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Full Length Research Paper

Influence of environmental factors and salinity on phosphate solubilization by a newly isolated *Aspergillus niger* F7 from agricultural soil

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Most agricultural soils contain large reserves of phosphorous, a considerable part of which accumulates as a consequence of regular applications of phosphate fertilizers. Nearly 95 - 99% is present in the insoluble form and hence cannot be utilized by plants. Fungi have been reported to possess greater ability to solubilize insoluble phosphates than bacteria. In the present study, fungal strains isolated from agriculture soil, having potential to solubilize insoluble inorganic phosphates on Pikovskya's (PVK) medium with 0.5% (w/v) tricalcium phosphate (TCP) were characterized. *Aspergillus niger* (F7), *A. niger* (F4), *A. niger* and *Penicillium* sp. showed 107.7, 108.3, 112.7 and 110.3% phosphate solubilisation efficiency on PVK medium with 0.5% (w/v) TCP and 285, 187.5, 258 and 70.5 µg/ml phosphate, respectively from 0.5% (w/v) TCP in liquid broth in 5 days of growth. *A. niger* (F7), showed 107.7% phosphate solubilization efficiency on PVK agar medium and 285 µg/ml phosphate, in solid and liquid medium respectively from 0.5% (w/v) TCP in 5 days of growth and hence was selected for further studies. F7 showed diverse levels of phosphate solubilization activity in both solid and liquid broth culture in presence of various carbon and nitrogen sources and different media. Presence of soluble phosphates, in terms of different concentrations of KH_2PO_4 supplemented in PVK agar media, suppressed TCP solubilization activity by F7. F7 showed different levels of phosphate solubilization under different saline conditions tolerating maximum salinity up to 2% NaCl concentrations. The strain *Aspergillus* sp. F7 can thus be of great benefit in maintaining the available phosphate level for crops in saline alkaline soils. Phosphate solubilizing microorganisms convert insoluble phosphates into soluble forms generally through the process of acidification, chelation and exchange reactions. Thus such microorganisms may not only compensate for higher cost of manufacturing fertilizers in industry but also mobilize the fertilizers added to soil.

Key words: *Aspergillus* sp. F7, phosphate solubilization, environmental factors, salinity.

INTRODUCTION

Phosphorus plays a vital role in plant nutrition (Hayman, 1975) but its concentration in soil solution is approximately 0.05 mg/l (Ozanne, 1980; Goldstein, 1994). For this reason, the possibility of using rock phosphate as a fertilizer has received significant interest in recent years. Unfortunately, rock phosphate is not available to plants in soils with pH greater than 5.5 ± 6 and, even when conditions are optimal, yields are as a rule lower than those obtained with soluble phosphate (Khasawneh and Doll, 1978). One very attractive approach for rock phosphate solubilization is the application of microorganisms able to excrete organic acids. It has been repeatedly shown that low-molecular-mass organic acids can strongly increase the concentration of phosphorus in solution by mechanisms involving chelation and exchange reactions (Earl et al., 1979; Fox and Comerford, 1990; Gerke, 1992). Filamentous fungi are widely used as producers of organic acids (Mattey, 1992; Vassilev and Vassileva, 1992) and particularly *Aspergillus niger* and some *Penicilium* spe-

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cies have been investigated in fermentation systems or inoculated directly into soil in order to solubilize rock phosphate (Kucey, 1987; Asea et al., 1988; Cerezine et al., 1988; Cunningham and Kuiack, 1992; Omar, 1998; Seshadri et al., 2004; Wakelin et al., 2004). Presently, rock phosphate is being chiefly employed to sustain soil phosphorus level in available form for plants 16. Fungi have been reported to possess greater ability to solubilize rock phosphate than bacteria (Illmer and Schinner, 1992). In India it is estimated that 260 million tonnes of rock phosphate deposits are available and this material should provide a cheap source of phosphate fertilizer for crop production (Fai, 2002).

In the present study fungal strains from agricultural soils in and around Bangalore having potential to solubilize insoluble phosphates were isolated and checked for their ability to solubilize insoluble phosphates under solid and liquid growth conditions. Influence of certain environmental factors like media, carbon and nitrogen sources, availability of free phosphorous and salinity on the phosphate solubilizing activity of the selected fungal strain was also determined. The present paper describes screening of indigenous agricultural soil fungi for solubilization of tri calcium phosphate. The promising isolate was characterized and solubilizing activity was studied using various parameters such as like media, carbon and nitrogen sources, availability of free phosphorous and salinity.

MATERIALS AND METHODS

Microorganisms

Fungal strains were isolated from the rice field soil of Bangalore, India after serial dilution of soil solution on Potato Dextrose Agar plates. Isolated, predominant, morphologically distinct colonies were selected, purified by repeated culturing and maintained on PDA slants at 4°C. Isolates were identified by their colony characteristics, spores morphology and microscopic observations. The isolates were checked for phosphate solubilising ability on Pikovskaya (PVK) medium (Pikovskaya, 1948) incorporated with tricalcium phosphate (TCP) [$Ca_3(PO_4)_5$]. Composition of all mediums used for this study is given in Table 1. Formation of a clear halo zone around the fungal growth after 5 days of incubation indicates phosphate solubilizing ability. Solubilization efficiency was tested on PVK plates by the formula:

Solubilization efficiency (%) = (diameter of clearance zone/ diameter of growth zone) x 100.

Fungal strains showing highest solubilization efficiency were selected for further studies.

Media and growth conditions

Phosphorus solubilizing ability of fungal strains was tested in six different types of liquid media reported in literature. Compositions of different media are given in Table 1. Rest of the experiments were performed using PVK medium with 0.5% TCP ('P' ~ 997 µg/ml). Flasks were inoculated with 5% (v/v) spore suspension and incubated on a rotary shaker at 30°C for 7 days. Effect of different carbon sources on P solubilization was done with addition of 1% of respective sugar in place of glucose. Similarly for determining effect of different nitrogen source, 0.5% of different nitrogen salts were added to medium instead of $(NH_4)_2SO_4$.

Solubilization of phosphorus from rock phosphate

Rock phosphate sample (RP-140) having P_2O_5 content about 18.8% was used for the study with *Aspergillus* sp. Quantitative estimation of phosphate solubilization activity was carried out in PVK medium amended with 0.25% (w/v) rock phosphate with other conditions same as for TCP solubilization for a duration of 7 days.

Estimation of phosphorus

Cultures were harvested after different growth periods in order to record the change in pH and concentration of P released in the medium. After centrifugation at 12,000 rpm for 20 min, the pH of the culture medium was measured with a pH meter equipped with a glass electrode. Dissolved phosphate concentration in the culture filtrate was determined by vanado-molybdate method as described in APHA (1995). It was expressed in terms of µg/ml phosphorus released in culture medium.

Effect of soluble KH_2PO_4 (10 - 150 mM) and salinity (NaCl 0.5 - 2%)

Effect of soluble P and salinity on phosphate solubilization ability of the fungi was carried out in PVK medium amended with KH_2PO_4 (10 – 150 mM) and NaCl (0.5 - 2%) with other conditions same as for TCP solubilization for a duration of 7 days. The broth was inoculated with fungal strains and incubated at 28°C. Phosphate solubilisation ability was determined as mentioned earlier.

Statistical analysis

All experiments have been conducted in duplicates and average mean of the values reported.

RESULTS AND DISCUSSION

Isolation of phosphate solubilizing fungi from agricultural soil

Out of all the 40 fungi isolated from the agricultural soil in and around Bangalore, only 4 fungi showed significant zone of P solubilization. A clear halo zone was formed around the colonies after 5 days of incubation on solidified PVK medium supplemented with calcium phosphate, indicating phosphate-solubilizing ability of the fungal isolates. The fungal strains were identified as *Aspergillus* sp. (F7), *Aspergillus* sp. (F4), *A. niger* and *Penicillium* sp. based on their colony morphology, spore characteristics and microscopic studies. In liquid PVK medium, F7 showed maximum P solubilization (285 µg/ml) (Figure 1) and therefore was selected for further studies (Figure 2: F7 on PVK plate).

Media and growth conditions

After confirming the phosphorus solubilizing ability on

Table 1. Composition of different media used in the study.

Media components (g/L)	Medium 1 (AYG; Halder et al., 1991)	Medium 2 (Kim et al., 1997)	Medium 3 (Vassilev et al., 1998)	Medium 4 (PVK; Pikovskaya 1948)	Medium 5 (NBRIY; Nautiyal, 1999)	Medium 6 (NBRIP; Nautiyal, 1999)
Glucose	20	10	100	10	10	10
$(NH_4)_2SO_4$	1	-	-	0.5	0.5	0.1
$MgSO_4.7H_2O$	0.5	0.4	0.2	0.1	0.1	0.25
Yeast Extract	0.2	0.5	-	0.5	-	-
KCl	-	-	-	0.2	0.2	0.2
NaCl	-	1	-	0.2	0.2	-
$FeCl_3$	Trace	-	-	-	-	-
$FeSO_4.7H_2O$				0.002	0.002	
$MnSO_4.H_2O$	Trace	-	-	0.002	0.002	-
$MgCl_2.6H_2O$	-	-	-	-	-	5
$CaCl_2$	-	0.2	-	-	-	-
NH_4NO_3	-	1.5	0.5	-	-	-
$ZnSO_4$	-	-	0.004	-	-	-
$Ca_3(PO_4)_5$	5	5	5	5	5	5
pH	6.8	7	5	7	7	7

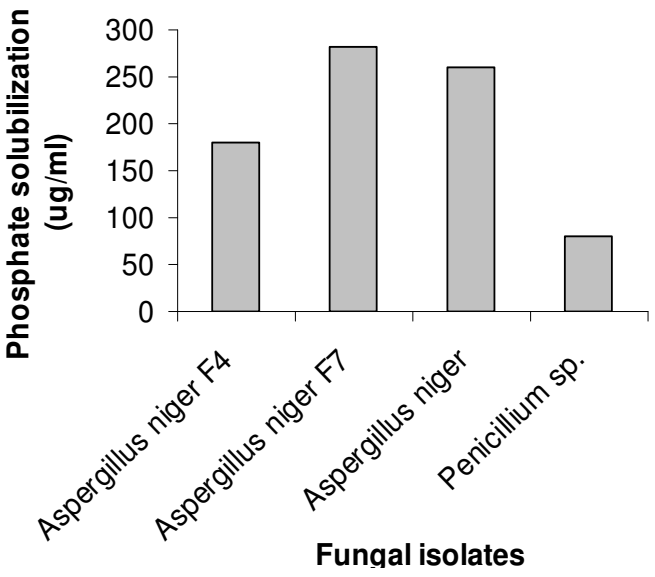

Figure 1. P solubilization by different fungal isolates in PVK broth.

solid medium, the phosphorus solubilization in liquid medium was carried out. Different researchers have used different media for studying phosphorus solubilization in liquid medium. Therefore we tried to find out which media formulation was best for new isolate *Aspergillus* sp. F7. Phosphate solubilization activity was accompanied by a decrease in pH of the medium by F7. F7 solubilized 285 µg/ml of P from 0.5% TCP in PVK with decrease in pH from 7.00 to 3.47. Medium 1 (AYG; Halder et al., 1991) with 20% glucose and 1% $(NH_4)_2SO_4$ showed maximum P solubilization (412.5 µg/ml) with drop in pH from 6.80 to 3.71. However, for other media pH drop did not correlate with P solubilization. Treatment of rock phosphate by F7 released 202.5 µg/ml of P in culture medium after 7 days of incubation. However, the slight decrease in rock phosphate solubilization as compared to TCP in PVK medium could be attributed to the complexity of the structure (Figure 3). Low level of P solubilization was observed in other media. Considering amount of glucose used in medium and corresponding efficacy of P solubilization, PVK medium proved to be most cost effective without compromising the solubilization. Therefore for further studies PVK medium was used.

Phosphate solubilization was accompanied by a decrease in the pH of the medium by F7. Phosphorus solubilizing microorganisms are reported to dissolve insoluble phosphates by the production of inorganic or organic acids and/or by the decrease of the pH (Whitelaw, 2000). Most of the previous reports state that calcium phosphates are dissolved by acidification. Therefore, any microorganism that acidifies its external medium will show some level of phosphorus solubilizing activity. In most soils, proton substitution reactions are driven by microbial production of organic acids, represented generically by the equation:

$$(Ca^{2+})m\ (PO_4^{3-})n + (HA) = (H^+)\ (PO_4^{3-}) + (Ca^{2+})\ (A^-)$$

There is no stoichiometry in the equation because of the complexity of CaP chemistry and the multiplicity of microbially produced organic acids (HAs) with differing numbers of dissociable protons (Goldstein, 1986).

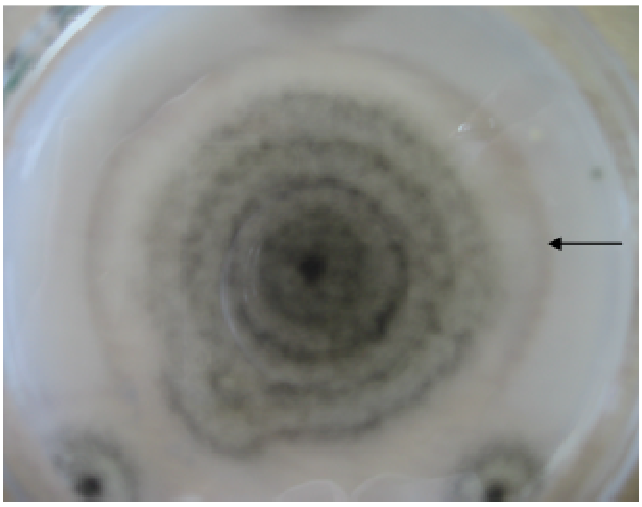

Figure 2. *Aspergillus* sp. F7 showing phosphate solubilization on PVK plate. Arrow indicates zone of phosphate solubilization.

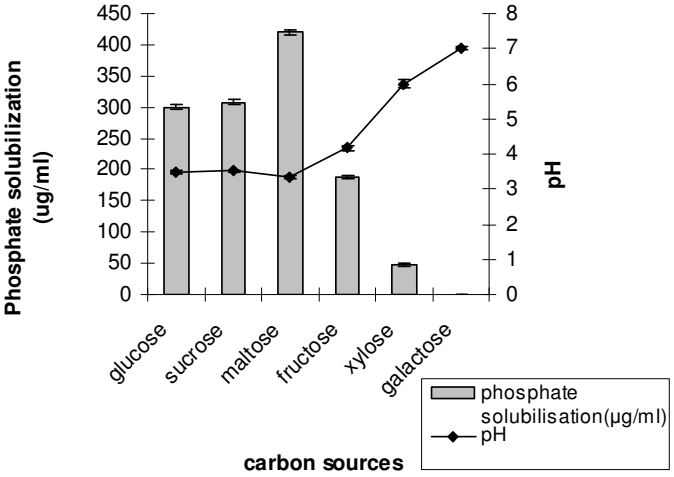

Figure 3. Effect of different media on 'P' solubilization.

Effect of carbon and nitrogen sources on phosphate solubilization by F7

The role of carbon source is important in phosphate solubilization as the production of acid which was commcommon mechanism of solubilization (Di Simine, 1998) was affected by the carbon source and the nature of acid produced is more important then the quantity of the acid (Agnihorti, 1970). Phosphate solubilization activity of *Aspergillus* sp. F7 was evaluated in the presence of five carbon and seven nitrogen sources, by replacing glucose and $(NH_4)_2SO_4$, respectively of the PVK medium. This strain demonstrated diverse levels of phosphate solubilization activity in the presence of various carbon and nitrogen sources. Production of acids was greatly affected by the nature of carbon sources. This strain demonstrated diverse levels of P solubilization activity in the presence of various carbon sources in the order maltose (420 µg/ml) > sucrose (307.5 µg/ml) > glucose (300 µg/ml) with drop in pH from 7.0 to 3.35, 3.52 and 3.47 respectively (Figure 4). The solubilization activity of a microorganism is related to its organic acid production, however, the nature of the acid produced is also important which is dependent on the carbon source supplied (Vassileva, 1998). Fasim et al. (2002) have reported such bacterial isolates, which solubilize only in presence of glucose. Simmine (1998) reported solubilization only in presence of glucose and slight solubilization in presence of mannose while no solubilization was detected in presence of gluconate, galactose, glycerol, sorbitol and fructose. While other workers have reported solubilization in presence of a wide range of carbon sources as well. Our results correspond to those of Nautiyal et al. (2000).

Nitrogen salts having either ammonium group or nitrate group or both were used as N source for the study. $(NH_4)_2SO_4$ was found to be best in reducing the medium pH to 3.75 and simultaneous solubilization of 397.5 µg/ml of P, out of the entire N sources used (Figure 5). It was thus observed that *A. niger* F7 was able to utilize $(NH_4)_2SO_4$ most efficiently to decrease the pH of the medium for P solubilization. This finding correlates with earlier reports (Pradhan and Shukla, 2005). The rest of the nitrogen sources except NH_4Cl and casein show very marginal increase in P solubilization when compared to control. It was thus observed that *Aspergillus* sp. was able to utilize $(NH_4)_2SO_4$ and NH_4Cl most efficiently to decrease the pH of the medium for P solubilization. This finding was also evident from effect of different media on P solubilization (Figure 3). AYG medium (Halder et al., 1991) and PVK medium (Pikovskaya, 1948) show maximum P solubilization and both happen to contain $(NH_4)_2SO_4$ as nitrogen source. Low level of P solubilization was observed in other media containing NH_4NO_3 (Kim et al., 1997; Vassileva, 1998) and NBRIP medium containing less $(NH_4)_2SO_4$ as nitrogen sources.

A number of fungi had been reported of being able to solubilize phosphate only in the presence of ammonium as the nitrogen source (Illmer and Schinner, 1992; Lapeyrie, 1991). Cerezine et al. (1988) reported that ammoniacal sources increased the solubilization of fluorapatite by *A. niger* more than organic sources of N. An increase in rock phosphate solubilization by *Penicillium bilaji* was also found when $NH4^+$-N was added to the medium (Asea et. al., 1988). Similar results have also been reported by Whitelaw et al. (1999) that found a higher acid production and P solubilization from ammonium assimilation by *Penicillium radicum.*

Previous reports on phosphorus solubilizing microorganisms (Lapeyrie, 1991; Carlile and Watkinson, 1994) have attributed the differences in phosphate solubilization (when ammonium and nitrate were used) to the use of different mechanisms for the generation of acidity in the culture. Our observation was also similar.

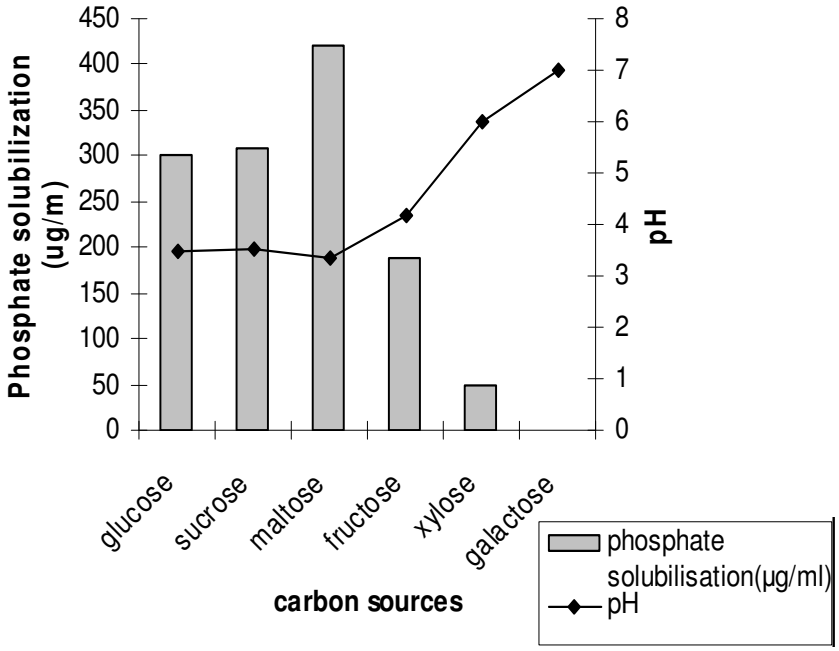

Figure 4. Effect of carbon source on 'P' solubilization using *Aspergillus niger* F7.

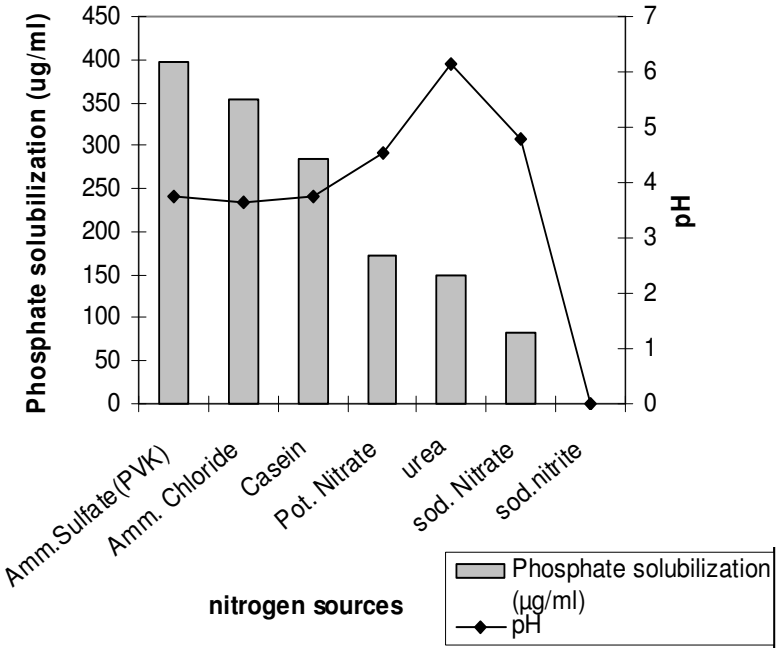

Figure 5. Effect of nitrogen source on 'P' solubilization using *Aspergillus niger* F7.

Effect of KH_2PO_4 on phosphate solubilization by F7

P solubilization ability of F7 was profoundly affected by the presence of free P (above 10 mM) in the form of KH_2PO_4 in the medium (Figure 6). This result indicates that P solubilization activity is stress-induced (Bagyaraj, 2000).

Effect of salinity on P solubilization ability of F7

F7 showed P solubilization up to 2% NaCl concentration (Figure 7). Similarly, the phosphate solubilizing ability of *Fomitopsis* sp. PS 102 was enhanced in the presence of 1% NaCl (Kang et. al., 2002). The strain *Aspergillus* sp. F7 can thus be of great benefit in maintaining the availa-

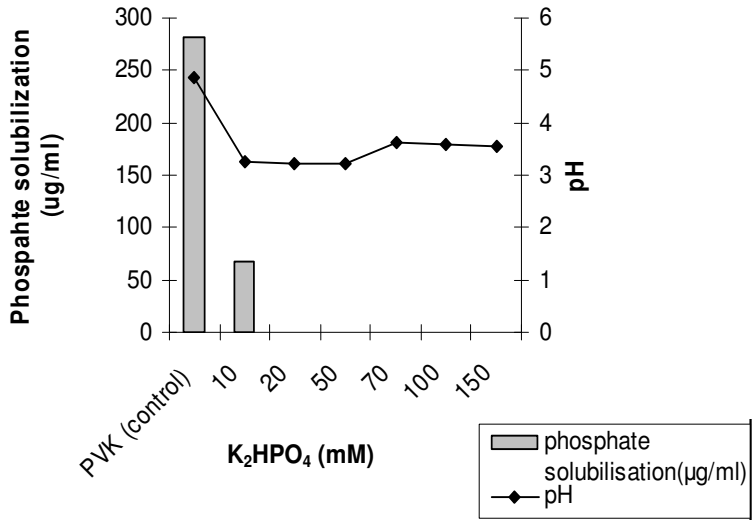

Figure 6. Effect of KH_2PO_4 on 'P' solubilization by *Aspergillus niger* F7.

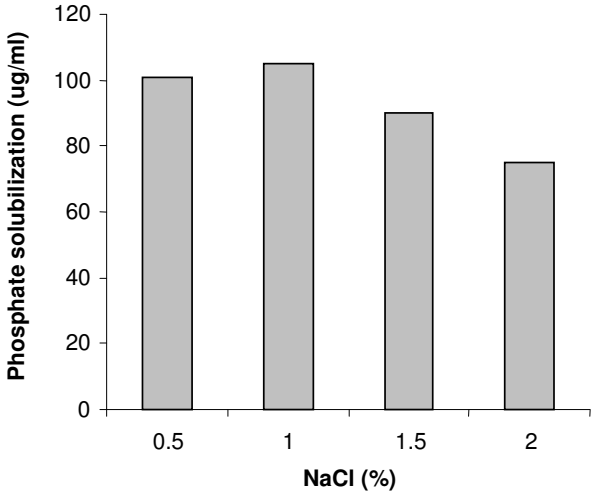

Figure 7. Effect of salinity (NaCl) on 'P' solubilization by *Aspergillus niger* F7.

ble phosphate levels for crops in saline alkaline soils. A large fraction of land in arid and semiarid regions is affected by salinity and in India alone about 7.5 million hectares of land are saline or alkaline (Kumar et al., 1999). The fungal strain *Aspergillus* sp. F7 can thus be utilized in land reclamation of the saline regions along with biological nitrogen fixers. However, since conditions in the soil are far more complex, further study is required to assess the ability of the fungal isolate, *in vivo*.

Kang (2002) and Kim et al. (1997) reported the enhancement of solubilization in presence of 1% sodium chloride. Johri et al. (1999) reported eighteen bacterial isolates out of fifty seven isolates in presence of 5% sodium chloride while two bacterial isolates lost the ability of phosphate solubilization in plate assay in absence of sodium chloride. Nautiyal (1999) and Rosado et al. (1998) reported solubilization in presence of 10% sodium chloride but there is a general trend of decrease in solubilization activity with the increase of sodium chloride concentration. This might have two reasons either two stresses at the same time may harm cell growth and proliferation which result in less efficiency of solubilization or it might be possible that too much chloride ions may chelate or neutralize proton ions or acid produced in the media.

Conclusion

In this study we have isolated a potential phosphate solubilizing fungi *Aspergillus* sp. F7 which solubilized TCP and rock phosphate and also showed diverse levels of phosphate solubilization under different growth conditions with an additional ability to solubilize phosphate in saline conditions.

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