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Optimization of inorganic phosphate solubilization by *Pseudomonas fluorescens* and *Bacillus* sp. isolated from wheat rhizospheric soil

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Abstract

Phosphate solubilizing bacteria play a vital role in soil fertility. They are used to promote the growth of a large group of plants. The aim of this work was to evaluate the bacterial isolates' capacity to release inorganic phosphate and optimizing their solubilization. Different culture media, nitrogen source and carbon source were used under varying culture conditions for optimizing solubilization of phosphate by two bacteria isolated from saline soil *Pseudomonas fluorescens* and *Bacillus* sp. Optimization of growth conditions was also tested using different incubation periods, temperature and pH. The comparison of amounts of phosphorus released by the isolates in different liquid cultures showed that the best solubilization was obtained in NBRIP medium for both *Pseudomonas* and *Bacillus* with quantities of 65.619 µg/mL and 560.667 µg/mL of free phosphorus, respectively. Glucose was found to be the best source of carbon for solubilization of phosphate by the two isolates. The effect of the variation of nitrogen source in the medium allowed to select ammonium sulfatas the most favorable nitrogen source for both bacterial isolates. The results showed that the pH = 5 and the incubation temperature of 30°C are optimal conditions for phosphate solubilization bybacterial isolates. The study of the effect of incubation time led to select the 6th day of incubation as an optimal time for phosphate solubilization by the two isolates.

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Introduction

Phosphorus (P) is one of the most crucial plant nutrients which intensely affect the whole plants growth (Wang *et al.,* 2009) by influencing various key metabolic processes such as cell division and development, energy transport, macromolecular biosynthesis, respiration and photosynthesis in plants (Shenoy and Kalagudi, 2005).Inorganic phosphorus is found in soils, mostly in insoluble mineral complexes such as tricalcium phosphate $Ca_3(PO_4)_2$, ion phosphate $FePO_4$, and aluminium phosphate $AlPO_4$ (Barber, 1995), which appear after repeated applications of chemical fertilizers. Plants have not the capacity to absorb these insoluble forms besides only 0.1% of total phosphorus is in soluble form and it is available for plant nutrition (Zhou *et al.,* 1992).Such P shortage in agronomic practices is, however, corrected through the application of synthetic phosphoric fertilizers which indeed is expensive and hazardous. Moreover, greater portion of P applied exogenously to soils is rapidly fixed into soil constituents (Borling *et al.,* 2001).

Considering the high cost of chemical phosphoric fertilizers and ability of P to form a complex with soil constituents, it has become imperative to find an inexpensive and viable alternative to chemical P fertilizers. Utilization of phosphate solubilizing bacteria to solve this problem for raison of their ability to solubilize phosphate in soil is supported by many researchers (Khan *et al.,* 2007).Recently; phosphate solubilizing microorganisms have attracted the attention of agriculturists as soil inocula to improve the plant growth and yield (Rodriguez and Fraga, 1999). There are various kinds of phosphate solubilizing bacteria (PSB) characterized that belong to different phylogenetic groups: *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agro-bacterium*, *Micrococcus*, *Aerobacter*, *Flavobacterium*, *Mesorhizobium*, *Azotobacter*, *Azospirillum* and *Erwinia* (Goldstein, 1986; Rodriguez and Fraga, 1999). PSB use different mechanisms to convert the insoluble forms of phosphate into soluble forms, but it is generally believed that the major mechanism of mineral phosphate solubilisation is the release of microbial metabolites such as organic acids (Lin *et al.,* 2006). PSB are capable of solubilizing inorganic phosphate from different compounds, such as dicalcium phosphate, tricalcium phosphate and rock phosphate.

The objective of this work is the study of the solubilization capacity of inorganic phosphorus by two rhizobacteria isolated from the saline soil and the optimization of the culture medium and growth conditions for this solubilization.

Materials and methods

Bacterial isolates

Two phosphate solubilizing bacteria used in the present study were isolated from wheat plant rhizosphere of Algerian saline soil previously identified as *Pseudomonas fluorescens* and *Bacillus* sp. and have the ability to solubilize high quantities of tricalcium phosphate.

Optimization of culture medium composition

Effect of different culture mediaon efficiency of phosphate solubilisation

Phosphate solubilizing ability of bacterial strains was tested in four different types of liquid media PVK (Pikovskaya, 1948), AYG (Halder *et al.,* 1990), NBRIY (Nautiyal, 1999) and NBRIP (Nautiyal, 1999) (Table 1).Flasks containing 50 mL of NBRIP medium was inoculated with 1 mL of bacterial suspension (2×10^9cfu/mL) and incubated at 28±2°C on a rotary shaker 180 rpm for 7 days.

Estimation of phosphorus

After incubation the contents of each flask were centrifuged at 6000 rpm for 30 min, the pH of the culture medium was measured. Dissolved phosphate concentration was determined by vanado-molybdate-yellow colour method as described by Jackson (1973).The total soluble phosphorus was calculated from the regression equation of standard curve. The values of soluble phosphate liberated were expressed as µg/mL.

Effect of various carbon sources on efficiency of phosphate solubilisation

The effect of various carbon sources such as glucose, fructose, lactose and sucrose on phosphate solubilisation capacity of bacterial isolates was investigated in favourable culture media containing tricalcium phosphorus as the sole source of phosphate. Flasks containing 50 mL of liquid medium was inoculated with 1 mL of bacterial suspension (10⁹cfu/mL) and incubated at 28±2°C on a rotary shaker 180 rpm for 7 days. Efficiency of phosphate solubilization was calculated as mentioned above.

Effect of various nitrogen sources on efficiency of phosphate solubilisation

The effect of various sources of nitrogen such as $(NH_4)_2SO_4$, urea, casein and $NaNO_3$ on phosphate solubilization capacity of bacterial isolates was investigated in favourable culture media. Flasks containing liquid medium was inoculated with bacterial suspension (2×10^9cfu/mL) and incubated at 28±2 °C on a rotary shaker 180 rpm for 7 days. The released phosphorus concentrations were estimated by the Vanado-molybdate-yellow colour method.

Optimization of growth conditions

Effect of incubation period on efficiency of phosphate solubilisation

The ability of bacterial isolates to solubilize the phosphate was tested for the optimal incubation time.50 mL of liquid medium was inoculated with 1 mL of each bacterial suspension. The inoculated media were incubated at 28±2 °C for 15 days under constant stirring. Every 3 days the amount of phosphorus released by the bacterial isolates was measured by the Vanado-molybdate-yellow colour method.

Effect of temperature on efficiency of phosphate solubilisation

Efficiency of phosphate solubilization by the bacterial isolates was tested by varying their incubation temperature. 50 mL of liquid medium was inoculated with 1 mL of each bacterial suspension. The bacterial cultures were incubated at a temperature of 25 °C, 30 °C, 35 °C and 40 °C in a stirring incubator.

Effect of pH on efficiency of phosphate solubilisation

The ability of bacterial isolates to solubilize phosphate was tested by varying the initial pH of the culture medium to 5, 6, 7 and 8. The culture medium was inoculated by bacterial suspensions and then incubated in a stirring incubator at 180 rpm at 28±2 °C.

Statistical analysis

The data obtained in this study was subjected to analysis of variance (ANOVA) and comparisons of means were performed by Newman and Keuls test at $p \leq 0.05$ using Statbox.

Results and discussion

Effect of different culture media on efficiency of phosphate solubilisation

The ability of studied isolates to solubilize ticalcium phosphate was tested using four liquid culture media: PVK,AYG, NBRIY and NBRIP. Two bacterial isolates were used in this study *Pseudomonas fluorescens* and *Bacillus* sp.

Table 1. Composition of different culture media.

Media Component (g/l)	PVK	AYG	NBRIY	NBRIP
Glucose	10	20	10	10
$(NH4)_2SO_4$	0.5	1	0.5	0.1
$MgSO_4.7H_2O$	0.1	0.5	0.1	0.25
Yeast extract	0.5	0.2	-	0.2
KCl	0.2	-	0.2	0.2
NaCl	0 .2	0.002	0.2	0.2
$FeSO_4.7H_2O$	0 .002	0.002	0.002	-
$MnSO_4.7H_2O$	0.002	-	0.002	-
$MgCl_2.6H_2O$	-	-	-	5
$Ca_3(PO_4)$	5	5	5	5
pH	7.2	6.8	7	7

The amount of soluble phosphorus released by the bacterial isolates in different culture medium ranged from 84.476 to 651.619 µg /mL where *Pseudomonas* was more efficient than *Bacillus*. The comparison of phosphorus quantities released in different liquid culture media revealed that the best solubilization was obtained in the NBRIP medium for both isolates followed by PVK, NBRIY and lastly AYG medium with the lowest phosphorus quantities (Fig. 1). Johri *et al.,* 1999 and Lins *et al.,* 2014 also found that NBRIP liquid medium is the best medium for solubilization of phosphate by rhizobacteria. This Classify NBRIP liquid medium as the best medium for solubilizing phosphate by most PSMs (Nautiyal *et al.,* 2000).

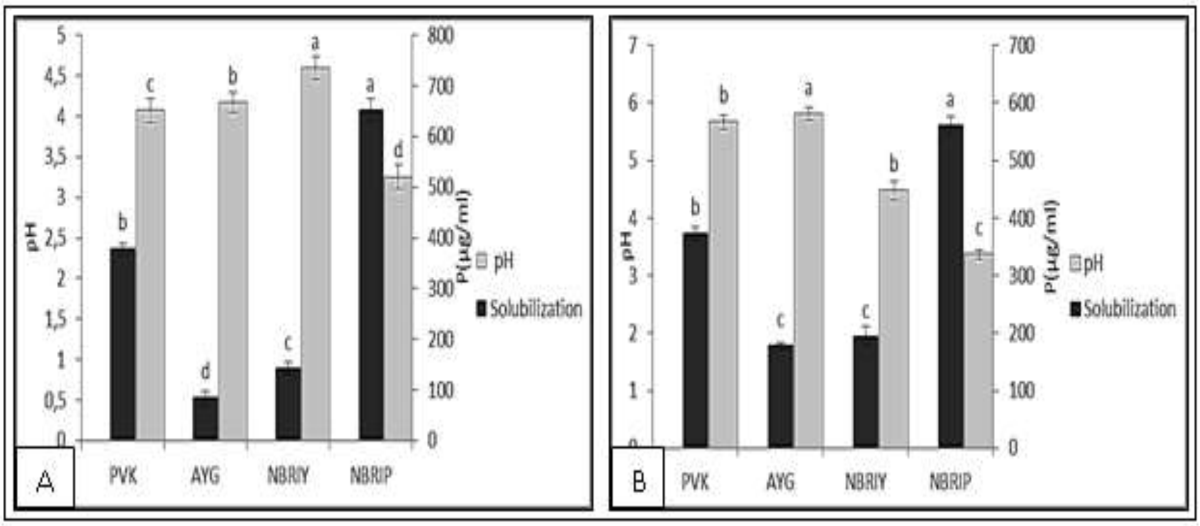

Fig. 1. Effect of various growth media on P solubilization. A: *Pseudomonas fluorescens,* B: *Bacillus sp.*

The pH is an essential factor in phosphate solubilization, pH values of the bacterial cultures decreased from initial value of 7.0 to 3.9 in NBRIP medium. Negative correlation was observed between the amounts of solubilized P and pH values. It was reported that the production of organic acids by the microorganisms is the major factor but not the sole factor responsible for phosphate solubilization by bacteria (Chen *et al.,* 2005, Khan *et al.,* 2014).*Bacillus* and *Pseudomonas* spp. Species are among the most preferment bacterial communities in phosphate solubilization (Kucey *et al.,* 1989; Wani *et al.,* 2007).

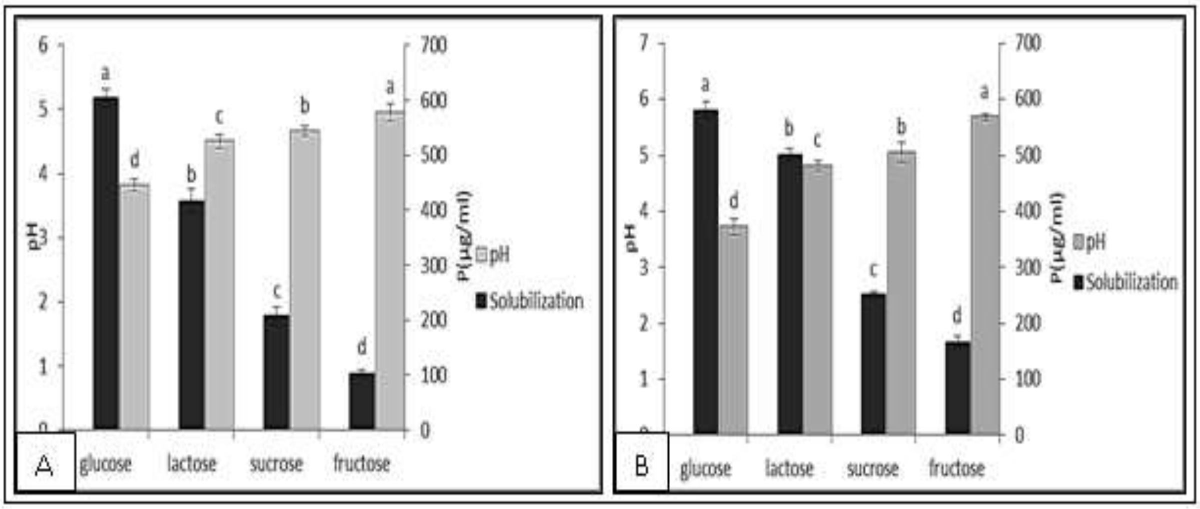

Fig. 2. Effect of various carbon sources on P solubilization. A: *Pseudomonas fluorescens*, B: *Bacillus sp.*

Effect of various carbon sources on efficiency of phosphate solubilization

Phosphate solubilisation by isolates was tested using different carbon sources and according to the results obtained it is remarkable that the most preferred source for the two bacterial isolates was glucose showing the best phosphate solubilization followed by lactose sucrose and fructose (Fig. 2).A decrease in pH values was accompanied by an increase in solubilization rate. Phosphate solubilization occurs in the presence of a carbon source with a maximum solubilization of tricalcium phosphate with glucose (Nautyal, 1999; Fasim *et al.,* 2002; Kumar and Ram, 2014). Phosphate release increases with increasing glucose concentrations, which can be attributed to increased energy source availability for strain growth and acid production (Yadav and Singh, 1991).

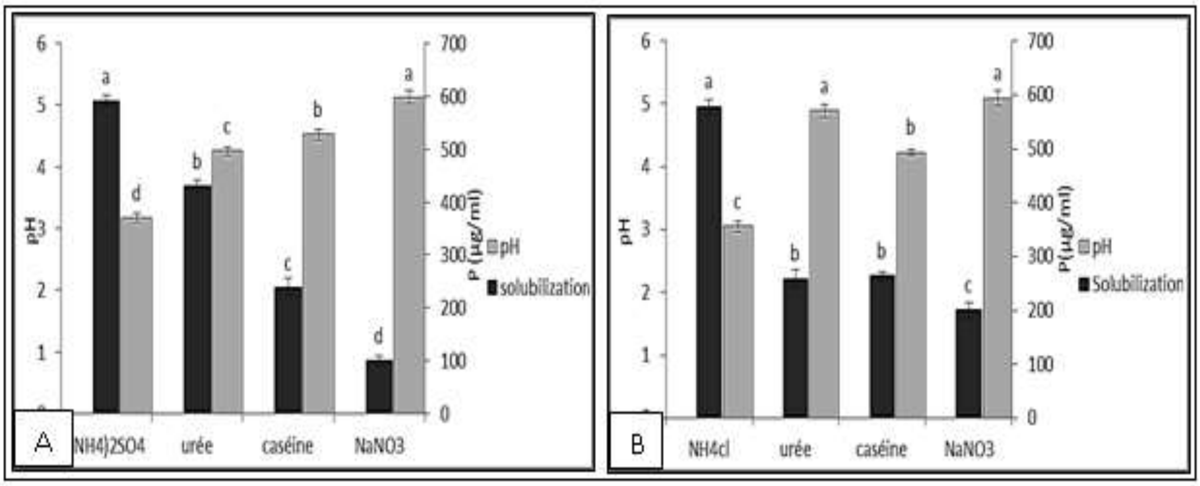

Fig. 3. Effect of various Nitrogen sources on P solubilization. A: *Pseudomonas fluorescens*, B: *Bacillus* sp.

Effect of various nitrogen sources on efficiency of phosphate solubilisation

To study the effect of the nitrogen source on the phosphate solubilization, different sources of nitrogen have been added to the medium. It was observed that utilization of ammonium sulfate $(NH_4)_2SO_4$ provided maximum phosphate solubilisation by both isolates followed by urea, casein and $NaNO_3$ (Fig. 3). These results are in agreement with the studies of Illmer and schinner (1992) and Kumar and Ram (2014).

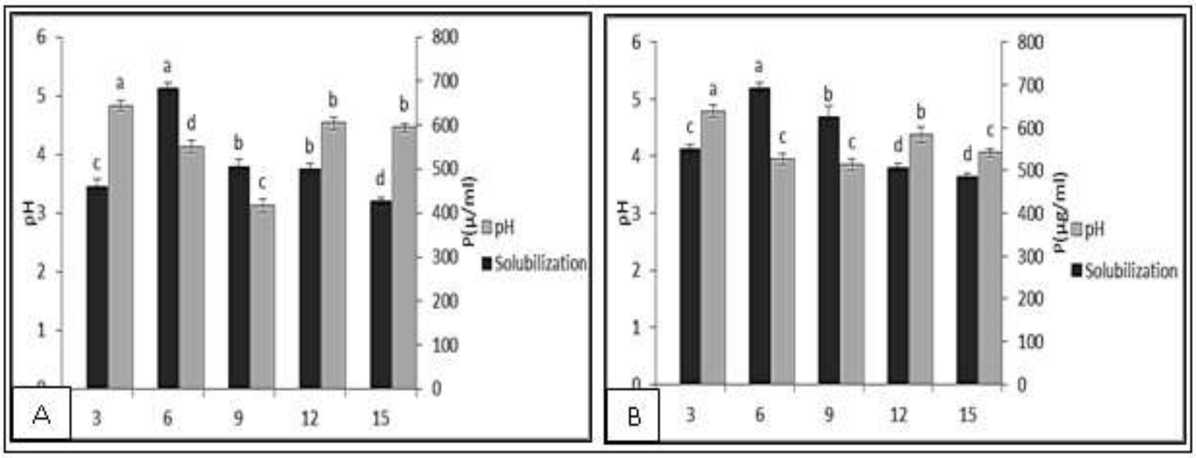

Fig. 4. Effect of incubation period on P solubilization. A: *Pseudomonas fluorescens*, B: *Bacillus sp.*

This shows that the soluble P concentration in the culture medium using ammonium salts as nitrogen source is higher than that with other nitrogen sources, suggesting that the acidification of culture medium by H^+ extrusion during $NH4^+$ assimilation may be involved in the TCP solubilization (Ahuja *et al.,* 2007; Xiao *et al.,* 2009).

Also, ammonium in most of the studies has been found as a better N source than nitrate (Wenzel *et al.*, 1994), and *P. fluorescens* utilized $(NH_4)_2SO_4$ most efficiently and significantly decreased the pH of the medium during P solubilisation as reported by Musarrat and Khan (2014).

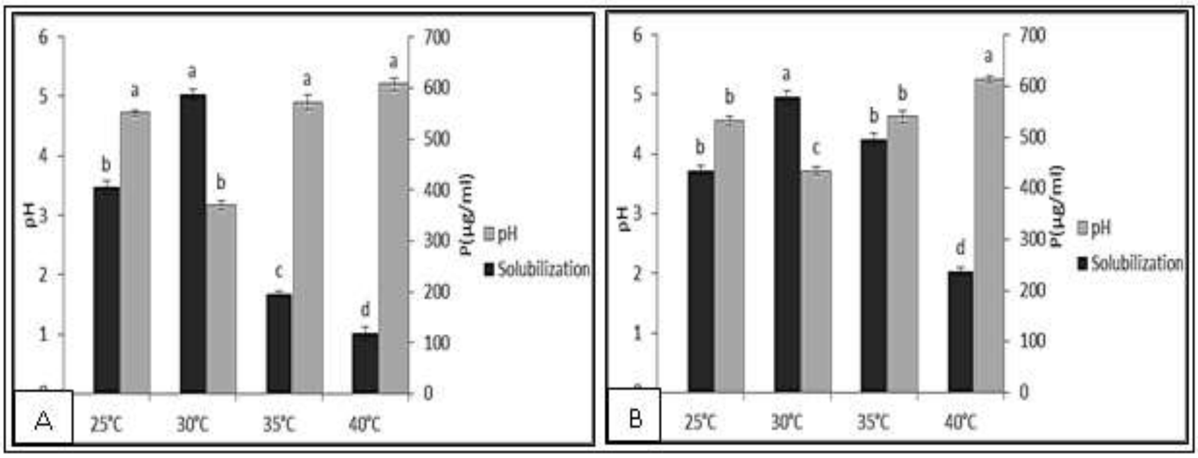

Fig. 5. Effect of temperature on P solubilization. A: *Pseudomonas fluorescens*, B: *Bacillus sp*.

Effect of incubation period on efficiency of phosphate solubilization

The ability of various bacterial isolates to solubilize phosphate was tested for optimal incubation time. The results obtained in Fig. 4 show that the maximum solubilization values of tricalcium phosphate were observed after 6 days of incubation for *Pseudomonas* and *Bacillus*. However, subsequently a significant drop in soluble phosphorus levels was observed on later days.

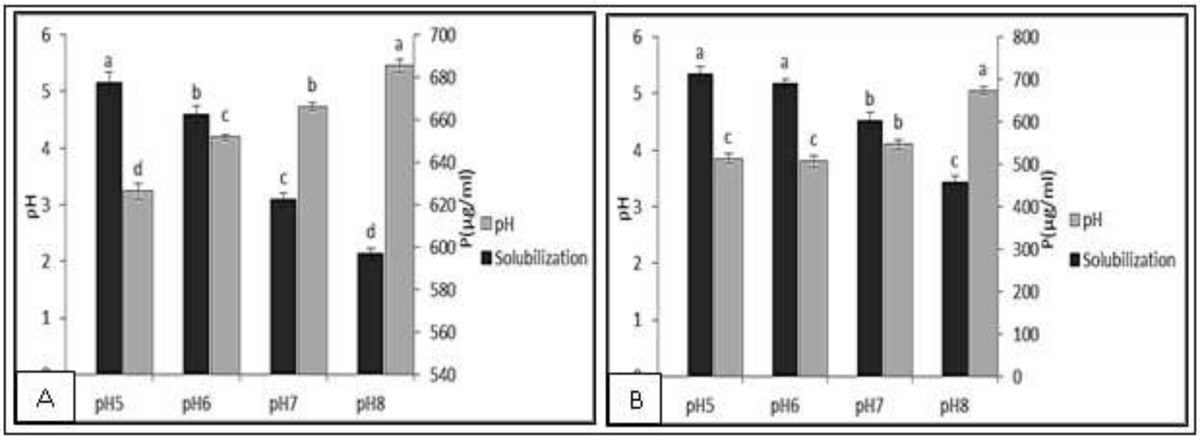

Fig. 6. Effect of pH on P solubilization. A: *Pseudomonas fluorescens*, B: *Bacillus* sp.

The decrease could be due to the availability of soluble form of phosphate, which has an inhibitory effect on further phosphate solubilization (Varsha-Narsian *et al.,* 1994) and the formation of an organo-phosphate compound induced by organic metabolites released, which in turn, reduce the amount of available phosphate (Illmer and Schinner, 1995).

The decrease in P solubilization, however, occurs after certain period of incubation which could be due to the depletion of nutrients, production of certaintoxic metabolites in the growth medium, or autolysis of cells (Khan *et al.,* 2013). According to the results obtained by Muleta *et al.* (2012), it is remarkable that the solubilization of tricalcium phosphate is maximum after the sixth day of incubation.

Effect of temperature on efficiency of phosphate solubilisation

The incubation temperature appears as a factor affecting the ability of isolates to efficiently solubilize tricalcium phosphate. For the two isolates studied, 30 °C was the optimum temperature for the growth and solubilization of the phosphate. At temperatures of 25, 35 and 40 °C low solubilization rates were observed for both strains (Fig. 5).

Similar results were obtained by Cherif-Silini *et al.* (2013) who report that the solubilization of tricalcium phosphate was maximum at a temperature of 30 °C with *Bacillus* strain.Generally, the PS microbes identified and considered so far belong to mesophilic group (Khan *et al.,* 2007), suggesting that they could only be utilized under mesophilic environment.This shows that bacteria adapt to their indigenous environment so their metabolic activities are linked to the temperature of the environment. (Shahab and Ahmed, 2008).However *Bacillus* was able to solubilize high levels of phosphorus even at 35°C and 40°C compared to *Pseudomonas*which was due to the ability of their enzyme systems to tolerate higher temperatures (Musarrat and Khan, 2014).

Effect of pH on efficiency of phosphate solubilisation

The ability of bacterial isolates to solubilize phosphate was tested by varying the initial pH of the culture medium to 5, 6, 7 and 8.The results obtained show that the highest amounts of soluble phosphorus were observed at pH 5 for both strains followed by pH 6, however pH 8 exhibited the lowest solubilized tricalcium phosphate levels (Fig. 6). Nahas (1996) reported that solubilization of insoluble phosphate depends on a multitude of factors, of which the decrease in pH is the major factor.

Conclusion

In this study, bacteria isolated from saline soil had a high potential of phosphate solubilisation under different growth conditions. An optimization of culture medium composition and growth conditions was achieved and both isolates *Bacillus* and *Pseudomonas* preferred almost the same medium (NBRIP) and growth conditions such us 6th day of incubation, temperature at 30°C and pH 5.

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