*Full Length Research Paper*

# Isolation and characterization of a fungal isolate for phosphate solubilization and plant growth promoting activity

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A fungal species namely F1 was isolated from the rhizosphere on the basis of its ability to form halos (zone of solubilization) on Pikovskaya's agar. F1 was assessed for phosphate solubilization, titratable acidity (TA), gluconate concentration and change in pH over incubation period of 21 days and other plant growth promoting traits. F1 solubilized maximum inorganic phosphorus (662 µg P ml$^{-1}$) from tricalcium phosphate present in the Pikovskaya's broth on 18th day. The TA followed a similar trend as that of P solubilized, except on day 21 when the value that for TA was highest. A similar pattern was also observed with production of gluconic acid, for which a constant value of $8.96 \times 10^{-4}$ g% was observed till 18th day of incubation. However, gluconic acid was not the only organic acid produced in the culture broth, because the amount of gluconic acid produced did not relate to the high values observed for P solubilized and TA. Efficiency of the F1 to solubilize phosphate from organic reserves was determined by performing assays of phosphatases and phytases. The culture F1 produced 1.86 and 1.90 EU of enzymes alkaline and acid phosphatase, respectively and phytase activity was 28 mU. The concentration of catechol and hydroxymate type siderophores produced by F1 was 4.50 and 4.55 µgml$^{-1}$ respectively and it also produced 11.45 µgml$^{-1}$ of IAA which is significantly high. Some fungi may possess traits associated with biocontrol of plant pathogens such as production of enzyme chitinase which was 0.037 EU for F1. On the basis of cultural and microscopic features, the isolate F1 could be *Absidia* spp. and has potential of being a competent bioinoculant.

**Key words:** Phosphate solubilization, bioinoculant, plant growth promoting fungus (PGPF).

## INTRODUCTION

Use of voluminous amounts of chemical fertilizers and fungicides has been an impediment to development of sustainable agriculture. Employment of biofertilizers and biopesticides may be able to side-step some of the deleterious effects caused by chemical fertilizers. Root associated bacteria and fungi have been known to benefit plants and are hence referred to as plant growth promo-

ting rhizobacteria (PGPR) (Kloepper et al., 1990) and plant growth promoting fungi (PGPF). It is reported that PGPF may make use of one of the several mechanisms to promote plant growth like production of phytohormones, solubilization of minerals and antagonism to phytopathogens.

Phosphorus in the soil is essentially unavailable to the plants and use of plant associated organisms may help in solubilization of mineral P for easy uptake by the plants. Fungi are reported to solubilize P by production of organic acids and are known to have a higher efficiency of solubilization than bacteria. The other plant growth promoting trait of siderophore production is widespread among fungi. Arbuscular mycorrhizal fungi (AMF) are known to enhance Iron (Fe) uptake of associated host plants (Haselwandter, 2008). Siderophores are also known to function as virulence factors and are commonly

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**Abbreviations: TA**, titratable acidity; **MU**, milli units; **P**, phosphate; **µg P ml$^{-1}$**, microgram phosphate per ml; **g%**, gram percent; **EU**, enzyme units; **PGPR**, plant growth promoting rhizobacteria; **PGPF**, plant growth promoting fungi; **AMF**, arbuscular mycorrhizal fungi; **IAA**, indole acetic acid.

produced by pathogenic fungi especially the hydroxymate type. However, siderophore production is not restricted only to pathogenic fungi. Other laboratory studies have emphasized on use of PGPF as biocontrol agents (Hossain et al., 2007) and the role of auxin production (Contreras-Cornejo et al., 2009) in plant growth promotion.

Our aim for the present study was therefore to isolate and characterize fungi for greater phosphate solubilization efficiency and other plant growth promoting activities like production of phytohormones, siderophores and antagonism. Such a PGPF with the ability to promote plant growth by more than one mechanism may be a potential bioinoculant particularly for nutrient deficient soils and for redemption from pathogens.

## MATERIALS AND METHODS

### Isolation

A fungal species was isolated from the rhizospheric soil by plating appropriate dilutions on Pikovskaya's agar. The isolate formed clear halo around the colony after 3 days of incubation and was maintained on the Pikovskaya's agar at 30°C.

### P solubilization, titrable acidity and Gluconic acid

Phosphate solubilization was estimated quantitatively by inoculating the isolates in Pikovskaya's broth at initial pH adjusted to 7 and incubated at 30°C for 21 days. After every 3rd day inorganic P concentration was estimated as described (Ames, 1964). The amount of P solubilized (Psol) was reported after deducting the values of soluble P concentration of the uninoculated control (that is, P released by autoclaving). Titratable acidity (TA) was estimated by titrating 1 ml of the culture supernatant against 10 mM NaOH in presence of phenolphthalein (Whitelaw et al., 1999).

The concentration of gluconate produced by the isolate was estimated titrimetrically against 0.05 M EDTA in presence of 0.05 ml ammonia-ammonium chloride, 0.05 ml magnesium sulphate (0.5 M) and a pinch of Eriochrome T dye at regular intervals as mentioned (Welcher, 1958).

### Phytase and phosphatase activity

The activity of phytase and phosphatases (acid and alkaline) produced by the isolate was estimated by the methods given by Richardson and Hadobas (1997) and Tabatabai and Brimner (1969), respectively.

### Siderophore production

Microorganisms usually produce two different types of sidero-phores: hydroxymate type and catechol type. The production of hydroxymate type siderophore estimated by growing the isolate in medium composed of 4.25 g $Na_2HPO_4$, 30 g $KH_2PO_4$, 0.95 g $NH_4Cl$, 0.35 g KCl and 0.65 g NaCl, 0.9 ml of trace element solution, 1.3 ml of succinic acid solution (0.1 gl$^{-1}$), 3.7 ml of 1 M KOH added to 1000 ml distilled water. The final pH of the media was adjusted to 7.25. For providing iron limiting condition, the media was prepared in deionised distilled water and all the glassware were also washed thoroughly with deionised distilled water. 1 ml of $MgSO_4.7H_2O$ (0.1

gml$^{-1}$) was autoclaved separately and added to the media afterwards. The isolate was grown in the medium for 24 h. The hydroxymate type siderophore from the culture supernatant was estimated by the formula OD x 1500 x 1000/16500 and expressed as mgl$^{-1}$ as described (Mayer and Abdallah, 1978).

To determine catechol production, Arnow's method was used (Arnow, 1937). The isolate was grown in Fiss glucose minimal media (Vellore, 2001) prepared in deionized distilled water. The assay was performed by mixing the following in order: 1 ml culture supernatant, 1 ml 0.5 M HCl, 1 ml nitrite-molybdate reagent and 1 ml 1 M NaOH. These were allowed to incubate for 5 min for the reaction to fully occur. Absorbance was measured at 500 nm with uninoculated media serving as the blank.

### Antifungal activity

To check whether our fungal isolate had antifungal activity, activity of chitinase was determined by inoculating the isolate in the medium composed of 10 g colloidal chitin (Tanaka et al.,1999), 0.78 g $NH_4NO_3$, 0.80 g $K_2HPO_4$, 0.20 g $KH_2PO_4$, 0.20 g $MgSO_4 \cdot 7H_2O$, 0.06 g $CaCl_2$, 0.10 g NaCl, 0.002 g $Na_2MoO_4 \cdot 2H_2O$, 0.00024 g $ZnSO_4 \cdot 7H_2O$, 0.00004 g $CuSO_4 \cdot 5H_2O$, 0.010 g $CoSO_4 \cdot 7H_2O$, 0.003 g $MnSO_4 \cdot 4H_2O$, 0.028 g $Na_2FeEDTA$, 0.005 g $H_3BO_3$ and 15 g agar in 1000 ml distilled water. Magnesium sulfate and calcium chloride solutions were autoclaved separately and added to the medium after autoclaving. Biotin (5 µgl$^{-1}$) and p-aminobenzoic acid (10 µg l$^{-1}$), were filter-sterilized (0.2 µm membrane filter) and added to the medium after autoclaving. Quantitative estimation of chitinase was performed by the colorimetric method given by Ressig et al. (1955) after growing the isolate in the media containing nutrient broth and 0.5% colloidal chitin and incubated at 30°C for 48 h. β- 1, 3-glucanase production was determined in the above medium, except the carbon source was β-1, 3-glucan (5 gl$^{-1}$). The plates containing this media were spot inoculated with the cultures and incubated at 30°C for 5 days (Renwick et al., 1991).

### Phytohormones

The nutrient broth M-26 (5 g NaCl, 10 g peptone, 10 g beef extract) and MS medium (6 g $Na_2HPO_4$, 3 g $KH_2PO_4$, 0.5 g NaCl, 1 g $NH_4Cl$, 15 g agar, 10 ml of 100 mM $MgSO_4$, 10 mM $CaCl_2$ and 100 mM sucrose, 10 ml of 100 mM thiamine in 1000 ml water) were used for the production of indole acetic acid (IAA) (Husen, 2003). The isolate was inoculated in M-26 media and incubated for 24 h.

It was then grown in MS media containing 100 µl L-tryptophan stock solution (1%) for 48 h. After the incubation period, the ino-culated medium was centrifuged and supernatant was used for the estimation of IAA by Ferric chloride-perchloric acid assay (Gordon and Weber, 1951).

## RESULTS AND DISCUSSION

The fungal isolate F1 produced a clear halo around its colony in Pikovskaya's agar (Figure 1). Quantitative estimation of phosphate solubilization, carried out after incubation from 3 to 21 days (at 3-day intervals), is pre-sented (Figure 2). The fungal isolate showed a gradual increase in values for phosphate solubilized reaching a peak followed by a steep decline. Gaind and Gaur, 1991 reported that the drop in solubilization after a maximum value might be attributed to deficiency in nutrients in the culture medium. The fungus F1 discharged maximum amount of soluble phosphates (662 µg Pml$^{-1}$) in the culture
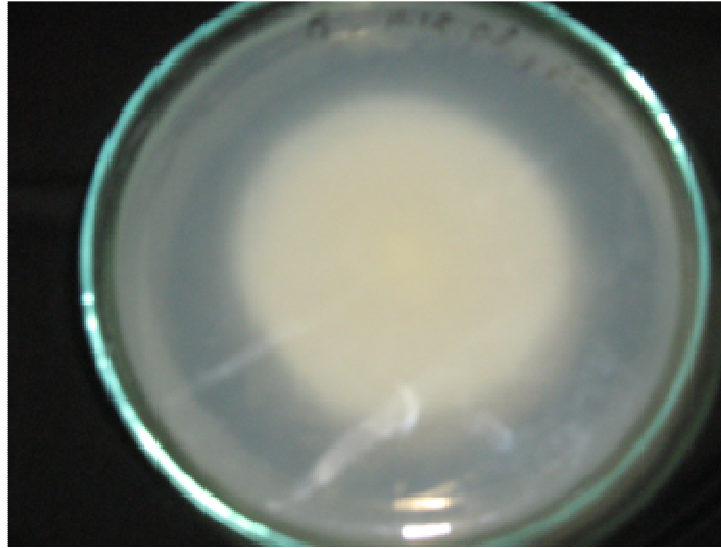
**Figure 1.** Formation of halo on Pikovskaya's medium by the isolate F1.
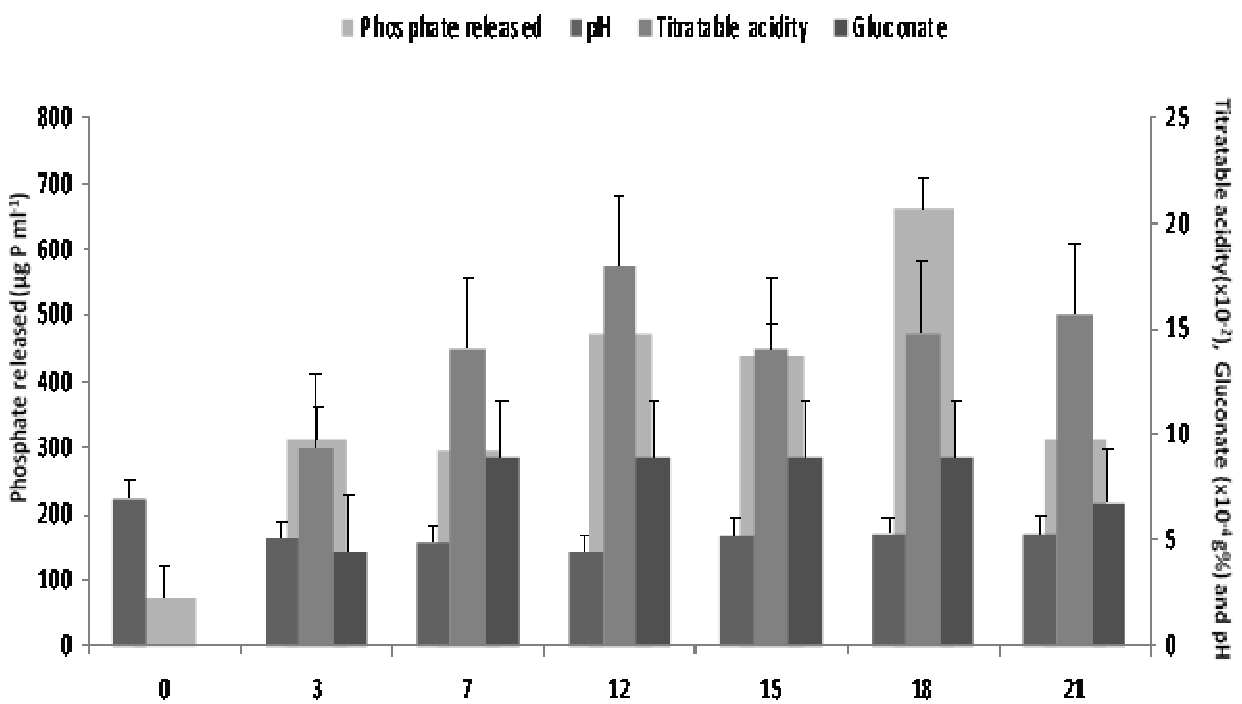


**Figure 2.** Changes in phosphate released, titratable acidity, gluconic acid and pH during solubilization over incubation period by F1 (Values are the mean ± S.D. of three replicates).

ture broth from tricalcium phosphate on the 18th day of incubation. The TA also followed a similar trend as that of P solubilized, except on day 21 when the value for P solubilized decreased whereas that for TA increased.

A similar pattern was also observed with production of gluconic acid, for which a constant value of 8.96 × 10^{-4}

g% was observed till 18th day of incubation (Figure 2). Since glucose was used in the medium as the carbon source, the concentration of gluconate produced by the isolate within was estimated. However, gluconic acid was not the only organic acid produced in the culture broth, because the amount of gluconic acid produced does not relate to the high values observed for P sol and TA. Pro-

**Table 1.** Plant growth promoting attributes of F1.

| Plant growth promoting trait | |
| --- | --- |
| Acid Phosphatase activity[a] | $1.90 \pm 0.07$ EU[*] |
| Alkaline Phosphatase activity[a] | $1.86 \pm 0.05$ EU[*] |
| Phytase activity[b] | $28.00 \pm 1.00$ mU |
| Siderophores(Catechol) | $4.50 \pm 0.05$ ($\mu g\ ml^{-1}$) |
| Siderophores(Hydroxymate) | $4.55 \pm 0.06$ ($\mu g\ ml^{-1}$) |
| IAA | $11.45 \pm 0.05$ ($\mu g\ ml^{-1}$) |
| Chitinase activity[c] | $0.037 \pm 0.07$ EU |

*Activity is sum of extracellular and intracellular enzyme activity; [a]EU= One unit of phosphatase activity is defined as the amount of enzyme which hydrolyses 1mM of p-nitrophenylphosphate$sec^{-1}$ at pH 5.4(acid phosphatase) or pH 9.4(alkaline phosphatase) at $35^{o}C$; [b]mU= One unit of phytase activity is defined as the amount of enzyme that released 1mmol of inorganic phosphate in 1min; [c]EU= One unit of chitinase activity is defined as the amount of enzyme, which produces 1 $\mu$ mole of N-acetylglucosamine in 1 ml of reaction mixture under standard assay condition (Values are the mean ± S.D. of three replicates).

duction of organic acids other than gluconic acid, such as oxalic acid, citric acid and succinic acid have been reported for the fungi *Aspergillus flavus*, *Aspergillus niger*, *Penicillium canescens* (Maliha et al., 2004). The mechanism of P solubilization can thus be a drop in pH due to production of organic acids. Wenzel et al. (1994) reported several potential mechanisms for phosphate solubilization, which include modification of pH by secretion of organic acids and protons or cation dissociation.

Phosphatases play a key role in transforming organic forms of phosphorus into plant available inorganic forms and are found to be active in microorganisms, plants and roots. A significant correlation had been noticed between the depletion of organic P and phosphatase activity in the rhizosphere soil of wheat (Tarafdar and Jungk, 1987). The culture F1 produced the enzymes alkaline and acid phosphatase in the range, 1.86 and 1.90 mU respectively (Table 1). Simultaneous exudation of organic acids and phosphatases could increase both P solubility, by re-leasing bound organic phosphates and its mineralization by increasing the rate of hydrolytic cleavage (George et al., 2002).

Despite the existence of phytase-positive soil organisms, which may account for 30 - 50% of total soil microbial flora, phytate remains the most recalcitrant pool of organic phosphorus in soils (Ognalaga et al., 1994) because of the inability of the phytase to act upon adsorbed and insoluble form. Therefore developing agricultural inoculants with high phytase production would be of great interest for improving plant nutrition and reducing P pollution in the soil. The culture F1 showed 28 mU of phytase activity (Table 1). This culture may serve as an efficient inoculant for solubilization of organic phosphates. The efficiency of hydrolysis of different organic P compounds of different fungi varied from 0.92 - 2.10 $\mu gmin^{-1}g^{-1}$ for phytin for isolated strains of *Aspergillus*, *Emmericella* and *Penicillium* (Yadav and Tarfdar, 2003).

Most fungi excrete ferric iron-specific chelators, siderophores, to mobilize this metal in response to low iron availability in the environment. Hence, the isolate was characterized for production of catechol and hydroxymate siderophores. The concentration of catechols and hydroxymates produced by F1 was 4.50 and 4.55 $\mu gml^{-1}$ respectively. In a study performed on *Absidia corymbifera*, only siderophores of the hydroxamate type were found (Holzberg and Artis, 1983). One of the traits of plant growth promoting microorganisms (PGPM) is their ability to produce or change the concentration of phytohormones (Husen, 2003). Therefore, the PSF was characterized for production of the phytohormone indole acetic acid. The isolate F1 was found to produce 11.45 $\mu gml^{-1}$ of IAA which is significantly high (Table 1). PGPM may possess traits associated with biocontrol of plant pathogens such as production of enzyme chitinase which can lyse fungal cells (Anjum et al., 2007). The chitinase activity of F1 was found to be 0.037 EU (Table 1).

## Identification of the unknown fungus F1

The fungal isolate was studied for cultural and microscopic features for identification. On these basis isolate F1 could be *Absidia* spp., which belongs to the phylum Zygomycota and family Mucoraceae (Dube, 2005). The strain was rapid growing and sporulated within 4 days. From the surface, the colony was grey in color. The reverse side was uncolored and there was no pigment production. Under microscope, the hyphae were broad and aseptate. Sporangiophores produced arches. The columella, the tip of the sporangiophore that extends into the sporangium, was semicircular in shape (Figure 3). Branched sporangiophores were present which a distinguishing characteristic of *Absidia* species. Circinate appendages arising from one or more sporangiosphores were observed (Figure 3).
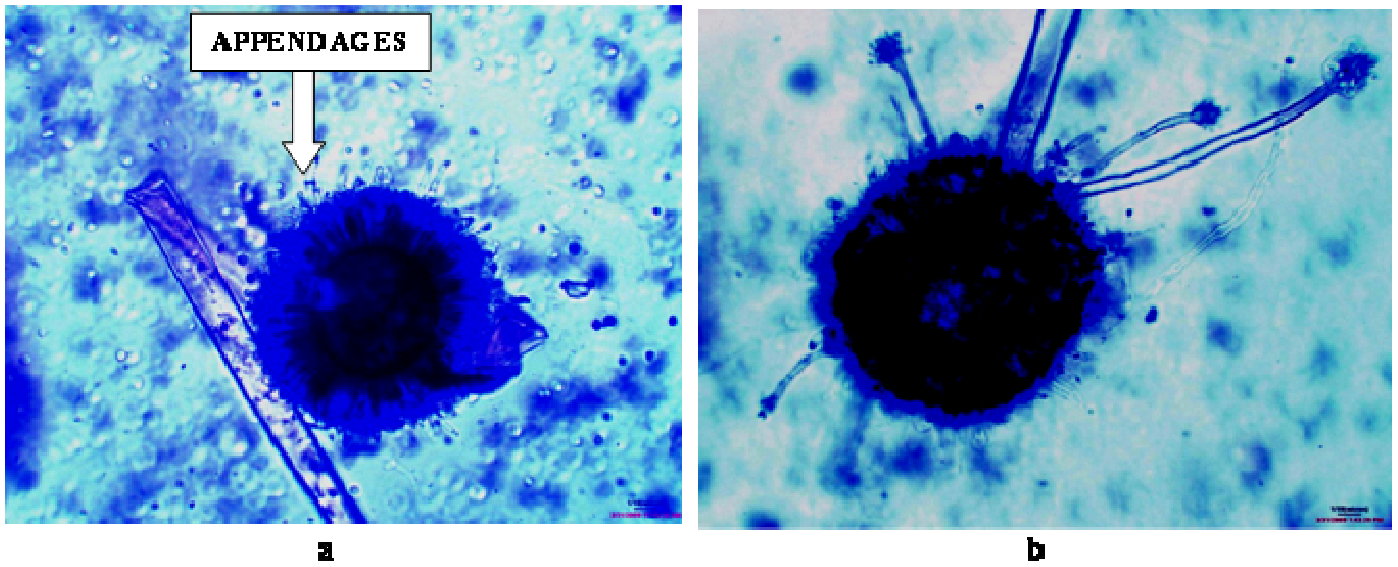
**Figure 3.** Microscopic features of F1: a) Sporangium with appendages; b) Several sporangia in a cluster.

To our knowledge, the fungus *Absidia* has not been reported in the literature as a phosphate solubilizer. However, considering the results obtained here for this fungus, the need to explore its potential as efficient inoculants for plant growth promotion arises.

## REFERENCES

Ames BN (1964). Assay of inorganic phosphate, total phosphate and phosphatases. Met. Enzymol. 8: 115-118.

Anjum MA, Sajjad MR, Akhtar N, Qureshi MA, Iqbal A, Jami AR and Mahmud-ul-Hasan (2007). Response of cotton to plant growth promoting rhizobacteria (PGPR) inoculation under different levels of nitrogen. J. Agric. Res. 45: 135-43.

Arnow LE (1937). Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. 118: 531-537.

Contreras-Cornejo H, Macı́as-Rodrı́guez L, Corte´s-Penagos C, Lo´pez-Bucio J, (2009). *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in Arabidopsis. Plant Physiol. 149: 1579–1592.

Dube H, (2005). An introduction to Fungi. Vikas Publishing House, New Delhi.

Gaind S, Gaur AC (1991). Thermotolerant phosphate solubilizing microorganisms and their interaction with mung bean. Plant Soil. 133: 141-149.

George T, Gregory P, Wood M, Read D, Buresh R (2002). Phosphatase activity and organic acids in the rhizosphere of potential agroforestry species and maize. Soil Biol. Biochem. 34: 1487-1494.

Gordon SA, Weber RP (1951). Colorimetric estimation of indoleacetic acid. Plant Physiol. 26: 192.

Haselwandter K (2008). Structure and function of siderophores produced by mycorrhizal fungi. Mineral. Mag.72: 61-64.

Holzberg M, Artis W (1983). Hydroxamate siderophore production by opportunistic and systemic fungal pathogens. Infect. Immun. 40: 1134–1139.

Hossain M, Sultana F, Kubota M, Koyama H, Hyakumachi M (2007). The plant growth-promoting fungus *Penicillium simplicissimum* GP17-2 induces resistance in *Arabidopsis thaliana* by activation of multiple defense signals. Plant Cell Physiol. 48: 1724-1736.

Husen E (2003). Screening of soil bacteria for plant growth promotion activities *in vitro*. Indonesian J. Agric. Sci. 4: 27-31.

Kloepper JWE, Young S, Lifshitz R, Zablotowicz RM, (1990). Description of a coryneform plant growth promoting rhizobacteria (PGPR) strain and its effects on plant development. In Klement, A.Z., (Eds) Plant Pathogenic Bacteria. Akademia: Kiado, Budapest, pp. 613-618.

Maliha R, Samina K, Najma A, Sadia A, Farooq L (2004). Organic acids production and phosphate solubilization by phosphate solubilizing microorganisms under *in vitro* conditions. Pakistan J. Biol. Sci. 7:187–196.

Mayer JM, Abdallah MA (1978). The fluorescent pigment of *Pseudomonas fluorescens* biosynthesis, purification and physico-chemical properties. J. Gen. Microbiol. 107:319–32.

Ognalga E, Frossard E, Thomas F (1994). Glucose-1-phosphate and myo-inositol hexaphosphate adsorption mechanisms on goethite. Soil Sci. Soc. Am. J. 58: 332-337.

Ressig JL, Strominger JL, Leloier LF (1955). A modified colorimetric method for the estimation of N- acetylamino sugars. J. Biol. Chem. 217: 959-966.

Richardson AE, Hadobas PA (1997). Soil isolates of *Pseudomonas* spp. that utilize inositol phosphates. Can. J. Microbiol. 43: 509-516.

Renwick A, Campbell R, Coe S (1991). Assessment of *in vivo* screening systems for potential biocontrol agents of *Gaeumannomyces graminis*. Plant Pathol. 40: 524–532.

Tabatabai MA, Bremner JM (1969). Use of *p*-nitrophenol phosphate for assay of soil phosphatase activity. Soil Biol. Biochem. 1: 301-307.

Tanaka T, Fujiwara S, Nishikori S, Fukui T, Takagi M, Imanaka TA (1999). Unique chitinase with dual active sites and triple substrate binding sites from the hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1. Appl. Environ. Microbiol. 65: 5338–5344.

Tarafdar J, Jungk A (1987). Phosphatase activity in the rhizosphere and its relation to the depletion of soil organic phosphorus. J. Biol. Fert. Soils 3: 199-204.

Vellore J (2001). Iron acquisition in *Rhodococcis erythropolis* strain IGTS8: isolation of a non-siderophore producing mutant [Dissertation]. East Tennessee State University, Johnson City, TN.

Welcher FJ (1958). The analytical uses of ethylene diamine tetraacetic acid (EDTA). D. Van Nostrand company, Inc. Princeto, New Jersey.

Wenzel L, Ashford E, Summerell A (1994). Phosphate solubilizing bac-

teria associated with protoid roots of seedings of waratoh (*Telopea speciosissama*). N. Phytol. 128: 487–449.

Whitelaw MA, Harden, TJ, Helyar KR (1999). Phosphate solubilisation in solution culture by the soil fungus *Penicillium radicum.* Soil Biol. Biochem. 31: 655-665.

Yadav R, Tarafdar J (2003). Phytase and phosphatase producing fungi in arid and semi-arid soils and their efficiency in hydrolyzing different organic P compounds. Soil Biol. Biochem. 35(6): 745-751.