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Phosphate solubilization by a few fungal strains belonging to the genera *Aspergillus* and *Penicillium*

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Many phosphate solubilizing fungi (PSF) are found in soil and their introduction in the rhizosphere of crops not only increases the availability of phosphorus from insoluble sources of phosphate but also increases the efficiency of phosphate fertilizers such as superphosphate and rock phosphate. Studies on 2 strains belonging to *Aspergillus niger*, and 1 strain each of *Aspergillus flavus*, *Penicillium aurantiogriseum* and *Penicillium claviformis* with special reference to phosphate solubilization were performed in this communication. All the fungal strains showed halo zone around them on Pikovskaya plates. Quantitative estimation of 3 indigenous strains along with the other 2 non-indigenous strains taken as reference strains was done in two nutrient broths namely: Czapek's Dox and Pikovskaya containing dicalcium phosphate (DCP) as insoluble source of phosphorus. Out of the two media selected for the study, Pikovskaya broth supported better solubilization. The insoluble phosphates DCP, TCP (tricalcium phosphate) and hypt (hydroxyapatite) were tested, DCP was least solubilized by all strains but there was no significant difference in solubilization of TCP and hydroxyapatite. There was significant negative correlation between pH and phosphate solubilized in all forms of insoluble phosphates. Types of nitrogen sources and metal ions were also screened during the study. $(Na_4)_2SO_4$ supported more solubilization than $NaNO_3$, whereas phosphate solubilizing activity decreased (except in *A. niger* ATCC 282) in presence of Mn^{+2} and Fe^{+3} but presence of Al^{+3} did not have statistically significant effect on solubilization. Though various species of *Penicillium* have been reported for phosphate solubilization in literature but to the best of our knowledge this is first report of phosphate solubilization by *P. claviformis.*

Key words: *Aspergillus, Penicillium,* phosphate solubilisation, dicalcium phosphate, tricalcium phosphate, hydroxyapatite.

INTRODUCTION

Phosphorus (P) is the second most important macro-nutrient required for plant growth next to nitrogen. In particular, Indian soils are poor to medium in available phosphorus (Hasan, 1994). Besides, there is also the problem of its fixation in nature. As soon as it is applied to soil in the form of fertilizer, a large amount of it quickly combines with other chemicals forming compounds which do not release the phosphorus for plants. In acidic soil it is fixed by free oxides and hydroxides of aluminium (Al) and iron (Fe), while in alkaline soil by calcium (Ca). In order to reduce P deficiencies and ensure plant productivity, large quantities of expensive chemical phosphate

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fertilizers are applied worldwide every year.

Microorganisms play critical role in natural P cycle, and the use of phosphate-solubilizing microorganisms (PSMs) has been proposed as a low-cost input to increase the agronomic effectiveness of insoluble phosphates. Several scientific reports showed that microorganisms such as bacteria, fungi, and actinomycetes were indeed able to promote the P solubilization and increase crop yields (Whitelaw, 2000; Oberson et al., 2001; Hamdali et al., 2008; Minaxi et al., 2010). These PSMs render insoluble phosphate into soluble form through the process of acidification, chelation, and exchange reactions (Assailed et al., 2003).

Out of all microbes, fungi are superior to their bacterial counterpart for P solubilzation both on precipitated agar and in liquid (Kucey, 1983a; Banik and Dey, 1982; Singhal et al., 1994; Whitelaw et al., 1997; Sheshadri and Ignacimuthu, 2004). Fungal hyphae are able to reach greater distances in soil more easily than bacteria. Furthermore, it has been observed that PSB upon repeated sub-culturing lose the phosphate solubilizing activity (Halder et al., 1990a; Illmer and Schinner, 1992) but such losses have not been observed in PSF (Kucey, 1983b).

Among the fungal genera with the phosphate solubilization ability are *Aspergillus, Penicillium, Trichoderma, Mucor, Candida, Yeast, Discosia, Eupenicillium* and *Gliocladium* (Xiao et al., 2008; Rahi et al., 2009). The strains from the genera *Aspergillus* and *Penicillum* are among the most powerful phosphate solubilizers. Solubilization of PSF depends on the insoluble inorganic phosphate source, type of carbon, nitrogen and metal ions in soil, as well as on culture conditions (Kucey, 1983b; Nahas, 2007; Jain et al., 2012).

Thus, in view of above facts, there is an increasing realization to explore the possibilities of utilizing a viable alternative for chemical fertilizers for sustainable agriculture. The present study is an effort to see the solubilization pattern of a few fungi belonging to Aspergilli and Penicillia on insoluble forms of phosphate salts of calcium and the effect of different nitrogen sources and metal ions on P solubilization.

MATERIALS AND METHODS

Organisms

Two known organic acid producing fungal strains namely: *Aspergillus niger* ATCC 282 and *Penicillium aurantiogriseum* MTCC 2285 were obtained from IMTECH, Chandigarh, India in lyophilized state and maintained in laboratory on Czapek's Dox Agar slants at 4°C in a refrigerator. The other 3 strains namely: *A. niger, A. flavus* and *P. claviformis* were isolated from the rhizosphere region of wheat fields of Banasthali, India.

The soil was collected from rhizosphere region of 5 wheat plants of 3 agricultural fields in sterilized polythene bags and composite mixtures of soil were prepared from each field. Serial dilution and enrichment methods were used for isolation of fungi. The phosphate solubilizing species were identified based on morphological characteristics by manuals and expertise available in the department. These were designated as *A. niger* bv, *A. flavus* bv and *P. claviformis* bv. Due to lowest solubilization capacity of different phosphate sources, *A. flavus* was not selected for further studies.

Media used

The following media have been used:

i) Pikovskaya agar medium obtained from Hi Media Labs was used to determine the halo formation.
ii) To see the effect of 3 different forms of insoluble phosphatesnamely: DCP, TCP and hypt, Pikovskaya broth having following composition Glucose 10.0, Tricalcium phosphate 2.0, $(NH_4)_2SO_4$ 0.5, KCl 0.2, $MgSO_4$. $7H_2O$.1, $MnSO_4$ Trace, $FeSO_4$ Traces, Yeast extract 0.5, Distilled wáter 1000 ml (pH 7.0) was used. All these salts being insoluble in water, 0.5 g of each form of phosphate was added in 100 ml of broth in separate flasks.
iii) For experiments on effect of different nitrogen sources and metal ions modified Czapek's Dox broth was used (composition: Sucrose 30.000, Sodium nitrate 2.000, Dipotassium phosphate 1.000, Magnesium sulphate 0.500, Potassium chloride 0.500, Ferrous sulphate 0.010, Final pH (at 25°C) 7.3 ± 0.2 in which soluble KH_2PO_4 was replaced by insoluble $CaHPO_4.2H_2O$ as it is dominant in alkaline soils of study area). Czapek's being a synthetic medium, it is easy to replace or add different ions. To see the effect of nitrogen source, $NaNO_3$ was replaced by equal amount of $(NH_4)_2SO_4$. For metal ions viz. Fe^{+3}, Al^{+3} and Mn^{+2}, 0.1 g of $FeCl_3$, $Al_2(SO_4)_3$ and $MnSO_4$, respectively were added separately to modified Czapek's Dox broth.

Solubilization of phosphate on solid medium

The strains were subjected to screening test for their phosphate solublization potential. Pikovskaya's medium containing TCP was prepared and poured into sterilized Petri plates. The isolates were spotted on these plates and incubated at 28°C for 4 to 7 days. Those showing halo zones around the colonies were supposed to be phosphate solubilizing ones. Solubilizing efficiency (S.E) was calculated according to Nguyen *et al.* (1992) by following formula:

$$\% \text{ Solubilization efficiency (SE)} = \frac{\text{Diameter of solubilization zone (S)}}{\text{Diameter of the colony}} \times 100$$

Microdetermination of phosphorus:

Two ml of 10^5 spores/ml of sterilized water was used for inoculation of flasks. Control flasks were also prepared with 2 ml of sterilized water instead of spore suspension. The flasks were incubated at 28 ± 2°C and 120 rpm in an incubator shaker for 2, 4, 6, 8, 10 and 12 days. After completion of respective incubation periods, cultures in triplicate were harvested and filtered with Whatman filter paper no. 42 into separate 250 ml beakers to separate the broth containing phosphate from fungal mat. Chen's method (Chen et al., 1956) was used for quantitative estimation of solubilized phosphate. Absorbance at 820 nm was read by spectrophotometer. Standard curve was plotted to determine the values. The pH of fungal filtrate was also taken using a glass electrode pH meter.

All the glassware used for biochemical studies were soaked in chromic acid overnight. Thereafter they were washed with distilled water, rinsed with dilute HCl, washed again with distilled water and then dried in glassware drying oven to remove organic matter from the glasswares, especially phosphorus, if present.

Statistical analysis

A statistical analysis for the comparison of phosphate solubilization

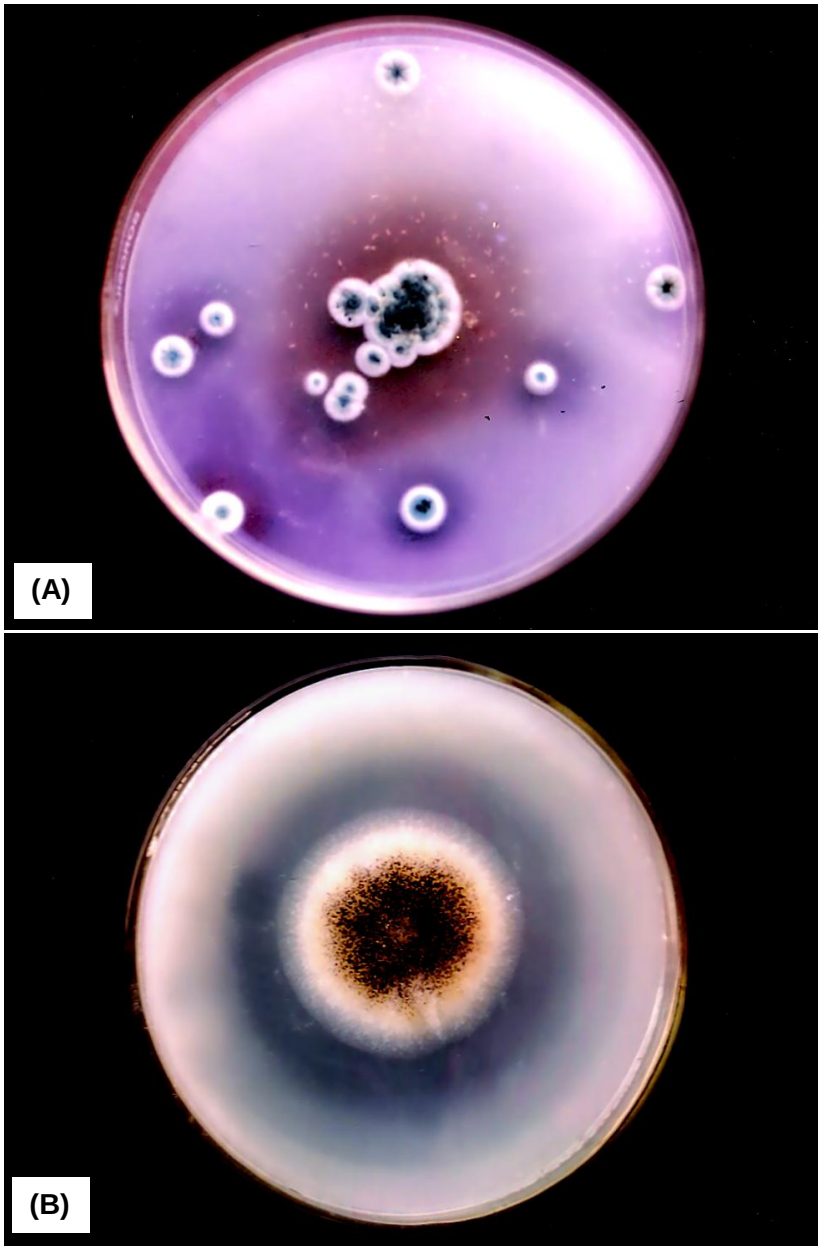

Figure 1. (A) *Aspergillus niger* bv showing halo zone. (B) *Penicillium aurantiogriseum* MTCC 2285 showing halo zone.

process by different strains has been done using two-way and three-way incomplete ANOVA. For the multiple pair wise comparisons Tukey test and l.s.d. were used.

RESULTS

Phosphate solubilization by different strains

The screening of phosphate solubilizing fungi was done on Pikovskaya agar plates. All the fungi selected for the study showed a clear halo zone around them (Figure 1 A and B). *A. flavus* solubilized 46.5 μg/ml DCP and nearly half the amount of TCP and hydroxyapatite, hence discarded for further studies. The percent phosphate solubilization efficiency of this strain was lowest i.e. 90% in comparison to other strains which was in between 300 to 380%, which shows that percent efficiency was directly related to P solubilized in medium.

Three forms of most commonly found insoluble phosphates namely: DCP, TCP and hydroxyapatite were chosen to see the effect of solubilization by different fungal strains in Pikovskaya broth. The results are depicted in Table 1. In general, the amount of soluble phosphate increased with the increase of incubation period in all types of insoluble phosphates solubilized by all the strains,

Table 1. Comparison of solubilization of different forms of insoluble phosphates.

Strain	Days	Dicalcium phosphate		Hydroxyapatite		Tricalcium phosphate	
		Amount of phosphate solubilized (μg/ml)	pH	Amount of phosphate solubilized (μg/ml)	pH	Amount of phosphate solubilized (μg/ml)	pH
A. niger ATCC 282	0	8.00	7.00	8.50	7.00	9.03	7.00
	2	26.50	5.11	19.00	5.03	16.51	4.78
	4	45.91	3.63	29.12	3.50	27.51	3.39
	6	54.25	3.10	52.14	3.25	32.48	3.14
	8	56.50	2.87	72.23	3.06	63.00	2.98
	10	57.12	2.75	77.40	2.71	67.50	2.62
	12	58.40	2.73	78.11	2.48	**80.22**	2.54
A. niger bv	0	8.00	7.00	8.52	7.00	9.04	7.00
	2	24.22	5.80	17.30	5.75	20.00	4.47
	4	40.12	3.72	25.51	3.43	31.40	2.94
	6	49.71	3.31	47.00	2.97	36.10	2.70
	8	55.73	3.25	62.33	2.75	64.00	2.59
	10	56.81	3.04	68.89	2.25	67.55	2.51
	12	57.41	2.96	72.00	2.20	**74.01**	2.50
P. aurantiogriseum MTCC 2285	0	8.00	7.00	8.51	7.00	9.02	7.00
	2	26.05	5.80	19.50	5.27	21.00	5.19
	4	31.63	4.61	30.50	3.85	32.51	3.70
	6	36.14	4.25	25.82	3.40	32.23	3.06
	8	39.74	3.80	56.90	3.05	60.90	3.22
	10	36.72	3.62	66.48	2.86	75.66	2.82
	12	40.21	4.32	72.33	3.16	**77.70**	3.35
P. claviformis bv	0	8.00	7.00	8.42	7.00	9.00	7.00
	2	24.07	5.78	21.42	5.68	20.03	5.25
	4	38.50	4.58	32.20	4.28	35.52	4.55
	6	35.11	3.99	30.05	3.80	28.51	3.47
	8	32.72	3.91	63.20	3.52	76.60	3.64
	10	46.01	4.25	83.51	3.32	69.70	3.03
	12	30.52	3.95	69.49	3.56	**75.01**	3.13
Control	0	8.00	7.00	8.52	7.00	9.01	7.00
	2	8.32	6.99	9.00	6.68	9.25	6.99
	4	8.50	6.98	9.25	6.72	9.75	6.98
	6	10.00	6.86	10.81	6.86	10.81	6.83
	8	11.41	6.74	9.75	6.74	11.75	6.72
	10	10.60	6.72	11.50	6.68	10.30	6.65
	12	10.50	6.63	11.65	6.61	12.11	6.62

although the amount varied with the phosphate type and the fungal strain. The pattern of solubilization of different forms of insoluble phosphates was significantly different in all four strains with F= 2.579 and p-value < 0.1. The solubilization of TCP was highest, closely followed by hydroxyapatite, while DCP was most difficult to solubilize. However, there was no significant difference in solubilization of TCP and hydroxyapatite at 5% level of significance. *A. niger* strains solubilized phosphates efficiently for first 6 days, then the rate of solubilization finally attained an almost constant values. However, the values increased in DCP whereas; there were more fluctuations in case of TCP and hydroxyapatite. But the *Penicillium* spp. did not give constantly increasing values at all, as shown by more evident fluctuations in solubilized phosphate concentrations. Also, after 10 to 12 days of incubation

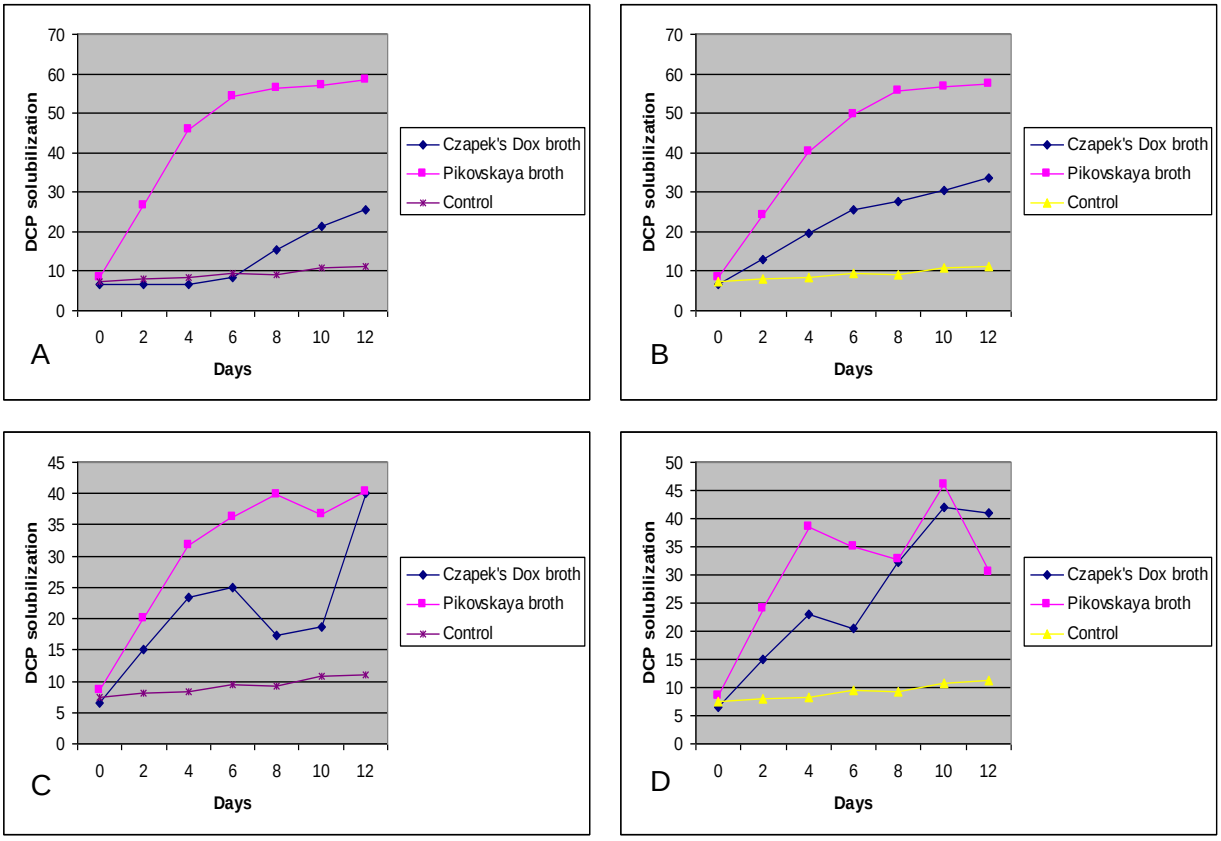

Figure 2. Solubilization pattern of Dicalcium Phosphate by (A) *A. niger* ATCC 282 , (B) *A. niger bv*, (C) *P. aurantiogriseum* MTCC 2285, (D) *P. claviformis bv* in two nutrient media.

there was a slight increase in pH.

Table 1 also showed that the rise in the available phosphate concentration was accompanied by concomitant decrease in pH of the medium. It decreased from 7 at day zero to 2.20 at 12th day in case of *A. niger* bv solubilizing hydroxyapatite. In other strains also the decline in pH from 7 to 2 to 4 was observed. In all, statistical analysis revealed a significant negative correlation (-0.879 with p value<0.01) between amount of phosphate solubilized and pH values.

Microdetermination of phosphorus in liquid medium

Phosphate solubilizing activity of four strains was observed on Czapek's Dox and Pikovskaya broth containing DCP as the insoluble phosphate source (Figure 2 A-D). There was a significant difference between amounts of phosphate solubilized in vitro by all the four strains in Czapek's Dox and Pikovskaya broth with t = 3.370 and p-value<0.01. It was found that Pikovskaya broth overall supported better solubilization than Czapek's Dox broth.

The results of effect of $NaNO_3$ and $(NH_4)_2SO_4$ as nitrogen sources and Mn^{+2}, Al^{+3} and Fe^{+3} as metal ions on phosphate solubilization by all four strains is depicted in Figures 3 to 4. There was a significant difference between amounts of phosphate solubilized by all the four strains in case of nitrate and ammonium ions as nitrogen source with t=-2.471 and and p value<0.05. $NaNO_3$ proved inferior nitrogen source to $(NH_4)_2SO_4$ in supporting phosphate solubilization activity.

Mostly, in all the experimental fungal strains, phosphate solubilizing activity decreased (except in *A. niger* ATCC 282) in presence of Mn^{+2} and Fe^{+3} as compared to control but presence of Al^{+3} did not have statistically significant effect at 1% level of significance on these strains. The pattern of solubilization of different forms of insoluble phosphates in presence of metal ions was significantly different in all four strains. Al^{+3} did not make any remarkable difference but Fe^{+3} and Ca^{+2} had an inhibitory effect on phosphate solubilization.

DISCUSSION

In general, TCP supported highest phosphate solubilizing activity, closely followed by TCP and hydroxyapatite while DCP was least solubilized. Calcium phosphates are the dominant insoluble inorganic phosphates present in neutral to alkaline soils. The soil of study area is also alkaline (Anamika et al., 2007), therefore, locally isolated strains are expected to solubilize these forms of phosphates well. Out of three insoluble inorganic phosphate sources,

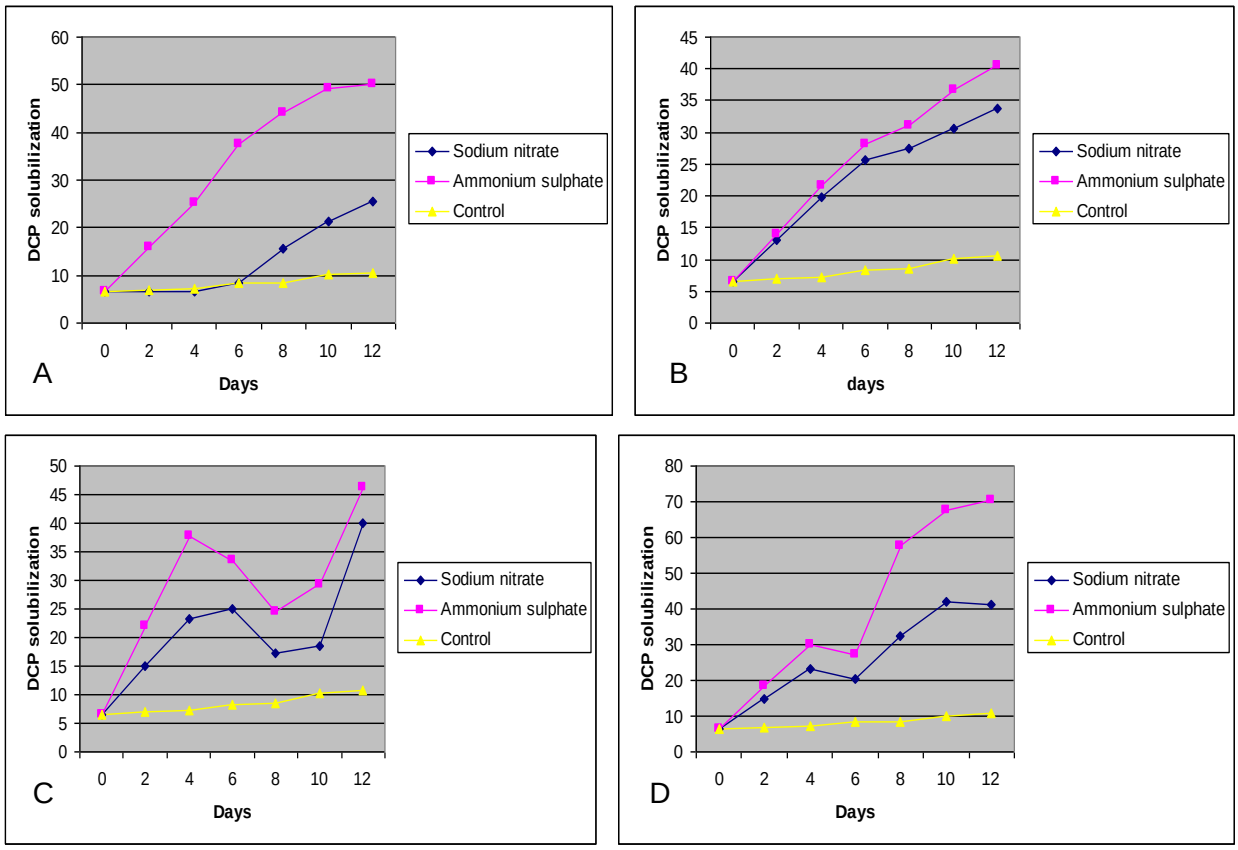

Figure 3. Solubilization pattern of Dicalcium Phosphate by (A) *A. niger* ATCC 282 , (B) *A.niger bv*, (C) *P.aurantiogriseum* MTCC 2285, (D) *P.claviformis bv* in the presence of NO_3^- and NH_4^+ as nitrogen sources.

TCP was solubilized the most which is in agreement with Narsian et al. (1995). This could be due to the difference in chemical structure and charge of three forms of phosphates. $CaHPO_4^-$ gives HPO_4^- after release of phosphate while $Ca_3(PO_4)_2$ gives PO_4^{-3} which bears more negative charge. This would react more readily with or have higher affinity for H^+ present in water in dissociated form, thus go into solution more easily. This was further supported from the data obtained from control samples which did not have inocula. For all three forms of insoluble phosphates, the control showed a very small and gradual increase in available phosphorus concentration with increase in number of days of incubation. This may be explained by the phenomenon of leaching, an abiotic process, caused due to formation of traces of organic acids due to chemical interaction between the components of media (Johnston, 1954).

The rise in the available P concentration was accompanied by decrease in pH of the medium. This drop in pH indicates the production of organic acid (Pradhan and Sukla, 2005; Jain et al., 2011). *A. niger* is known to produce citric, oxalic and gluconic acids (Fomina et al., 2004), thus lowering the pH of medium and facilitating the solubilization. The rate of fall in pH was sharp and uniform during the first 8 days and then it gradually lowered to attain a more or less stable value. These results coincide with Halder and his team mates (Halder et al., 1990b). The lower values of pH in case of *Aspergillus* spp. in comparison to *Penicillium* spp. may be due to less organic acid produced by Penicillia. After 10 to 12 days of incubation there was a slight increase in pH in all the cases which can be explained by the start of autolysis phase (Halder et al., 1990a; b).

In the present study, NH_4^+ was found to be a good nitrogen source in comparison to NO_3^-. Our results corroborate with Jain et al. (2011) who also reported NO_3^- as inferior nitrogen source for phosphate solubilization. The possible reason could be the acid production in form of H^+ release in response to the assimilation of cations such as NH_4^+, which has been reported as a well known phenolmenon in fungi (Kucey, 1983a; b; Roos and Luckener, 1984). Asea et al. (1998) attributed higher phosphate concentration in plant amended with NH_4^+ to better development of roots and acidification of rhizosphere. Roos and Luckner (1984) have reported that the presence of NH_4^+ in growth medium of *P. cyclopium* resulted in the development of inorganic acid following an operation of NH_4^+/H^+exchange mechanisms.

The phosphate solubilizing activity decreased (except in *A. niger* ATCC 282) in presence of Mn^{+2} and Fe^{+3} as

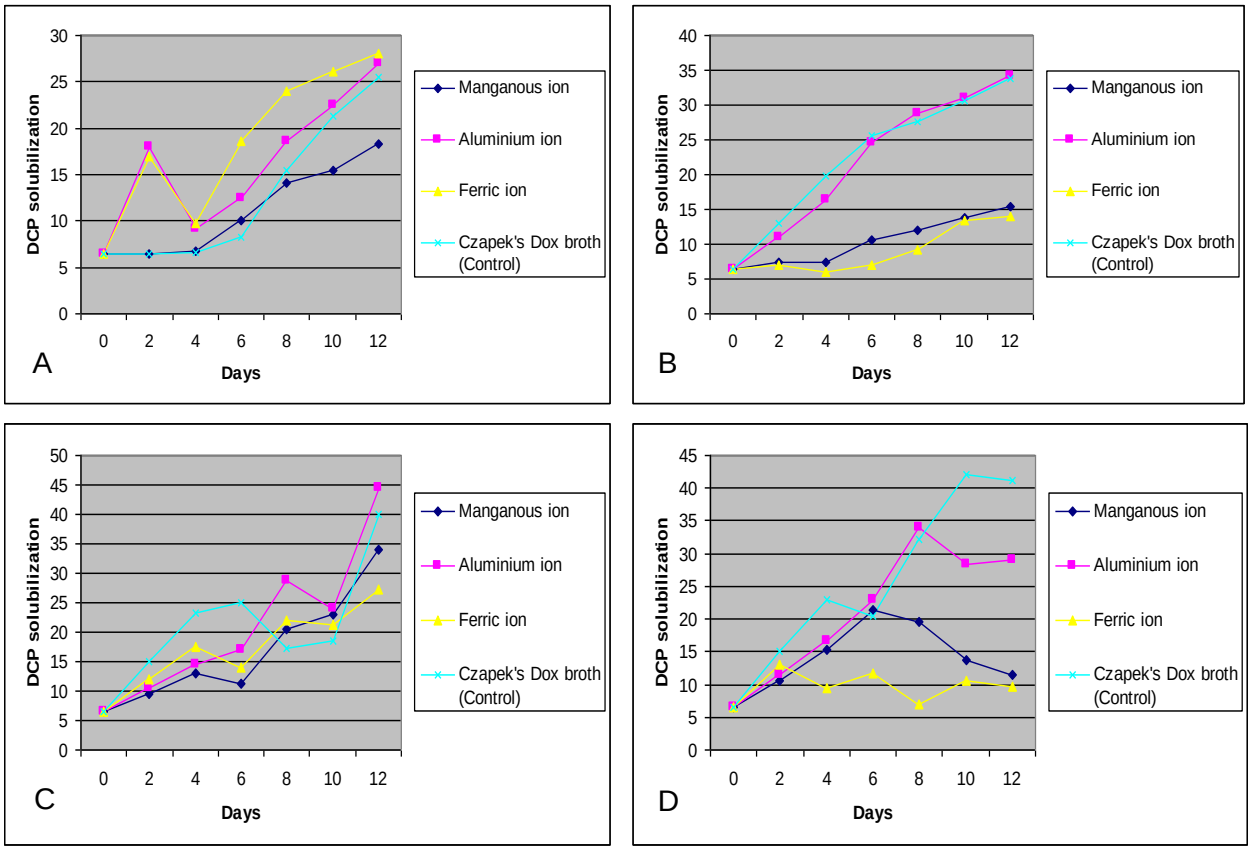

Figure 4. Solubilization pattern of Dicalcium Phosphate by (A) *A. niger* ATCC 282 , (B) *A.niger bv*, (C) *P.aurantiogriseum* MTCC 2285, (D) *P.claviformis bv* in the presence of Mn^{+2}, Al^{+3} and Fe^{+3} as metal ions.

compared to control but presence of Al^{+3} did not have statistically significant effect on these strains. Gaur and Sachar (1980) found that Al^{+3}, Fe^{+3} and Ca^{+2} were inhibitory to growth and activity of fungi or caused a change in pH of the medium, which in turn affected phosphate solubilization. In the present study Al^{+3} did not make any remarkable difference but the other two ions had inhibitory effect. Sayer et al. (1995) observed the activity of *P. simplicissimum* and *A. niger* in presence of 8 insoluble metal compounds. Cobalt phosphate was the most toxic whereas, zinc phosphate was the least toxic and most resistant to solubilisation, while zinc oxide was the most readily solubilized compound.

The type of insoluble phosphate present in the soil of a particular locality seems to affect the efficiency of phosphate solubilization by microbes present locally or applied as biofertilizer. Also, NH_4^+ which was proved to be a better source of nitrogen, could be considered while choosing nitrogen fertilizer if these microbes were simultaneously applied in the same soil as a part of integrated management of nutrients. More studies are in offing with regard to metal ions which could play important role in selecting the biofertilizer, if any of the salts containing these ions were present in the soil.

The advantage of using fungi as bioinoculant includes their tolerance to high concentrations of potentially toxic metals (Sayer et al., 1995), and better acid and alkali tolerance than bacteria (Chuang et al., 2007), although fungi might be inferior to bacteria in their ability to colonize plant root. Overall, fungi may have a much better potential to serve as an agent to convert insoluble inorganic P into a soluble form (for example, HPO_4^{2-}, $H_2PO_4^-$) usable by plants in low or high soil pH.

The present study has generated useful information about the phosphate-solubilizing fungi under control and stressful conditions *in vitro*. It is expected that these fungal strains could serve as suitable candidates for solubilizing P in rigorous environments. However, since the conditions in soil are much more complex than those in vitro, further study of environmental factors affecting phosphate solubilization by these strains in soil should be of practical importance for crops.

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