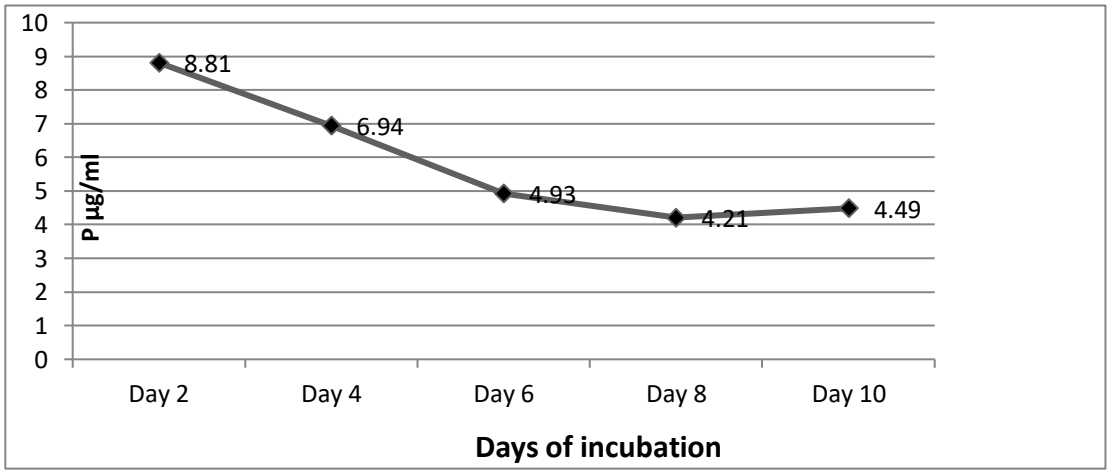

Figure (2) Phosphate-solubilization by *A.lipoferum* (H3) free bacterial isolates in liquid (PVK) medium.

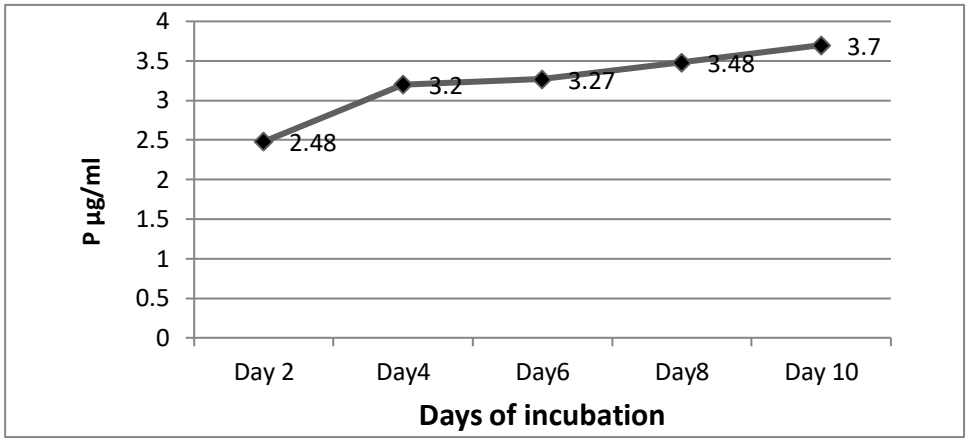

Figure (3) Phosphate-solubilization by *A.lipoferum*(H3) immobilized bacterial isolates in liquid (PVK) medium

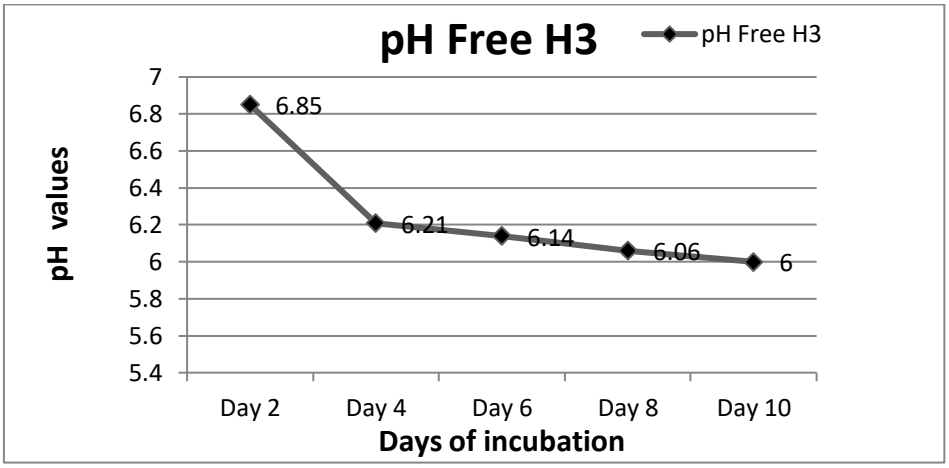

Figure (4) pH values of free *A.lipoferum* (H3) in (PVK) broth

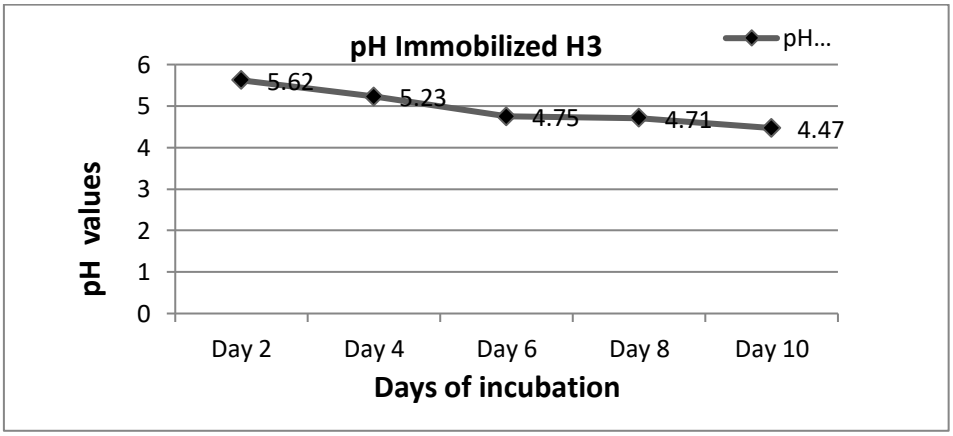

Figure (5) pH values of immobilized *A.lipoferum* (H3) in (PVK) broth

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التقدير الكمي لذوبان الفوسفات المعقد بواسطة بكتيريا *Azospirillum Lipoferum* H3 الحرة والمكبسلة كلقاح حيوي في مرق Pikovskaya (PVK)

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الملخـــص

تم جمع أنواع من بكتيريا الأزوسبيريلم من منطقة ريزوسفير والتربة الحرة لمختلف النباتات في مناطق الجبل الأخضر، تم تعريف العزلات وفقًا للاختبارات الكيميائية والحيوية، عزلة (H3) الذي أظهر أعلى كفاءة ذوبان (SE) على بيئة(PVK) (Pikovskaya) يحتوي على فوسفات غير قابل للذوبان (فوسفات غير عضوي) $Ca_3(po4)_2$ تم تعريف عزلة (H3) باسم (*Azospirillum Lipoferum*)، والذي يستخدم كلقاح في صورة الحرة أو المكبسلة، تم قياس ذوبان الفوسفات بواسطة (*A.lipoferum* H3) كخلايا حرة أو مكبسلة في وسط سائل (PVK) وتسجيل درجة pH في نفس الوقت، أظهرت النتائج في محتوى الفسفور بواسطة البكتيريا المكبسلة في وسط مرق السائل (PVK) من اليوم الثاني من التحضين (2.48 ميكروغرام / مل) إلى اليوم العاشر(3.70 ميكروغرام / مل) والبكتيريا الحرة من اليوم الثاني (8.81 ميكروغرام / مل) إلى اليوم العاشر (4.49 ميكروغرام / مل)، تم تسجيل درجة pH في وسط مرق السائل (PVK) من اليوم الثاني إلى اليوم العاشر بعد التحضين التي انخفضت بواسطة عزلة (*A.lipoferum* H3) المكبسلة (H3) من 7.00 إلى 4.47 pH في اليوم العاشر من التحضين, وبواسطة (*A.lipoferum* H3) الحرة إلى pH6.00 مما يحسن إنتاج الأحماض العضوية من السكريات التي كانت مستجابة لانخفاض في درجات درجة pH، تساهم هذه الدراسة في جعل الزراعة أكثر إنتاجية مع تقليل الأضرار التي تلحق بالبيئة وبالبلدان النامية حيث يكون استخدام الأسمدة مكلفًا، وفي تشجيع استخدام "نمو النبات للأسمدة البيولوجية" في تعزيز استخدام البكتيريا الجذرية المستحثة لنمو النبات (ريزوبكتيريا) "PGPR" بدلاً من الأسمدة الكيماوية لتعزيز نمو النبات الذي هو الهدف الرئيسي لزيادة إنتاج الأغذية بطريقة صحية.