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Inorganic phosphate solubilization by phosphate solubilizing fungi isolated from acidic soils

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The experiment was conducted to evaluate the native phosphate solubilizing fungi on inorganic phosphate solubilization in the acidic soils of Jharkhand, India. Mineral phosphate solubilizing activities of three fungal isolates were tested in tricalcium phosphate and ferric phosphate medium by analyzing the possible phosphorus release and phosphatase activity from 5th, 10th and 15th day of incubation. Maximum P release was observed on 5th and 10th day of incubation in ferric phosphate and tricalcium phosphate medium, respectively, by all the isolates. Among the isolates, *Aspergillus niger* induced highest P release of 635 and 695 µg ml^{-1} and resulted in 75 and 77% P solubilisation in tricalcium phosphate and ferric phosphate medium, respectively. The pH of the culture filtrate medium gradually decreased with the progress of incubation and maximum decrease was observed between 5-10th day of incubation by all the fungal species. The highest peak of acid phosphatase activity in culture filtrates of fungal isolates were observed on 5th and 10th day of incubation in ferric phosphate and tricalcium phosphate medium, respectively. The highest acid phosphatase activity of 73 U was induced both by *Aspergillus niger* and *Trichoderma viride* in tricalcium phosphate medium. However, the highest acid phosphatase activity in ferric phosphate medium was 51 U induced by *A. niger*. Among the isolates, *A. niger* was more efficient in solubilising the inorganic P followed by *T. viride* and *Penicillium chrysogenum*.

Key words: Acid phosphatase, fungi, phosphorus, phosphate solubilization.

INTRODUCTION

Phosphorus (P) is one of the major essential macronutrients for biological growth and development (Ehrlich, 1990). Phosphorus is considered to be one of the major nutrient elements limiting agricultural production in Jharkhand, India. Deficiency of phosphorus is one of the important chemical factors restricting plant growth. Phosphorus contributes remarkably to photosynthesis, energy and sugar production, nucleic acid synthesis, and promotes N_2 fixation in legumes (Saber et al., 2005). Though soil constitutes 0.5% phosphorus, only a minute amount is available for plant absorption, others remain as insoluble salts and cannot be absorbed by plants (Rodriguez and Fraga, 1999). Besides this, a large portion of chemical fertilizers with high phosphorus content applied to soil is immobilized rapidly and becomes unavailable to plants (Goldstein, 1986).

Phosphorus is added to soil in the form of phosphatic fertilizers, part of which is utilized by plants and the remaining converted into insoluble fixed forms. A greater part of soil phosphorus, approximately 95 to 99% is present in the form of insoluble phosphates and hence cannot be utilized by the plants (Vassileva et al., 1998).

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Table 1. Fungal strains isolated from different rhizospheric soils of plant.

Rhizospheric soils of plant	Strain code	Fungal strains identified
Dolichos bean	F1	*Aspergillus niger*
Mango	F2	*Penicillium chrysogenum*
Cauliflower	F3	*Trichoderma viride*

Use of phosphatic fertilizers has become a costly affair and there is a need for alternative sources. Availability of phosphorus to plant is limited due to fixation as free oxides and hydroxides of aluminum and iron in acidic soils and of calcium in alkali soils (Goldstein, 1986, 1994; Jones et al., 1991). The principle mechanism for mineral phosphate solubilization is the production of organic acids and acid phosphatases play major role in the mineralization of organic phosphorus in soil. It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganism. Production of organic acids results in acidification of the microbial cell and its surrounding.

Phosphate solubilizing microorganisms (PSMs) play an important role in supplementing phosphorus to the plants, allowing a sustainable use of phosphate fertilizers. Application of PSMs in the field has been reported to increase crop yield. Several mechanisms like lowering of pH by acid production, ion chelation and exchange reactions in the growth environment have been reported to play a role in phosphate solubilization by PSMs (Abd-Alla, 1994; Whitelaw, 2000).

Species of *Aspergillus, Penicillium* and yeast have been widely reported to solubilize various forms of inorganic phosphates (Whitelaw, 2000). Fungi have been reported to possess greater ability to solubilize insoluble phosphate than bacteria (Nahas, 1996). The fungi and probably all living organisms, synthesize a number of phosphatases which are necessary to scavenge phosphates (Pi) from medium containing bound phosphorus. We examined the potentiality of three fungal species isolated from the acid soils of Jharkhand, India, for their efficiency of release of phosphorus from inorganic phosphorus compounds and acid phosphatase activity.

MATERIALS AND METHODS

Isolation of fungal species from rhizospheric soil

Different fungal species namely *Aspergillus niger*, *Penicillium chrysogenum* and *Trichoderma viride* were obtained from the rhizospheric soils (acidic soils, pH 5.5) of Pea, Mango and Cauliflower (Table 1), respectively from PLANDU, Jharkhand, India and screened for their ability to solubilize inorganic phosphate in Pikovskaya (PVK) medium (Pikovskaya, 1948). The cultures were maintained on potato dextrose agar slants at 30°C inside an incubator.

Rhizosphere soil samples from pea, mango and cauliflower were separated and the pH of the soil samples was determined and air dried at room temperature (30 ± 2°C). Samples of 10 g of the soil were dispersed in 90 ml of sterile water in 250 ml conical flasks. The flasks were incubated at 28 ± 2°C at 200 rpm in an incubator cum shaker (Scigenics Orbitech, India). The supernatant was serially diluted in sterile water with dilution to 10^{-4} and plated in 10-cm Petri dishes containing Pikovskaya's agar medium: (g l^{-1}), glucose- 10; $(NH_4)_2SO_4$- 0.5; NaCl- 0.3; $MgSO_4.7H_20$- 0.1; K_2SO_4- 0.2; yeast extract- 0.5; $FeSO_4 \cdot 7H_2O$- 0.03; $Ca_3(PO_4)_2$- 5.0; $MnSO_4.7H_20$- 0.02: Agar- 20 (Pikovskaya, 1948) by spread plate technique. The pH of the medium was adjusted to 6.8-7.0 before sterilization. The fungal discs were incubated at 28 ± 2°C for 5-7 days in an incubator.

Screening of the isolates for phosphate solubilisation

The fungal colonies showing a clear zone of solubilization and their growth were subcultured on potato dextrose agar slants. The species were morphologically characterized after staining with lactophenol cotton blue (Himedia, India) under microscopic observation. Clear zones around the colonies indicated the capacity of phosphate solubilization (Gour, 1990).

Quantitative estimation of phosphate solubilization

The Pikovskaya broth was amended with 5 g l^{-1} each of tricalcium phosphate (17% P) and ferric phosphate (18% P) separately in two different set. Fungal discs from four day old potato dextrose agar cultures each of *Aspergillus*, *Penicillium* and *Trichoderma* (4 mm diameter) were inoculated to all the above media containing Pikovskaya broth with tricalcium phosphate and ferric phosphate separately in 250 ml conical flasks. Each inoculation was replicated 5 times. The flasks were incubated at 30°C for 5, 10 and 15 days under shaking conditions. Uninoculated flasks were kept for each set of treatment. After the recommended days, the contents of the flasks were filtered through Whatman No. 42 filter paper.

Water soluble P in the culture filtrates was estimated by the chlorostanous reduced molybdophosphoric acid blue method described by Jackson (1967). Two milliliters of five, ten and fifteen day's old cultures were centrifuged at 10, 000 rpm for 10 min and the supernatant was used to estimate the P release. One milliliter of this supernatant was mixed with 10 ml of chloromolybdic acid and the volume was adjusted to 40 ml with distilled water. To this, 1 ml of chlorostannous acid was added and the volume was made up to 50 ml with distilled water. Potassium di hydrogen phosphate was used as standard. The P released in the supernatant was measured at 600 nm wave length with UV- VIS spectrophotometer (ECIL: UV5704M). The pH of the culture medium was measured by a pH meter.

Acid phosphatase was assayed using p-nitrophenyl- phosphate (pNPP) by microtiter plate method as described by Tabatabai and Bremner (1969). The reaction mixture (750 µl) containing 150 µl of the culture filtrate, 120 µl of pNPP (0.05M) and 480 µl of 0.1M universal buffer pH 6.5 for acid phosphatase was incubated at

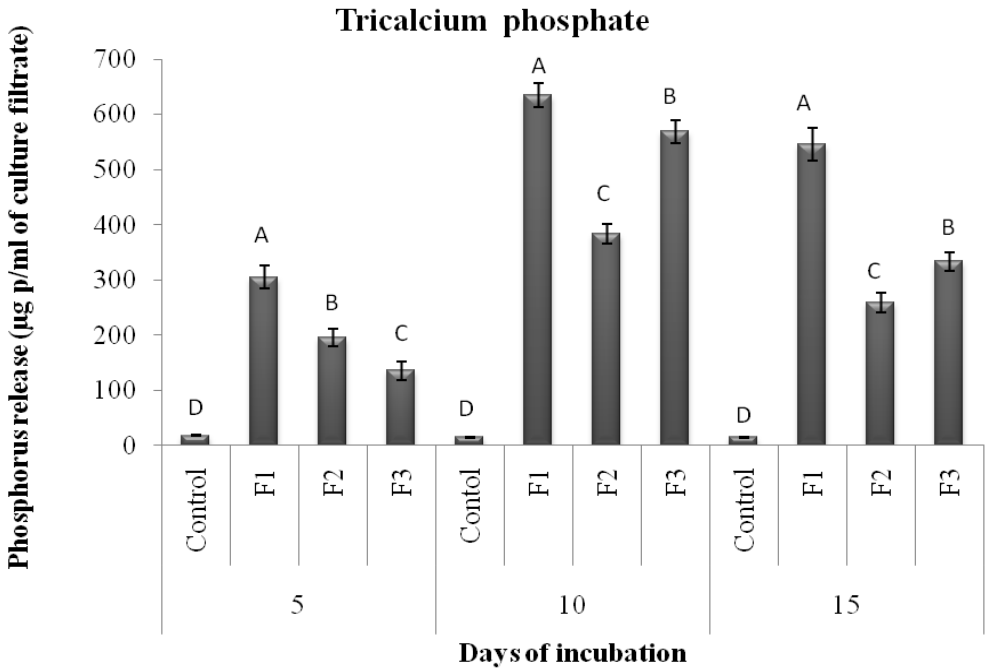

Figure 1. Tri-calcium phosphate solubilization by different fungal isolates. Bars are ±SE. Bars followed by the same letter are not significantly different at the 0.05 level of probability by Duncan's multiple range test (DMRT). Control: uninoculated, F1: *A. niger*, F2: *P. chrysogenum*, F3: *T. viride.*

37°C for 60 min. The reaction was terminated by the addition of 100 µl of NaOH-glycine buffer (0.4 M, pH 10.8). The yellow colour developed was measured at 405 nm using UV- VIS spectrophotometer (ECIL: UV5704M). The amount of p-nitrophenol released was quantified using the pNP standard. One unit (U) of phosphatase activity is the amount of enzyme required to release 1 µg of pNP per hour per ml of culture filtrate under the assay conditions. All the data were analyzed by analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Phosphorus release and pH

The P release from the tricalcium phosphate containing broth inoculated with the fungal species showed variation among the different species over the incubation period of 15 days (Figure 1). The P content varied significantly [$F_{(3, 16)}$ = 3.24, p≤0.05] among the culture filtrates of different fungal isolates throughout the period of incubation. The P release from uninoculated control was in trace amount throughout the incubation period. The P content was estimated from 5th day of incubation to the 15th day. Initially, the P content was less in all the culture filtrates and gradually increased with the progress of incubation. The P content in the culture filtrates of *A. niger* ranged from 305 to 635 µg ml^{-1} with maximum P content at 10th day of incubation and subsequently the P content declined gradually. Similarly, the P content in the culture filtrates of *P. chrysogenum* varied from 195 to 383 µg ml^{-1} with maximum P content at 10th day of inoculation. The culture filtrates of *T. viride* recorded maximum P content of 569 µg ml^{-1} at 10th day of incubation. The decrease in P content with the advance of incubation period could be attributed to the utilization of P by fungal species resulting in the fluctuating levels of P release (Deepa et al., 2010). However, the P content in the uninoculated control was in trace amount throughout the incubation period. Among the different isolates, *A. niger* was more efficient in the solubilization of tricalcium phosphate followed by *T. viride* and *P. chrysogenum*. The P content in the culture filtrate of *A. niger* resulted in 66 and 11.6% increase over *P. chrysogenum* and *T. viride*, respectively, at their highest availability on 10th day of incubation. Microorganisms through secretion of different types of organic acids (Deubel et al., 2000) and rhizospheric pH lowering mechanisms (Hinsinger, 2001) increased solubility of Ca-phosphates.

The P content in the culture filtrate of ferric phosphate amended broth varied significantly [$F_{(3, 16)}$=3.24, p≤0.05] among the various fungal species (Figure 2). The unincoculated control showed trace amount of P throughout the period of incubation. The maximum solubilization of ferric phosphate was observed on 5th day

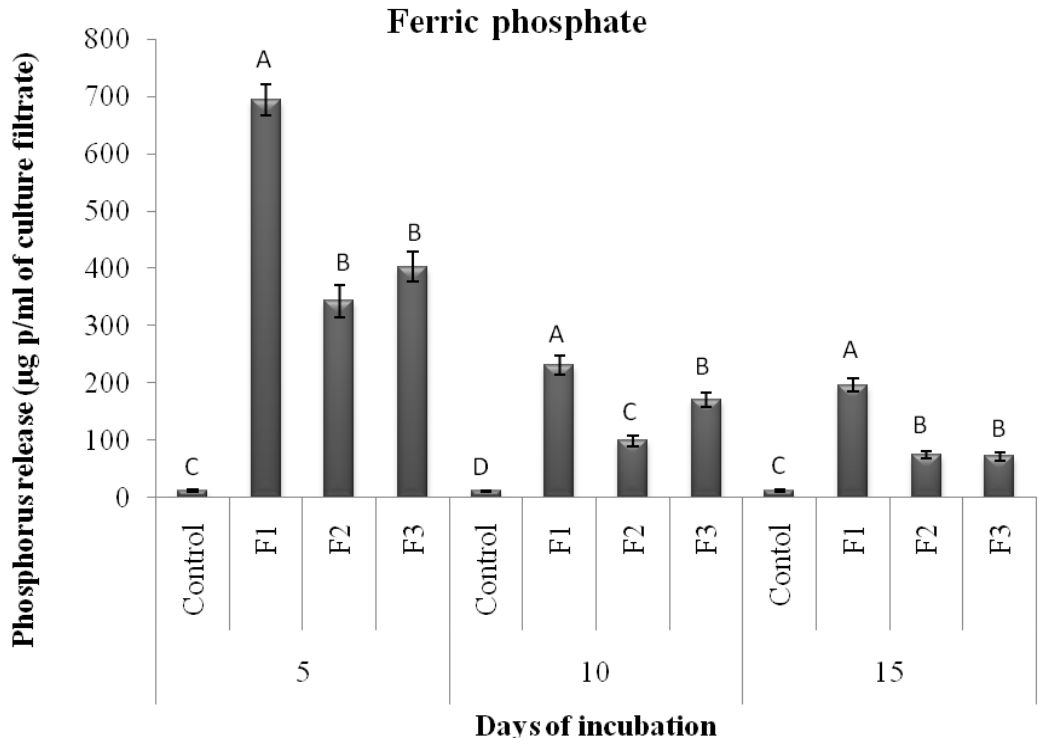

Figure 2. Ferric phosphate solubilization by different fungal isolates. Bars are ±SE. Bars followed by the same letter are not significantly different at the 0.05 level of probability by Duncan's multiple range test (DMRT). Control: uninoculated , F1: *A. niger*, F2: *P. chrysogenum*, F3: *T.viride*

Table 2. pH of Pikovskaya broth culture supplemented with tricalcium phosphate and ferric phosphate.

Phosphate solubilizing fungi	pH of culture filtrate							
	Tricalcium phosphate				Ferric phosphate			
	Initial	5th day	10th day	15th day	Initial	5th day	10th day	15th day
Uninoculated control	7.0[a]	7.0[a]	7.2[a]	7.0[a]	7.0[a]	6.8[a]	7.0[a]	7.0[a]
Aspergillus niger	7.0[a]	3.9[d]	4.0[d]	5.8[b]	7.0[a]	3.0[b]	3.1[b]	3.8[b]
Penicillium chrysogenum	7.0[a]	4.5[c]	5.1[c]	6.7[a]	7.0[a]	2.5[c]	2.6[c]	3.5[c]
Trichoderma viride	7.0[a]	5.7[b]	5.0[b]	5.8[b]	7.0[a]	2.5[c]	2.4[c]	3.1[d]

Means followed by the same letter are not significantly different at the 0.05 level of probability by Duncan's multiple range test (DMRT).

of incubation by all the fungal isolates under study and subsequently the P content gradually decreased with the progress of incubation. The culture filtrate of *A. niger* recorded highest P content of 695 µg ml^{-1} and was significantly [$F_{(3, 16)}$ = 3.24, p≤0.05] better than other fungal isolates throughout the incubation period. However, the P content in the culture filtrate of *P. chrysogenum* and *T. viride* was statistically at par among themselves and significantly [$F_{(3, 16)}$ = 3.24, p≤0.05] better than control. The P content in the culture filtrate of fungal species followed the trend, *A. niger* > *T. viride* > *P. chrysogenum* throughout the period of incubation. Phosphate solubilizing fungi convert the insoluble phosphates into soluble forms through the processes of acidification, chelation and exchange reactions (Earl et al., 1979; Gerke, 1992).

The pH of the broth culture was measured from 1st to 15th at an interval of 5 days. Initially, the pH of all the broth was adjusted to pH 7.0. A drop in pH of the broth culture was observed on 5th day and thereafter gradually increased to 15th day of incubation (Table 2). It was observed that the decrease in pH of the broth culture was more in ferric phosphate amended as compared to tricalcium phosphate. The phosphate solubilization was

Table 3. Simple correlation and linear regression equation that describe the P content in the culture filtrate(y) as a function of pH of the culture filtrate (x).

Days of incubation	Correlation coefficients	Linear regression equations	r^2
Tricalcium phosphate			
5	-0.880**	y = -78.87x + 578.8	0.774
10	-0.940**	y = -192.5x + 1425	0.884
15	-0.739**	y = -251.2x + 1884	0.546
Ferric phosphate			
5	-0.716**	Y=-97.70x + 726.0	0.513
10	-0.698**	y = -32.57x + 252.0	0.487
15	-0.509*	y = -22.66x + 188.0	0.259

** Significant at the 0.01 level, *significant at the 0.05 level.

accompanied by decrease in pH of the broth by all the species. The decrease in pH values was in agreement with the findings of Illmer and Schinner (1992), who have reported a decrease in pH upto four days followed by a gradual rise during P-solubilization by *Penicillium* and *Pseudomonas* in liquid cultures. The correlation between P-solubilization and pH was significantly negatively correlated in both sources of inorganic P (Table 3). The correlation studies indicated that the lower the pH of the culture filtrate, the higher the P availability in the broth. Further, the correlation between P content and pH was recorded highest (-0.940**) at 10 days of incubation in tricalcium phosphate broth, whereas the maximum correlation of -0.716** was recorded at 5 days of incubation in ferric phosphate broth. Phosphorus solubilizing microorganisms are reported to dissolve insoluble phosphates by the production of organic acids thus by decrease of the pH (Whitelaw, 2000). The potential mechanism for phosphate solubilization is by organic acids production and proton extrusion resulting in decrease of pH (Dutton and Evans, 1996; Jones, 1998).

Acid phosphatase activity

The acid phosphatase enzyme was induced by tricalcium phosphate (5 g l^{-1}) in the PVK broth inoculated with different fungal species as shown in Figure 3. Maximum enzymatic activity was recorded at 10^{th} day of incubation by all the PSF thereafter gradually decreased. The amount of acid phosphatase activity by the *A. niger* varied from 28 to 73 U, *P. chrysogenum* ranged from 21 to 66 U and *T. viride* ranged from 27 to 73 U throughout the incubation period of 15 days. Among the species, *A. niger* and *T. viride* produced more amount of acid phosphatase enzyme as compared to *P. chrysogenum*. The acid phosphatase activity was statistically non significant [$F_{(2, 12)} = 3.88$, $p \leq 0.05$] among the *A. niger* and *T. viride* throughout the period of incubation. However, *A. niger* and *T. viride* showed significantly [$F_{(2, 12)} = 3.88$, $p \leq 0.05$] better acid phosphatase activity than *P. chrysogenum*. The results suggested that the *T. viride* produced more amount of acid phosphatase activity but it released less amount of phosphorus which is due to the non-specific binding activity of the phosphatase (Deepa et al., 2010). The high phosphatase activity of the fungal isolates is responsible for higher P-solubilizing potential subjected to its specific binding activity. The acid phosphatase activities decreased after 15 days of incubation, which might be due to the disappearance of tricalcium phosphate from the medium after 10 days of incubation.

The acid phosphatase activity induced by ferric phosphate (5 g l^{-1}) in the PVK broth inoculated with different native fungal isolates showed significant [$F_{(2, 12)}=3.88$, $p \leq 0.05$] difference among them throughout the period of incubation (Figure 4). All the fungal isolates induced highest phosphatase activity at the early stage of incubation and were non significant among themselves. The phosphatase activity of different fungal isolates gradually decreased with the progress of incubation. At the later period of incubation, the acid phosphatase activity of *A. niger* was significantly [$F_{(2, 12)}=3.88$, $p \leq 0.05$] better than *P. chrysogenum* and *T. viride*. The highest phosphatase activity induced by *A. niger*, *P. chrysogenum* and *T. viride* was 51, 50 and 49U, respectively at 5 days of incubation. Although the acid phosphatase activity of *P. chrysogenum* and *T. viride* was more, they released less amount of phosphorus which is due to the non-specific binding activity of the phosphatase (Deepa et al., 2010).

Conclusions

The availability of P from the insoluble inorganic source of P was significantly better with the inoculation of isolated native fungal species from acid soils. Among the

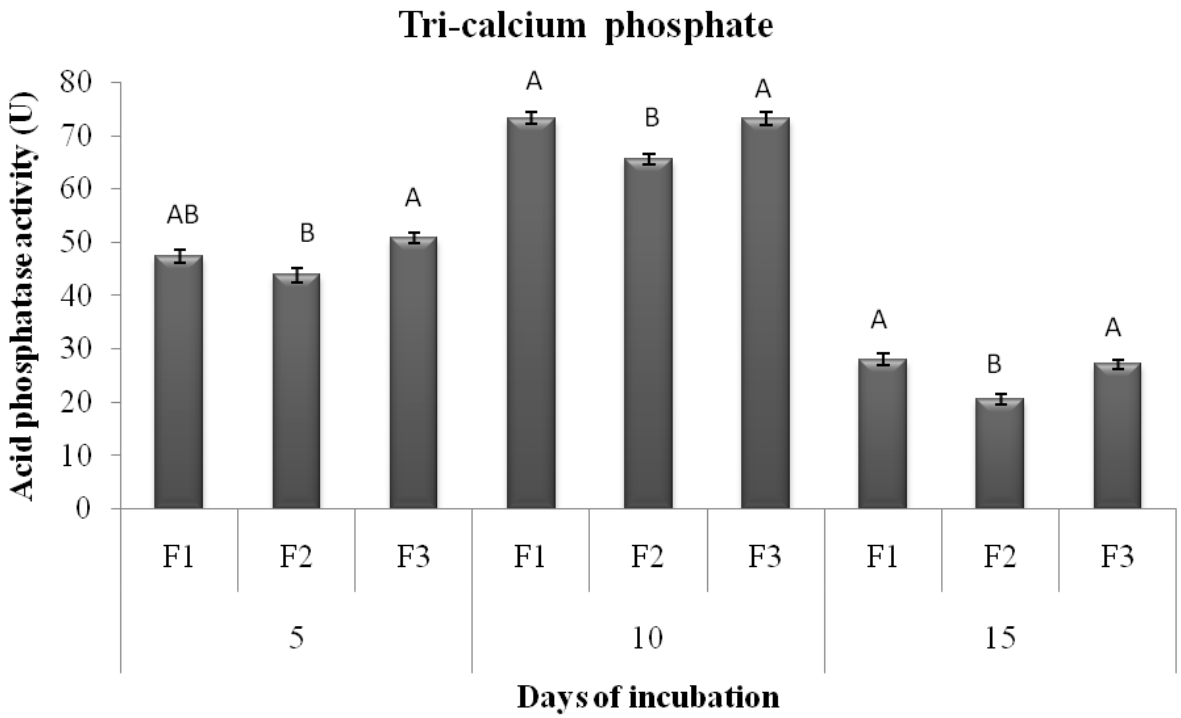

Figure 3. Acid phosphatase activity of the fungal isolates in tricalcium phosphate medium. Bars are ±SE. Bars followed by the same letter are not significantly different at the 0.05 level of probability by Duncan's multiple range test (DMRT). Control: uninoculated , F1: *A. niger*, F2: *P. chrysogenum*, F3: *T. viride.*

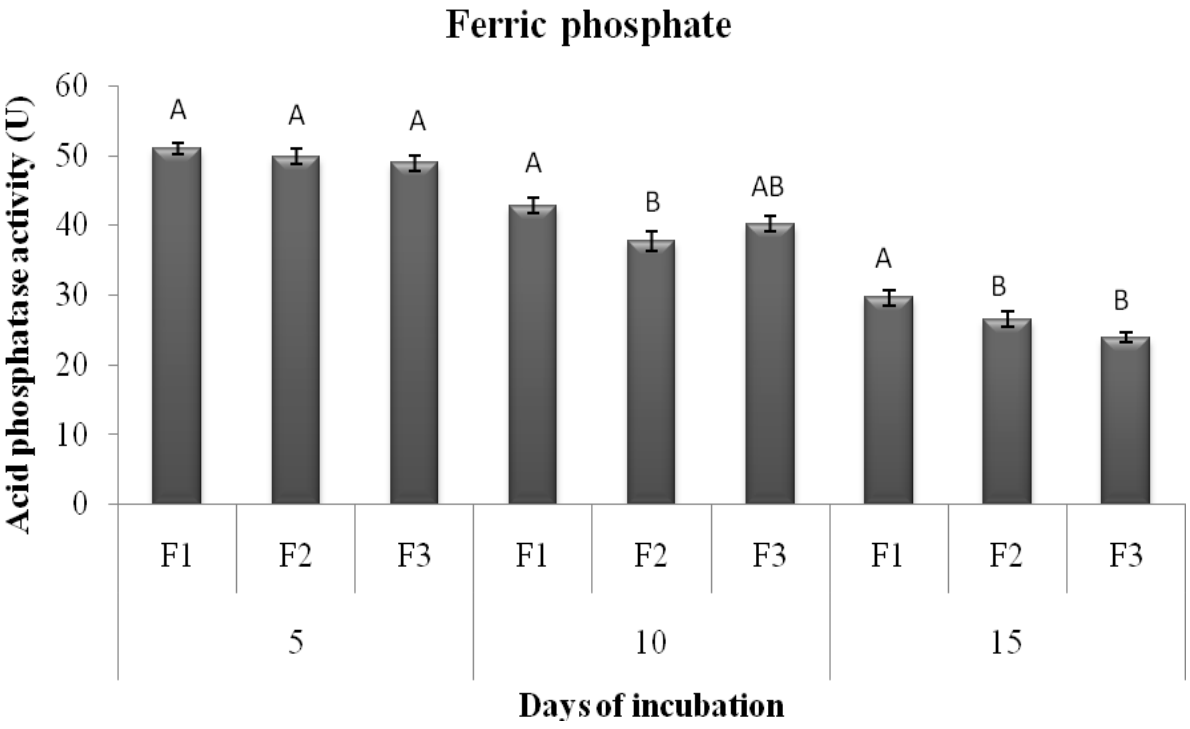

Figure 4. Acid phosphatase activity of the fungal isolates in ferric phosphate medium. Bars are ±SE. Bars followed by the same letter are not significantly different at the 0.05 level of probability by Duncan's multiple range test (DMRT). Control: uninoculated , F1: *A. niger*, F2: *P. chrysogenum*, F3: *T. viride*

species, *A. niger* was found to be most efficient in mobilizing P from tricalcium phosphate and ferric phosphate. The study revealed the highest P solubilization of 75 and 77% in tricalcium phosphate and ferric phosphate, respectively. The pH of the broth culture gradually decreased with the progress of incubation up to five days and thereafter increased. Further, the availability of P was significantly negatively correlated with the pH of the broth culture. The acid phosphatase activity induced by *A. niger* and *T. viride* was comparatively better than that of *P. chrysogenum* in tricalcium phosphate amended PVK broth. However, the acid phosphatase activity of all the native fungal species in ferric phosphate amended PVK broth was statistically at par at the highest activity.

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