

ROCK PHOSPHATE AND PHOSPHATE SOLUBILIZING MICROBES AS A SOURCE OF NUTRIENTS FOR CROPS

**A
DISSERTATION**

By

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for the award of the degree of Masters of Science in
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DEDICATED TOMY GUIDE

CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the dissertation entitled, “**Rock Phosphates and Phosphate Solubilizing microbes as a source of nutrients for crops**”, in partial fulfillment of the requirement for the award of the degree of Masters in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology, Patiala; is an authentic record of my own work during the period of five months from January 2003 to May 2003, under the supervision of Dr. M Sudhakar Reddy, Assistant Professor, Thapar Institute of Engineering and Technology. I have not submitted the matter embodied in this dissertation for the award of any other degree or diploma.

Place: Patiala

Date :

RICHA GROVER

This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

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DATE:

(RICHA GROVER)

1 INTRODUCTION

Phosphorous is second only to nitrogen as an essential macronutrient for plant growth and development (Scheffer *et al.*, 1998).

Soils are often high in insoluble mineral and organic phosphates but deficient in available orthophosphate (Pi) (Dadarwal *et al.*, 1997).

Soil amendment with phosphatic fertilizer, produced via chemical processing of rock phosphate ore, is therefore an absolute requirement in order to feed the world's population.

For over one hundred years, workers have recognized the ability of soil microorganisms to solubilize Pi from insoluble (i.e. nutritionally unavailable) organic and mineral phosphates (Whitelaw, 2000). Wide ranges of microbial biosolubilization mechanisms exist, so that much of the global cycling of insoluble organic and inorganic soil phosphates is attributed to bacteria and fungi. The genetic and biochemical mechanisms for this solubilization are as varied as the spectrum of P-containing soil compounds.

The limiting level of Pi in most soils provides the ecophysiological basis for positioning associations between plant roots and mineral phosphate solubilizing (MPS) and/or organic P solubilizing microorganisms. These associations are assumed to play an important role in phosphorus nutrition in many natural and agro-ecosystems. As a result, an enormous amount of research has been conducted involving isolation and characterization of MPS and organic P solubilizing microorganisms from a wide range of soils. In general, the goals have been to understand P cycling and/or to develop P biofertilizers analogous to biological nitrogen fixation. To date the results of these efforts have been problematic.

With respect to agriculture, bioprocessing of rock phosphate ore (RPO) to

inorganic phosphate may provide an energy efficient, environmentally desirable alternative to current technology for industrial P fertilizer production.

1.1 SIGNIFICANCE OF PHOSPHORUS

Phosphorus plays an indispensable biochemical role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and several other processes in the living plant.

An adequate supply of phosphorus in the early stages of plant growth promotes physiological functions including early root formation, and is important for laying down the primordia for reproductive parts of plants. It is vital to seed formation and its content is higher in seeds than in any other part of the plant. It helps plants to survive winter rigors and also contributes to disease resistance in some plants. Also known to improve quality of many fruits, vegetables and grain crops. Biological Nitrogen Fixation depends appreciably on the available forms of phosphorus.

Phosphorus (P) is an important structural constituent of nucleic acids, phytin and phospholipids.

1.2 PHOSPHATE AVAILABILITY IN SOILS

Mineral forms of phosphorus constitute the biggest reservoirs of phosphorus, represented primarily by rocks and deposits formed during geological age. The principal characteristic of these primary minerals (oxyapatite, hydroxyapatite, apatite) is their insolubility. A large portion of soluble inorganic phosphate applied to agricultural soil as chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to plants (Dadarwal *et al.*, 1997).

A second major component of soil P is organic matter, present largely in the forms of inositol phosphate (soil phytate), accounting for up to 50% of the total organic P. Other organic P in the soil is in the form of phosphomonoesters,

phosphodiesters including phospholipids and nucleic acids, and phosphotriesters.

Besides these, large quantities of xenobiotic phosphonates are released into the environment.

Despite of being so rich, the concentration of soluble P (that is, bioavailable P) is usually very low in soils due to the phenomenon of chemical fixation of phosphate.

1. 3 ROCK PHOSPHATES

To overcome the specific P nutrient deficiency, various forms of P, varying from processed rock phosphates (P-fertilizers) to ground phosphate rocks are applied. The use of commercial P-fertilizers is not cost effective. Among the alternative P sources, the most important are locally available Rock Phosphate (RP) resources (Rajan *et al.*, 1996).

Not all of the RP resources are readily plant available and agronomically reactive when applied directly to the soils. Reactivity is defined as the combination of RP properties that determines the rate of dissolution of RP in a given soil under given field conditions. The main factors influencing the agronomic effectiveness of rock phosphates are:

- 1) Mineralogy and chemistry of rock phosphates;
- 2) Reactivity/solubility of phosphate rocks;
- 3) Grain size and surface area;
- 4) Chemical and physical status of soil, especially pH and P fixing capacity of soil;
- 5) Type of crops and their nutritional requirements;
- 6) Management practices, including method and time of application, and liming.

1.4 PHOSPHATE SOLUBILIZING MICROORGANISMS (PSMs)

PSMs include different groups of microorganisms, which not only assimilate phosphorus from insoluble forms of phosphates, but they also cause a large portion of soluble phosphates to be released in quantities in excess of their requirements.

Species of *Aspergillus* and *Penicillium* are among fungal isolates identified to have phosphate solubilizing capabilities. Among the bacterial genera with this capability are *Pseudomonas*, *Azospirillum*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Arthrobacter*, *Alcaligenes*, *Serratia*, *Enterobacter*, *Acinetobacter*, *Flavobacterium* and *Erwinia* (Rodriguez *et al.*, 1996).

Seed or soil inoculation with PSMs is known to improve solubilization of fixed soil phosphorus and applied phosphates resulting in higher crop yields (Jones *et al.*, 1994)

PSMs are a low-cost solution that enriches the soil giving a thrust to economic development without disturbing ecological balance.

1.5 MECHANISMS OF PHOSPHATE SOLUBILIZATION AND MINERALIZATION

The phenomenon of fixation and precipitation of P in soil is highly dependent on soil type and pH. Thus, in acid soils, free oxides and hydroxides of aluminium and iron fix P while in alkaline soils Ca fixes it. Organic acid metabolite production and decrease of medium pH appear to be the major mechanisms for RP solubilization.

1.5.1 Effect of chelators on inorganic P solubilization

The principal underlying mechanism of action of chelators is formation of unionized association compounds with Ca^{++} , Fe^{++} , Al^{+++} and thus, increasing soluble phosphate concentration by scavenging phosphate from mineral phosphates.

The ability of low molecular weight organic acids to release P from ores or rocks, related to their ability to form stable metal complexes is well established (Mattey, 1992).

1.5.2 Role of phosphatase in organic phosphate solubilization

Mineralization of most organic phosphorus compounds that may constitute up to 30-50% of the total phosphorus in most soils is carried out by means of phosphatase enzymes, primarily acid phosphatases. These catalyze dephosphorylating reactions involving the hydrolysis of phosphoester or phosphoanhydride bonds.

1.6 APPLICATION OF IMMOBILIZED CELL TECHNOLOGY

Immobilized microbial P- solubilizers could be used for better understanding of the mechanisms of solubilization of insoluble phosphates. They could be used to substitute for chemical RP solubilization with inorganic acids, currently used in superphosphate production. Finally, such carrier-cell systems could be applied as soil microbial inoculants.

Immobilization methods are of particular importance for processes based on filamentous fungi. During cultivation, filamentous fungi demonstrate typical mycelial growth. At high biomass concentrations, mycelial suspensions constitute non-Newtonian fluids, with high viscosity (Braun and Vetch-Lifstichs, 1991).

This results in a decrease in mass transfer for oxygen and to a lesser degree for nutrients, such as carbon and nitrogen. Cultivation of fungi often is made difficult by the growth on the walls of the vessel. In general, immobilization methods provide an excellent protection of cells from adverse environmental effects.

1.7 SOIL AMENDMENT WITH ROCK PHOSPHATE & PHOSPHATE SOLUBILIZING MICROORGANISMS

Natural rock phosphates have been recognized as a valuable alternative source for P fertilizer. Common efforts in ways of manipulating such rocks to obtain a more valuable product include the use of chemico-physical means, that is, partially acidulating rock phosphates and decreasing particle size. (Hammond *et al.*,1986, Goenadi, 1990, Lewis *et al.*,1997, Rajan and Ghani, 1997, Babare *et al.*,1997).

By increasing soil microbial activities, bioavailability of P in a bioactive soil was remarkably enhanced (Thien and Myers, 1992).

- ♣ Bioconversion occurs at a low temperature and is more selective to phosphate extraction than conventional processes. This increased selectivity of attack may reduce the solubilization of undesirable ore contaminants such as radionuclides and toxic metals.
- ♣ The process uses carbohydrate as an energy/proton source as opposed to the 'wet-acid' chemical process that uses concentrated sulphuric acid and phosphoric acids.
- ♣ The bioprocessing of phosphate ore is not as sensitive to ore quality as are conventional approaches. This may allow lower grade ore deposits and tailings, not presently of any value, to be used.

Finally, appropriate formulation may allow the bioprocess to be utilized for *in situ* bioconversion of RP in the soil or even specifically in the rhizosphere of plant roots.

Looking towards the future it is reasonable to propose that, using the tools of biotechnology, Biophosphorous fertilization is an achievable goal that lends itself well to the global imperative of sustainable agricultural production.

2. REVIEW OF LITERATURE

Phosphorus (P) is an essential macronutrient for plants but in most soils its content is about 0.05% of which only 0.1% is plant-available (Scheffer *et al.*, 1998).

The optimal development of crops demands a high, often costly, input of P fertilizers. Current concepts in sustainability involve application of alternative strategies based on the use of less expensive natural sources of plant nutrients like rock phosphate. The beneficial effect of rock phosphate has made this material an attractive component for management in agriculture (Rajan *et al.*, 1996).

One traditional method of increasing P-availability is the acidulation of RP with small amounts of H_2SO_4 or H_3PO_4 to produce partially acidulated RP (Rajan and Watkinson, 1993). But this is uneconomical and environmentally nonviable.

Thien and Myers (1992) indicated that by increasing soil microbial activities, bioavailability of P in a bioactive soil was remarkably enhanced. The fact that certain soil microbes are capable of dissolving relatively insoluble phosphatic compounds (Nahas *et al.*, 1990, Bojinova *et al.*, 1997) has opened the possibility for inducing microbial solubilization of phosphates in soils.

It should be noted that filamentous fungi are among the most active and studied solubilization agents and a typical process for RP solubilization in submerged (single batch, shake-flask) fermentation conditions involves glucose based media and is performed for 7-20 days (Asea *et al.*, 1988, Cunningham and Kuiack, 1992; Illmer *et al.*, 1995; Nahas, 1996; Reyes *et al.*, 1999).

Filamentous fungi are widely used as producers of organic acids (Matty, 1992) and in particular *Aspergillus niger* and some *Penicillium* species have been tested in fermentation system or inoculated directly into soil in order to solubilize

rock phosphate (Kucey, 1987 and Vassilev *et al.*, 1995)

Reddy *et al.*, (2002) found that all the isolates of *Aspergillus tubingensis* and *A. niger* isolated from rhizospheric soils were found to be capable of solubilizing all the natural forms of rock phosphates. This is the first report of solubilization of rock phosphates by *Aspergillus tubingensis* and showed that this fungus might serve as an excellent rock phosphate solubilizer when inoculated into soils where rock phosphate is used as P fertilizer.

Goenadi *et al.*,(2000) determined the optimum incubation period and the optimum level of rock phosphate for a Phosphate Solubilizing Fungus (PSF), *Aspergillus niger* BCCF.194, isolated from tropical acid soils. They conducted a simple, effective, and environmentally sound process to improve P availability of phosphate rocks to crops by Phosphate Solubilizing Fungus.

Mechanism of Solubilization:

Phosphate solubilization by microbes is mediated by several different mechanisms including organic acid production and proton extrusion (Surange, 1995; Lapeyrie *et al.*, 1991; Burgstaller & Schinner, 1993; Cunningham & Kuiack, 1992; Dutton & Evans, 1996; Nahas, 1996). Increasing P concentration in the phosphate solubilizing fungal containing medium is related to the production of organic-acid-type metabolites, which should correlate with pH of the medium (Illmer and Schinner, 1992; Illmer *et al.*, 1995; Narsian *et al.*, 1995). It is generally recognized that organic acids solubilize RP through protonation and / or chelation reactions (Sagoe *et al.*, 1998). Besides the acid strength, the type and position of the ligand determine the effectiveness of the organic acid in the solubilization process (Kpombrekou and Tabatabai, 1994)

Illmer *et al* (1995) indicated the level of organic acids resulting in significant P dissolution were in the order of 3-30 μM /ml, distinctly below the efficiency of biotic leaching. Thus, the production of organic acids is an important mechanism for solubilizing insoluble P, but not the only one.

The principal mechanism for organic phosphate solubilization is acid phosphatase activity (McGrath *et al.*, 1995). Arbuscular Mycorrhiza (AM) can make use of organic phosphate (Balaz and Vosatka, 1997) and are able to acidify the environment, which facilitates inorganic P dissolution (Bago *et al.*, 1996).

Narsian and Patel (2000) studied the influence of chelators on phosphate solubilization by *Aspergillus aculeatus*, a rhizosphere isolate of gram. They concluded that different test chelators had differential behaviors in relation to phosphate solubilization. The chelator nitrilotriacetic acid (NTA) increased RP solubilization at 2mg/ml while diethylenetriaminepentaacetic acid (DTPA) enhanced PS only at 6 mg/ml while ethylenediaminetetraacetic acid (EDTA), aluminon and oxine inhibited PS at all concentrations tested. They also found that the highest PS activity, in presence of RP, was up to 50 mg P_2O_5 . Higher concentrations (ie. 100-250 mg P_2O_5) reduced fungal activity. Although higher concentrations of P were not effective for PS activity, growth, however, increased successively.

Immobilization technology:

Vassileva *et al* (1998) encapsulated spores of *Aspergillus niger* in agar, calcium alginate and k-carrageenan and further applied in citric acid production during six repeated batch cultivations. The highest average citric acid productivity was reached with alginate-bead-encapsulated on RP free culture medium while agar seemed to be the most suitable carrier on RP-supplemented medium.

Vassileva *et al.* (2000) found that cell encapsulation favored the acid-producing activity of *Yarrowia lipolytica* that ensured higher average acid productivity and solubilization levels as compared to treatments with free cells. The reuse efficiency of agar-encapsulated yeast cells for citric acid production was greater than that by freely suspended cells. Alginate and k-carrageenan appeared to be unsuitable carriers when rock phosphate was supplied to the medium solution in place of calcium carbonate.

Vassilev *et al.* (1997) immobilized *Aspergillus niger* on polyurethane foam. They found that immobilized cells were reused, with higher levels of acid formation being maintained for longer periods (at least 240 hours) than for free cell.

Vassilev *et al.* (2001) in the review paper summarized all available studies that involved immobilized microorganisms related to RP solubilization and P plant nutrition, and pointed out possible future trends in this field of research.

Phosphate Solubilizing Micro-organisms as inoculants:

Mineral solubilization by soil microorganisms is widespread and, with respect to agriculture, this process has been paid considerable attention. Microbial survival following introduction into, particularly natural soils depends on both abiotic and biotic factors (Van Loosdrecht *et al.*, 1990; Van Veen *et al.*, 1997).

In the USSR, a bacterial inoculant named phosphobacterin was the first prepared for application in agriculture. Phosphobacterin is a culture of *Bacillus megaterium* var. *phosphaticum*, phosphate solubilizing bacteria adsorbed on kaolin. IARI microphos culture (Gaur 1983)- a preparation of carrier based inoculant of *Pseudomonas striata*, *Bacillus polymyxa*, *Aspergillus awamori* was used in India.

Many free living and symbiotic fungi can solubilize rock phosphate and increase phosphorus availability for plants (Kucey, 1983, 1987; Lapeyrie, Ranger & Vairelles, 1991; Illmer & Schinner, 1992; Griffiths, Baham & Caldwell, 1994).

Asea, Kucey & Stewart (1988) found that *Penicillium billai* and *P. cf. fuscum* increased total plant phosphorus uptake by 14% and wheat dry matter yield by 16%. In Canada, the use of a commercial formulation of *P. billai* spores to increase the availability of phosphate to wheat has been documented (Cunningham & Kuiack, 1992).

Vassilev *et al.* (1996) observed a higher growth rate and shoot phosphorus concentration when microbially treated sugarbeet waste material and rock phosphate was applied to both mycorrhizal and non-mycorrhizal plants. However, combined introduction of both the filamentous and arbuscular fungi led to improved plant growth when degraded organic matter supplemented or not supplemented with RP was used.

Vanlauwe *et al.* (2000) studied the impact of RP application to Macuna and Lablab on grain yield, total N, and total P uptake of a subsequent maize crop for a set of non acidic soils in a representative toposequence. The studied legume-maize rotations supplied with RP during the legume phase and minimal amount of inorganic N during the maize phase are good examples of soil fertility management technologies alleviating N and P deficiencies.

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Fungal strains used:

Aspergillus tubingensis, *Aspergillus niger* (Reddy et al., 2002) isolated from rhizospheric soils of Patiala were used in this study and maintained on Potato Dextrose Agar.

3.1.2 Procurement of rock phosphate: Bilt Chemicals, Karnataka

3.1.3 Media used

3.1.3(a) Composition of Pikovskaya's medium (Pikovskaya, 1948)

Glucose	10.0g
Ca ₃ (PO ₄) ₂	5.0g*
(NH ₄) ₂ SO ₄	0.5g
NaCl	0.2g
MgSO ₄ .7H ₂ O	0.1g
KCl	0.2g
Yeast extract	0.5g
MnSO ₄	Trace
FeSO ₄ .7H ₂ O	Trace
Agar	15.0g
Water	1000ml
pH	7.0±0.2

*Stock suspension of 2.5% Ca₃(PO₄)₂ was prepared in distilled water and was autoclaved for preparation of plates or broth, 10 ml of stock suspension was added aseptically to the 90 ml of sterilized medium.

3.1.3 Reagents used in immobilization technology

Composition of growth medium (Vassilev et al., 2000)

Glucose	10.0g
NH ₄ NO ₃	1.0g
KH ₂ PO ₄	1.0g
MgSO ₄ .7H ₂ O	0.2g
ZnSO ₄ .7H ₂ O	0.007g
Corn Steep Liquor	1.0g
Water	1000ml
pH	5.5

Composition of production medium (Vassilev et al., 2000)

Glucose	10.0g
NH ₄ NO ₃	1.0g
MgSO ₄ .7H ₂ O	0.2g
ZnSO ₄ .7H ₂ O	0.004g
Water	1000ml
pH	5.5

Media were sterilized at 121°C for 15 minutes. Rock Phosphate (33.3% P) was sterilized separately and added to the production medium at a concentration of 3 g/l (Equivalent to 50 mg of P₂O₅. / 50 ml of medium).

3.1.4 Reagents for soluble P estimation in culture filtrate (Jackson, 1967)

- Chloromolybdic acid

Dissolved 15.0 g of ammonium molybdate in about 400 of warm distilled water. Filtered if it is necessary and added 342 ml of 12 N HCl, slowly with rapid stirring. Cooled and made the volume to one litre with distilled water and stored in amber glass bottle.

- Chlorostannous acid

Dissolved 10 g of SnCl_2 in 25 ml of conc. HCl. Kept it in a flask under airtight stopper. The solution was freshly prepared by taking 1 ml of above prepared stock solution and added 132 ml of distilled water.

- Standard P solution (100 ppm P)

Dissolved 0.4390 g of dried KH_2PO_4 in 400 ml of distilled water and added 25 ml of 7 N H_2SO_4 and made up to 1 Litre.

- Working P solution (10 ppm)

Diluted 10 ml of standard P solution to 100ml.

3.1.5 Reagents for Estimation of phosphatase activity (Eivazi & Tabatabai 1976)

- Universal Buffer (5X) (Skujins et al. 1962)

Tris (hydroxy methyl) amino methane	12.10g
Maleic acid	11.60g
Citric acid	14.00g
Boric acid	6.28g
NaOH (1N)	488ml
Distilled water	1000ml
pH	6.5

- 0.1 M disodium p-nitrophenylphosphate (in Universal Buffer)

- 0.5 M NaOH

3.1.6 Reagents used for Immobilization technology

- 3% Sodium Alginate
- 0.5 M Calcium Chloride
- Growth medium (3.1.3)
- Production medium (3.1.3)

3.2 METHODOLOGY

3.2.1 To find the optimum time for maximum solubilization (Goenadi et al., 2000).

- Separately sterilized and added Tricalcium Phosphate (TCP) in amounts equivalent to 100 mg P_2O_5 to 50-ml sterile Pikovskaya's broth.
- Inoculated with 4-mm spore discs from 4-day-old culture of *Aspergillus tubingensis*, *A. niger*.
- Incubated at 30°C under shaking conditions (150 rpm) for different time intervals such as 1, 2, 3, 4 and so on up to 8 days.
- Filtered the mycelial mass using Whatman no. 42 filter paper.
- The mycelia was repeatedly washed with distilled water and dried at 70°C for 48 hours. The dried mass served as the parameter for growth determination.
- The culture filtrate was analyzed for soluble P chlorostannous reduced molybdophosphoric acid blue method as described by Jackson (1967) (3.2.2©).

- All the experiments were carried out in triplicates.

3.2.2 To study the effect of increasing concentration of RP on P solubilization by the two strains (Goenadi, 2000; Narsian & Patel, 2000).

- TCP and Rock Phosphate (RP) in the Pikovskaya's medium were added in amounts equivalent to 50, 100 and 150 mg P₂O₅ in 50 ml of medium.
- Inoculated with 4-mm spore discs of 4-day old cultures of the two strains.
- Incubated at 30°C under shaking conditions (150 rpm) for 4 days.
- All the experiments were carried out in triplicates.
- Acid phosphatase activity, biomass of mycelia; pH and soluble P concentration of the filtrate was determined by the following procedures.

3.2.2(a) Growth determination

At the end of incubation, the contents of the flasks were filtered through Whatman no. 42 filter paper, washed repeatedly with distilled water and the mycelial mass was dried at 70°C for 48 hours. Dry weight of the mycelium represented growth.

3.2.2(b) Phosphorus estimation and pH

Water soluble P in the culture filtrate was estimated by the chlorostannous reduced molybdophosphoric acid blue method as described by Jackson (1967). The pH of the spent medium was measured by pH meter.

3.2.2 (c) Procedure for Estimation of Soluble P in Culture Filtrate (Jackson, 1967)

- Transferred 100 µl of filtrate to 50 ml volumetric flask.
- Added 10 ml of chloromolybdic acid reagent along the sides of the flask and diluted the contents of the flasks to 40 ml.
- Next, added 1 ml of chlorostannous acid reagent and after mixing made the volume upto 50-ml as quickly as possible.
- The blue color intensity of the solution was measured at 600 nm.
- To prepare standard curve, measured 0, 0.5, 2.5, 5.0, 7.5 and 10 ml of 10 ppm P solution in 50-ml volumetric flask and followed step 2 to 4.

3.2.2(d) Data analysis

All the data was analyzed by analysis of variance (ANOVA) and the means were compared with Duncan's Multiple Range Test (DMRT) at $P < 0.05$ level.

3.2.2(e) Estimation of acid phosphatase activity (Eivazi & Tabatabai 1976)

- Filtered mycelial mass was washed aseptically with sterile deionized water followed by a rinse with sterile universal buffer.
- Mycelium was placed in 30 ml screw cap tube with 4 ml of buffer solution plus 1 ml of filter sterilized, 0.1 M disodium p-nitrophenylphosphate.
- Incubated at 30 °C for an hour in the dark.
- 5ml of 0.5 M NaOH was added to stop the reaction.

Acid phosphatase activity was determined from the amount of p-nitrophenol released in the filtrate from the phosphatase substrate as measured by Vis/UV spectrophotometer at 410 nm.

3.2.3 To study the effect of chelators on P solubilization (Narsian & Patel, 2000): In order to understand the role of chelator in phosphate solubilization and

their influence on the behavior of our test cultures, the following experiment was designed.

- Ethylenediaminetetraacetic acid (EDTA) was added at the concentrations of 2, 4, 6 mg/ ml in Pikovskaya's medium amended with RP equivalent to 50mg of P₂O₅ in 50 ml of the medium.
- Inoculated with 4-mm spore discs of the two cultures.
- Incubated at 30°C for 4 days under shaking conditions (150 rpm).
- Biomass, pH, soluble P in the culture filtrate and acid phosphatase activity of the mycelia was determined for the two strains in the presence of varying concentrations of EDTA.
- Control treatments without the inoculation of cultures were maintained for all the concentrations of EDTA and analyzed for the above mentioned parameters.
- All the experiments were carried out in triplicates.

3.2.4 Application of immobilization technology to PSMs (Vassilev et al., 2000).: In order to test the efficacy of encapsulated cells with respect to free cells of *A. niger* and *A. tubingensis* in terms of soluble phosphorus, immobilization was carried out using calcium alginate as carrier.

- 50 ml of Potato Dextrose Broth was inoculated with 4-day-old *Aspergillus* spores of uniform density in a number of replicates.
- After 3 days, mycelia (almost equal mass) were washed with sterile distilled water and transferred to 50 ml of sterile deionized water, sterile 3% sodium alginate separately and homogenized.
- Followed by extrusion in 250 ml of 0.5M Calcium Chloride.

- Beads of 2-3 mm were allowed to harden for 30 minutes under gentle stirring at 28°C in CaCl₂ solution.
- A total of 12 beads (equivalent to 1 ml of free cell suspension in sterile distilled water) were transferred to 250-ml Erlenmeyer flasks containing 100ml of growth medium.
- Simultaneously, 1 ml of free cell suspension was inoculated separately under similar conditions.
- After 24 hours of incubation at 30°C on a rotary shaker (150-rpm), immobilized beads and free cells were washed with sterile distilled water and transferred to 100ml of production medium.
- RP solubilization was carried out for 4 repeated batch fermentations of 48 hours duration each.

Analytical methods:

At the end of each batch cycle, the culture broth free of encapsulated cells and samples from free cell experiments was used for analysis. The dissolved phosphorus concentration was determined by the chlorostannous reduced phosphomolybdic acid method as described by Jackson *et al.* (1967). The pH of the culture filtrate was determined using pH meter.

3.2.5 Nursery Plantation Experiment:

After in-vitro testing of solubilization of Rock Phosphates by *A. tubingensis*, *A. niger* in shake- flask fermentation conditions. These test cultures were amended in soils along with Rock Phosphate and evaluated on maize, in terms of shoot height, root dry weight, shoot dry weight as per control treatments.

- Soil collected from the agricultural field of Patiala was oven dried at 70°C for 12 hours duration with 12 hours incubation in between. The procedure was repeated twice in order to destroy inherent microflora.

- There were mainly 6 treatments: soil only, soil with *Aspergillus niger*, soil with *Aspergillus tubingensis*, soil amended with RP, RP amended soil with *Aspergillus niger*, RP amended soil with *Aspergillus tubingensis*.
- Similar treatments were undertaken for non sterile soil samples also.
- Inoculum preparation
 - } 100 g soilrite was autoclaved twice at 121°C for 45 minutes.
 - } Spores from 7-day old plates were scrapped off and uniformly dispersed in the soil rite.
- Filled approximately 250 ml of pot with soil and added 4.56 g of inoculum to each. (Added 2.964×10^9 cfu / pot of *Aspergillus tubingensis* and added 1.977×10^9 cfu / pot of *Aspergillus niger*).
- Added approx. 3.33% of rock phosphate in each pot receiving RP treatment.
- Inoculum was uniformly dispersed in the soil and approximately 50 g of soil was further added.
- Sowed 3 maize seeds equilaterally. Within a period of a week, two of the plantlets were pulled off.
- Harvested the plants after 45 days.
- Recorded shoot height, dry weight of shoot and root, after thorough washing, for each treatment after drying at 65° C for 48 hours.

4. RESULTS AND DISCUSSIONS

4.1 In the experiment carried out to determine the optimum time of activity of the two strains, *Aspergillus tubingensis* showed a significant increase in biomass up to third day only. In case of *Aspergillus niger*, the biomass increased significantly up to day fifth and thereafter decreased (Fig.1 a). Excessive sporulation was observed in the following days in both strains, owing to deficiency of either of essential macronutrients that could explain no significant increase in biomass thereafter.

Levels of soluble phosphorus in the culture filtrate, in case of *A. tubingensis*, increased significantly up to third day and remained high for fourth day. However, later on, a significant drop in soluble phosphorus levels was observed. In case of *A. niger*, the levels of soluble phosphorus detected in the culture filtrate increased significantly up to fourth day. Following which, a drastic drop in soluble P levels was observed (Fig.1 b).

A plausible reason for such an observation could be attributed to the availability of soluble form of phosphate, which has an inhibitory effect on further phosphate solubilization (Narsian et al., 1995). The negative effect of soluble P on microbial acid productivity (Rohr et al., 1983) might also be responsible for final soluble P concentration. Another explanation for this might be formation of an organo-P compound induced by organic metabolites released, which in turn, reduces the amount of available P (Illmer and Schinner, 1992).

4.2 Optimization of Tri-Calcium Phosphate (TCP) concentration:

In order to optimize the concentration of $\text{Ca}_3(\text{PO}_4)_2$ for maximum fungal activity, different amounts, viz., 50, 100, 150 mg of P_2O_5 equivalent $\text{Ca}_3(\text{PO}_4)_2$ were supplemented in 50 ml of Pikovskaya's broth.

4.2 (a) The pH of the culture filtrate inoculated with *A. tubingensis* dropped significantly as the amount of $\text{Ca}_3(\text{PO}_4)_2$ added (P_2O_5 equivalent) was increased from 50 mg to 100 mg. It further increased as the amount of P_2O_5 was further raised to 150 mg but not a significant difference was observed in the pH of culture filtrate amended with 50, 150 mg of P_2O_5 . The least pH was observed when 100 mg P_2O_5 was added. In case of *A. niger*, a significant rise in pH was observed only when the amount was increased from 100 mg to 150 mg of P_2O_5 . However, no significant increase in pH was observed when the amount was increased from 50 to 100 mg of P_2O_5 equivalent $\text{Ca}_3(\text{PO}_4)_2$ (Fig.2 a).

It is known that 0.1% RP added to the fermentation medium increases the pH by 0.5 units (Asea et al., 1988). No significant rise in pH could be attributed to neutralization by secretion of organic acids. It was also observed that the medium receiving lower quantity of RP remained more acidic than media with higher concentrations of RP. This was also reported by Gaur (1990) and Narsian and Patel (2000).

4.2 (b) A significant decrease in biomass of *A. tubingensis* was observed only when the amount of P_2O_5 was raised from 50 to 150mg/50ml.

A. niger showed a significant increase in biomass when the amount of P_2O_5 was successively raised from 50 to 100 mg, and then further from 100 to 150 mg/50 ml (Fig.2 b). Narsian and Patel (2000) also reported that at higher concentrations of P, growth, increased successively.

4.2 © Level of soluble P was increased significantly in case of medium inoculated with *A. tubingensis*, as the amount of P_2O_5 was increased from 50 to 100 mg. However, no significant increase was observed as the amount was further raised to 150 mg.

In case of *A. niger*, the level of soluble P increased significantly as the amount of P_2O_5 increased from 50 to 100, and then from 100 to 150 mg (Fig. 3a).

4.2 (d) Level of acid phosphatase activity increased significantly as the amount of P_2O_5 was raised from 50 to 100 mg in case of *A. tubingensis*. It, however, decreased as the amount was further raised to 150 mg. *A. niger* exhibited no significant difference in acid phosphatase activity as the amount of P_2O_5 was increased from 50 to 100 mg but it showed significant decrease as the amount was further raised to 150 mg (Fig. 3b).

In case of *A. tubingensis*, lowest pH, least biomass, highest acid phosphatase activity and maximum soluble P corresponded to 100 mg of P_2O_5 added, when tricalcium phosphate was used as the source of insoluble phosphate. *A. niger* exhibited lowest pH, least biomass, highest acid phosphatase activity corresponding to 50 mg of P_2O_5 added in the form of tricalcium phosphate. However, maximum soluble P level was observed when 150 mg of P_2O_5 was added. But the process efficiency, the percent level of soluble P in relation to total rock phosphate added, was found to be maximum in case of 50 mg of P_2O_5 amendment.

4.3 Optimization of Rock Phosphate concentration:

In order to determine the optimum concentration of Rock Phosphate (RP) for maximal activity of the fungus, rock phosphate was sterilized separately in amounts equivalent to 50, 100, 150 mg of P_2O_5 equivalent Rock Phosphate. The different parameters for different concentrations were analyzed.

4.3a *A. tubingensis*, when grown on RP as the sole source of phosphorus, exhibited significant increase in biomass only when the amount of P_2O_5 equivalent RP was increased from 100 to 150 mg/ 50ml. In case of *A. niger*, however, a significant increase in biomass was observed as the amount of P_2O_5 was successively increased (Fig.4 a).

4.3b A significant increase in pH of the culture filtrate was observed as the amount of P_2O_5 equivalent RP was increased from 100 to 150 mg in case of *A.*

tubingensis. However, in case of *A. niger*, no significant difference in pH of the culture filtrate was observed (Fig.4 b).

4.3c A significant increase in soluble P level was observed in case of medium inoculated with *A. tubingensis*, when the amount of P_2O_5 was increased from 100 to 150 mg (in 50 ml). However, a significant increase in amount of soluble P was observed in the media supplemented with 50, 150 mg of P_2O_5 equivalent RP, in case of *A. niger* (Fig5 a).

4.3d In case of *A. tubingensis*, the highest acid phosphatase activity was observed in the presence of 50 mg of rock phosphate. There was a significant decrease in acid phosphatase activity as the amount was increased from 50 to 100 and to 150 mg. Exactly, similar results were observed in case of *A. niger* (Fig. 5b).

Lower pH, least biomass and maximum acid phosphatase activity by *A. tubingensis* was observed when RP was amended in amounts equivalent to 50 mg P_2O_5 / 50ml. Maximum soluble P was observed in case of 150 mg of P_2O_5 , However, the process efficiency of solubilization was maximum when 50 mg of P_2O_5 was amended in 50 ml of medium. Similar results were illustrated by *A. niger*, that is, higher acid phosphatase activity, least biomass were observed when 50 mg of P_2O_5 equivalent RP was added. Soluble P level was higher in case of medium amended with 150 mg of RP equivalent P_2O_5 , but the process efficiency of solubilization was maximum when 50 mg P_2O_5 was amended.

*Amount of soluble P rendered by both strains, when tricalcium phosphate (TCP) was used, was found to be higher than the amounts solubilized from Rock Phosphate. TCP is more readily available P-source than rock phosphates.

*Acid phosphatase activity was found to be higher in RP supplemented medium than in tricalcium phosphate-enriched medium.

**A. tubingensis* exhibited higher acid phosphatase activity and thus, higher level of soluble P when compared to *A. niger* in the comparative fermentation conditions for both the sources of inorganic phosphates tested.

4.4 Effect of chelator on P solubilization by the two fungal strains:

In order to elucidate the influence of chelator on P solubilization by the respective strains, and the role of chelator alone, the following experiment was designed. 2, 4, 6 mg/ ml of EDTA was amended to 50 ml of Pikovskaya's broth. The flasks were inoculated and incubated under standard optimized conditions that is, 4days with 50 mg of P₂O₅ equivalent RP. The data was analyzed by Two Way Analysis Of Variance (ANOVA)

Table 1. Influence of varying concentration of EDTA, on soluble P by *A. tubingensis* and *A. niger*

Source of Variation	Df	Sum of Squares	Mean Sum of Squares	F value	P value
Interaction	3	42.09	14.03	9.175	P<0.01**
Fungi	1	121.5	121.5	79.44	P<0.001***
Conc.	3	89.45	29.2	19.50	P<0.001***
Residual	16	24.47	1.529		

Two way analysis of variance revealed that there is a significant difference in the level of P solubilization by the two fungal strains. Also, significant difference was observed in soluble P levels at varying concentration of EDTA (Table 1).

4.4a The pH of culture filtrate in case of *A. tubingensis*, decreased significantly when EDTA was added at the rate of 2 mg/ml. Thereafter, with subsequent rise in concentration of EDTA added, pH rose. Similarly, a significant decrease in pH was observed when 2mg/ml EDTA was added in case of *A. niger*. (Fig.6a).

4.4b A significant decrease in biomass was observed in case of *A. tubingensis* as it was successively amended with 2, 4 and 6 mg/ml of EDTA. However, in case of *A. niger*, the biomass significantly decreased up to 4mg/ml and thereafter no significant decrease was observed (Fig.6 b).

EDTA was found to inhibit the mycelial growth significantly. Higher the concentration of EDTA was added, lower the biomass was observed for both the strains. Also, the least pH of the culture filtrate was noted in case of medium amended with 2 mg/ml of EDTA for both cases.

4.4c In case of *A. tubingensis*, maximum level of soluble P was observed in the culture filtrate without EDTA. The level of soluble P decreased significantly when EDTA was amended at the rate of 2, 4 mg/ml. However, no significant difference in soluble P levels was observed between EDTA free medium and medium amended with 6 mg/ml of EDTA. *A. niger* showed a decrease in soluble P level in all the concentrations of EDTA added (Fig. 7 a).

Narsian and Patel (2000) reported that the increasing concentrations of various chelators showed different behavior on solubilization of rock phosphate by *A. aculeatus*. They reported that chelators like nitrilotriacetic acid (NTA) at 2mg/ml and diethylenetriaminepentaacetic acid (DTPA) at 6mg/ml increased the rock phosphate solubilization whereas EDTA, Aluminon and Oxine inhibited the solubilization activity in all the concentrations. In our study, EDTA at 6mg/ml concentration did not inhibit the solubilization of rock phosphate in case of *A. tubingensis*.

4.4d Acid phosphatase activity significantly dropped in case of both the strains as the concentration of EDTA amended in the medium was successively increased.

The maximum acid phosphatase activity was observed in case of medium free of EDTA. EDTA, in case of control treatments, was found to increase progressively soluble P levels in the medium. A very interesting observation was made. Soluble P level was found to be non significantly different in case of EDTA-free medium inoculated with *A. tubingensis* and control amended with 6mg/ ml of EDTA. Confirming chelation to play an important role in rendering fixed forms of phosphate free.

Thus, EDTA not only inhibited mycelial growth, but also acid phosphatase activity. The observed phenomenon could be attributed to increase in the level of solubilization by increasing the concentration of chelator as observed. The higher levels of soluble form of phosphate might have an inhibitory effect on further phosphate solubilization by acid phosphatase enzyme. The negative effect of soluble P (liberated via the action of chelator) on the microbial acid productivity (Roger's et al. 1983) could be another reason that may cause changes in the final soluble P concentration by lowering enzymatic activity. It can also be concluded that under stress conditions, that is, when the level of soluble P is low, the activity of acid phosphatase is remarkably enhanced.

4. 4.5 Immobilization technique:

In situ encapsulation of the two strains, *A. tubingensis* and *A. niger* was carried out using calcium alginate as carrier. The evaluation of encapsulated cells in comparison to free cells in terms of soluble P level over repeated batches in laboratory shake flask experiment was undertaken. RP was amended in amounts equivalent to 100 mg P₂O₅/100 ml of the production medium (3g/l of RP).

Table 2: Levels of soluble P in case of free and immobilized cells over 4 repeated batches in case of *A. tubingensis*, *A. niger*.

	1 st batch	2 nd batch	3 rd batch	4 th batch
<i>Aspergillus tubingensis</i>				
FC	14.58 ± 0.4215 a	12.26 ± 0.9393 a	13.47 ± 0.504 a	13.94 ± 0.7742 a
IC	11.47 ± 0.0154 b	10.74 ± 1.159 a	13.39 ± 0.2211 a	6.971 ± 0.7188 b
<i>Aspergillus niger</i>				
FC	18.77 ± 1.369 a	17.42 ± 0.1579 a	14.08 ± 0.0433 a	8.504 ± 0.7188a
IC	11.96 ± 1.039 b	12.45 ± 1.434 b	11.95 ± 0.3871 b	5.672 ± 1.766a

*The columns sharing a common letter within fungus are not significant at $P < 0.05$ (FC- Free Cell; IC- Immobilized Cell).

Statistical analysis revealed that in case of *A. tubingensis*, soluble P level in the first batch was found to be significantly lower in case of encapsulated cells. In the next two batches, no significant difference was observed in the level of soluble P on the basis of mode of biocatalyst application. The level of soluble P observed in second, third batches in free as well immobilized cells was almost comparable. In the fourth batch, a significant decrease in soluble P level was observed in case of immobilized cells (Table 2).

In case of *A. niger*, over the first three batches, we observed considerable difference in the level of soluble P between free and encapsulated cells. Soluble P was found to be higher in case of free cells as compared to immobilized cells. While in the fourth batch, no significant difference could be observed in terms of the level of soluble P (Table 2).

Bead destruction and cell outgrowth was observed in the course of time in both the strains. The destruction of alginate was not surprising bearing in mind that the phosphate ions even at concentration of 0.015% were reported to provoke destruction of alginates. Vassileva et al. (2000) evaluated free and encapsulated yeast cells of *Yarrowia lipolytica* in terms of citric acid production. They also reported alginate to be an unsuitable carrier when rock phosphate was supplied to the medium solution.

Level of solubilization by the cells was found to decrease over the batches in case of both immobilized and free cells. This might be due to the mechanical (abrasion) effect of the RP particles on the surface of culture. Vassilev et al., (1996, 1997a); Vassileva et al., (1998, 2000) concluded from biomass measurement that a part of soluble P was obviously consumed by the immobilized cells. Martin & Steel (1955) reported that the presence of more than 0.07 g PO₄⁻/ l in a submerged fermentation process with freely suspended cells was reported to reduce acid formation and to stimulate the mycelium growth.

Another explanation for reduced soluble P levels in case of encapsulated fungus as compared to free cells could be the limitation of activity caused by reduced oxygen transfer into the gel carrier. Such limitation could be overcome by the preparation of microbeads, that is, by decreasing the size of the beads and thereby increasing the surface area. Further, the addition of sources of nutrients such as skim milk and protective materials may enhance the stability and provide a protective and nutrient source. Clay and skim milk powder are among the most applied amendments for gel entrapped soil microbial inoculants (Jung et al., 1982; Van Elsas et al. 1992).

4.6 Nursery Experiment:

In order to determine the potential application of our strains as biofertilizers along with RP amendment as an important aspect of sustainable agriculture, the following experiment was designed.

Two sets of soil samples were taken, one of which was sterilized in order to study the effect of our test cultures alone. The other set was to study the efficacy of our test culture in natural conditions, and to understand the interactions with microbial community in the native ecosystems. Different treatments were undertaken in each set of soil sample, including amendment of rock phosphates with and without inoculation with *A. niger*, *A. tubingensis*. Destructive samplings were made after 45 days of plant growth and various growth parameters were recorded. Six replicates were maintained for each treatment.

The data was analyzed using two-way analysis of variance (ANOVA) for the soil types and the treatments. The various treatments within each soil type were analyzed for significant difference by one way-ANOVA.

Maximum shoot dry weight was observed in case of RP amended sterile soil inoculated with *A. tubingensis*, followed by un-amended soil with *A. tubingensis* as inoculant. Shoot dry weight observed in case of plants sown on RP amended soil inoculated with *A. tubingensis* was significantly higher in comparison to the soil where *A. niger* was used as microbial inoculant (Table 3). By two-way analysis of variance, we found no significant variation in shoot dry weight of sterile and unsterile soil (Table 3).

In case of unsterile soil, maximum shoot weight was observed in case of RP amended soils inoculated with *A. tubingensis*, *A. niger*. Significant increase in shoot biomass was observed in case of unsterile soil amended with or without RP, but inoculated with either of the strains (Table 4).

This directly reflects the effect of the test fungus on shoot biomass with and without RP amendment, indicating huge reserves of fixed phosphates added in the form of chemical fertilizers and precipitated, concluding soil to be a rich reservoir and the potential of microbial inoculants as mini-fertilizer factories.

Table 4-Two way ANOVA table showing the variation between soil types and treatments on shoot dry weight.

Source of Variation	Df	Sum of Squares	Mean sum of Square	F value	P value
Interaction	5	1.855	0.3709	3.055	P<0.05*
Soil type	1	0.1287	0.1287	1.06	ns
Treatment	5	1.053	0.2107	1.735	ns
Residual	36	4.371	0.1214		

By two-way analysis of variance, root dry weight was observed to be significantly different between sterile and unsterile soils (Table 3). The maximum root biomass was observed in case of sterile soil inoculated with *A. tubingensis*. Maximum root biomass was observed in case of non sterile soils, inoculated with *A. niger* (Table 5). As even high-quality rock phosphate has a relatively low solubility compared to soluble phosphate fertilizers, immediate effects of applied RP on maize yield on non-acid soils are very often not visible.

In case of shoot height, significant differences were observed in between the various treatments undertaken for each soil set. However, no significant difference could be established in shoot height parameter for the set of soil samples (Table 6). Significantly higher shoot height was observed in case of RP amended sterile soil inoculated with *A. tubingensis*. Highest shoot height was observed in plants grown in soils amended with rock phosphate, and test cultures, followed by plants amended with rock phosphate without addition of microbial inoculants and lowest height was noted in case of control.

Table 5-Two way ANOVA table showing the variation between soil types and treatments on root dry weight.

Source of Variation	Df	Sum of Squares	Mean sum of Squares	F value	P value
Interaction	5	0.2439	0.04878	0.3906	ns
Soil type	1	2.027	2.027	16.23	P<0.001***
Treatment	5	0.6394	0.1279	1.024	ns
Residual	36	4.497	0.1249		

Higher shoot height was observed in case of unsterile soils amended with rock phosphate inoculated with our strains (. The data reflects the potential of the phosphate solubilizing fungus in mobilizing large reservoirs of fixed phosphate deposits in the agricultural soils. As phosphorus is known to initiate cell division and enlargement processes, the increase in shoot height could be probably due to increased mobilization of phosphorus rendered soluble by our test organisms from soil reserves and Rock Phosphates.

Table 5. Two way ANOVA showing the variation between soil types and treatments on shoot height.

Source of Variation	Df	Sum of Squares	Mean sum of Squares	F value	P value
Interaction	5	70.36	14.07	6.128	P<0.001
Soil type	1	7.208	7.208	3.138	ns
Treatment	5	216.8	43.36	18.88	P<0.001
Residual	36	82.68	2.297		

From these results, it is clear that both these isolates, namely, *Aspergillus tubingensis* and *A. niger* are able to improve the growth of maize seedlings when grown in presence of Rock Phosphate and also in non amended soils.

SUMMARY

Phosphorus is an essential nutrient limiting plant growth. Despite of its wide distribution, it is deficient in most soils, owing to high P-fixation capacities of soils. Regular addition of this element in bioavailable forms is, thus, a prerequisite for maintaining and enhancing crop yields. Rock Phosphate is theoretically the cheapest fertilizer and most abundant, but its direct application in the soils is not always agronomically effective due to its low reactivity. An environmentally acceptable and economically sound alternative to traditional processing of these deposits is the use of Phosphate Solubilizing Microorganisms (PSMs).

The focus of the present study was to use Rock Phosphate and Phosphate Solubilizing Microorganisms in consortium as a source of P-nutrient for crops. Preliminary experiments were performed in order to characterize the phosphate solubilizing activity using rock phosphates as the sole source of P-nutrient to *Aspergillus tubingensis*, *A. niger* in laboratory conditions. An attempt was made to understand the underlying mechanisms of phosphate solubilization by the test cultures. The observations could be of significance while inoculating soils for enhanced crop yields with the test cultures.

Four days of incubation were found to be optimum for maximal activity of both the strains in terms of solubilization of phosphorus. When grown in the presence of varying amounts of Rock Phosphate, TriCalcium Phosphate. The results clearly indicated maximum soluble P level in the case when minimum amounts of phosphate bearing material was added. Maximum acid phosphatase activity was also found to be corresponding to the same concentration. We found that *Aspergillus tubingensis* performed better in fermentation conditions than *A. niger* in terms of soluble P.

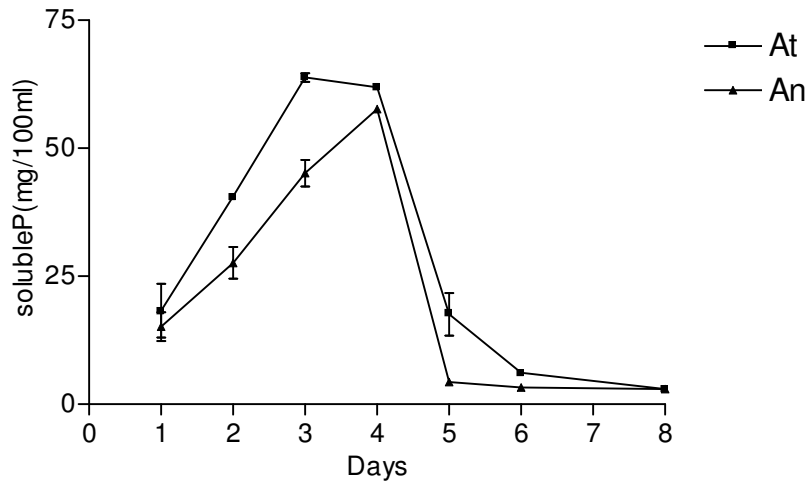
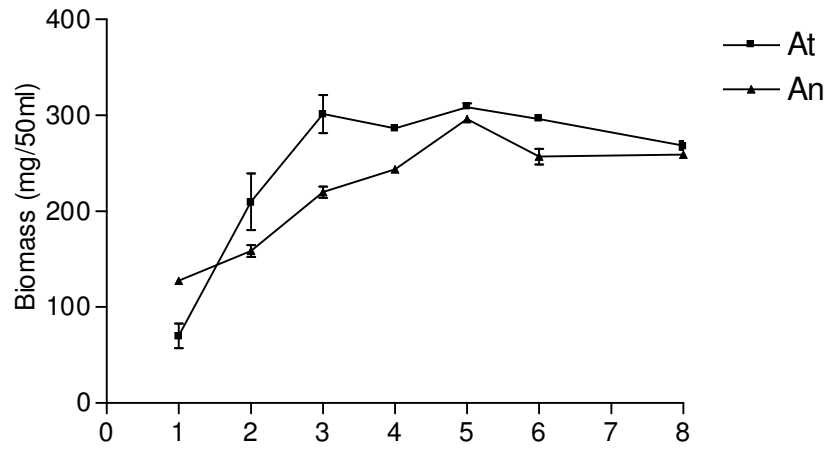
One to one correspondences were observed in the concentration of soluble P, acid phosphatase activity. Acid phosphatase enzyme was found to be highly

inducible, induced in response to stressful conditions, that is, lower levels of soluble P.

Increasing levels of soluble P were observed on increasing the concentration of chelator in the control treatments. However, chelator in the presence of test organism decreased the level of soluble P. All these observations lead to a conclusion that high soluble P levels inhibited further phosphate solubilization. Action of chelators, acid phosphatase activity and low pH were confirmed to be the major players in phosphate solubilization.

The principle of manipulation of microbial populations, including microorganisms related to plant P nutrition, can be applied as a promising practice. In the plantation experiments, significant improvements in terms of shoot biomass, shoot height were observed in case of soils amended with rock phosphates, with the test cultures over the control. The observations can direct us to a point that maximum effect of these phosphate solubilizers would be most remarkably observed in case of P-deficient soils. Further, the application of phosphatic fertilizers or superphosphates would decrease the activity of the test cultures. The following experiments proved *Aspergillus tubingensis* to be more efficient in both fermentation and soil plant experiments than *A. niger*. However once introduced into the soil, they behave entirely differently depending on the biotic and abiotic factors of the soil ecosystem. More research needs to be focussed on the performance of these organisms in soil ecosystems, where they have to be potentially inoculated. It has also been reported that growth of introduced suspended cells in soils, characterized by unaffected microbial activity, is a rare phenomenon.

ANNEXURE



Biomass proliferation, solubilization kinetics of *Aspergillus tubingensis* (At), *Aspergillus niger* (An)

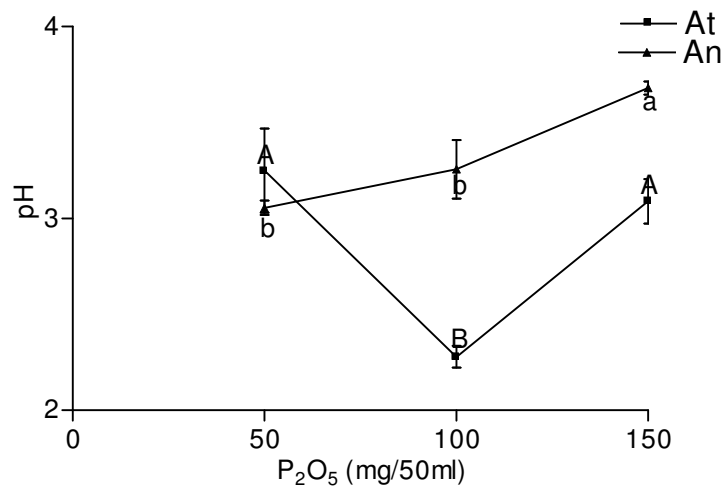
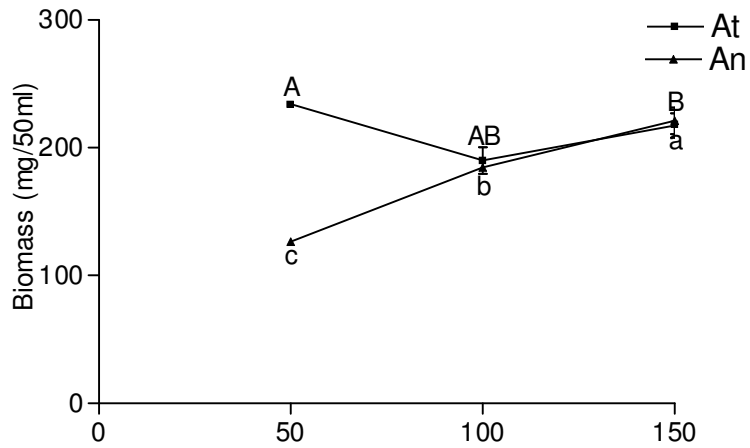


Fig.2(a, b) Effect of varying Ca₃(PO₄)₂ on biomass and pH of *Aspergillus tubingensis*(At), *Aspergillus niger*(An).

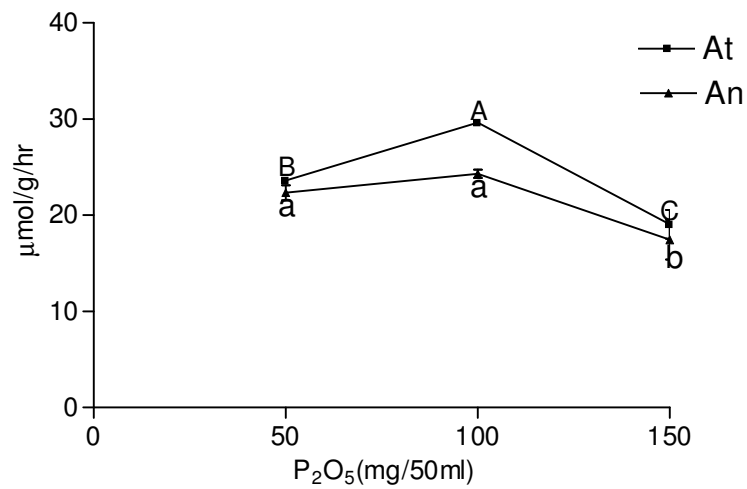
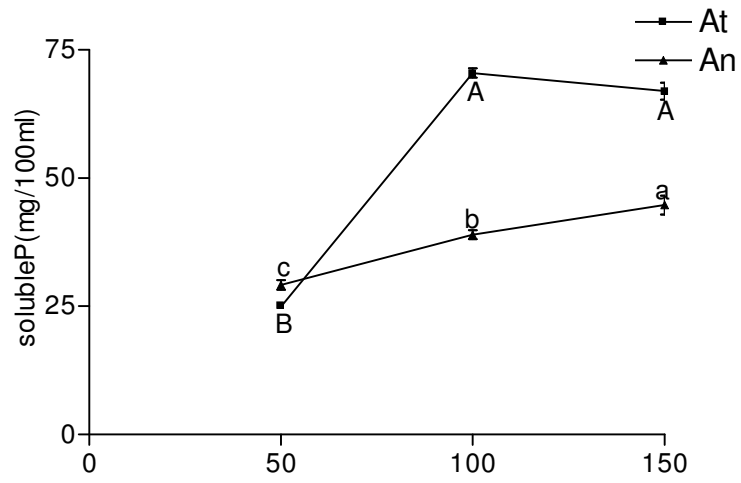


Fig3(a, b) Effect of varying $\text{Ca}_3(\text{PO}_4)_2$ on soluble P and acid phosphatase activity of At, An.

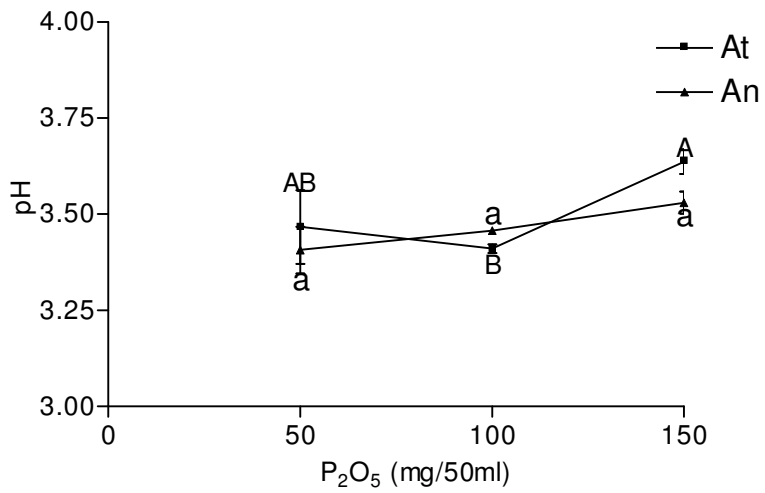
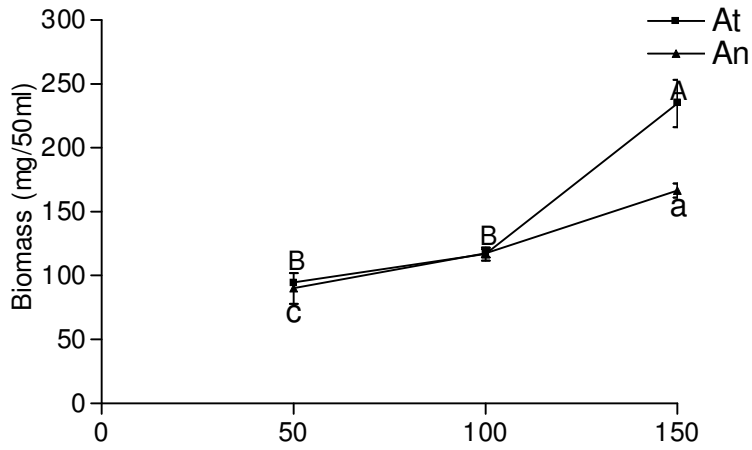


Fig.4(a, b) Effect of varying Rock phosphate on biomass and pH of At, An

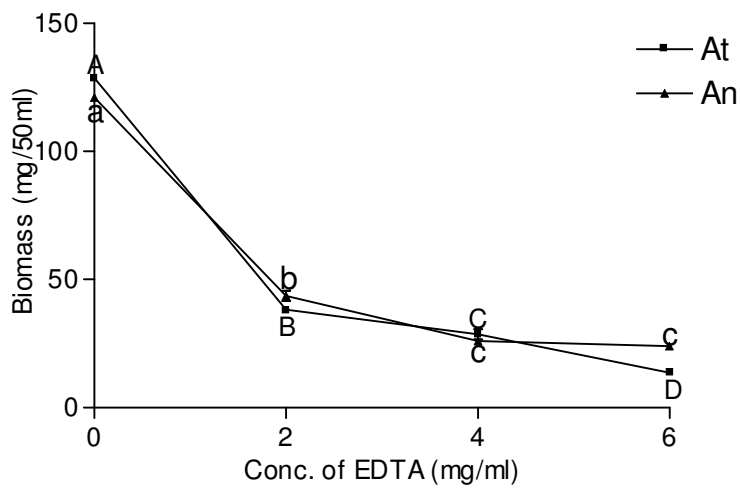
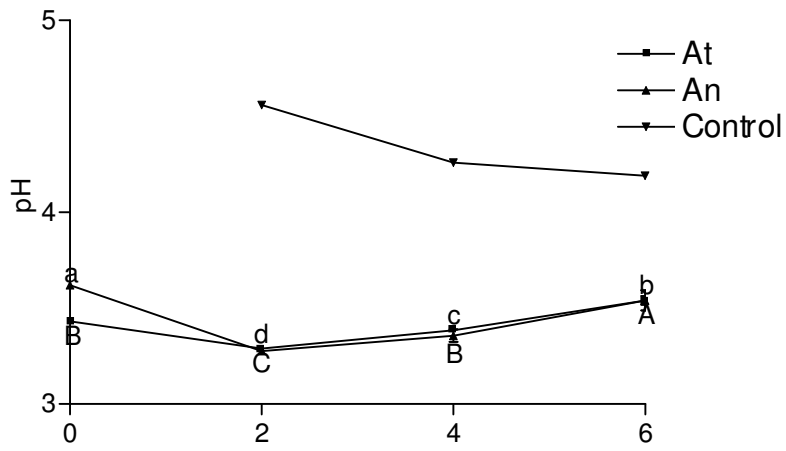


Fig.6(a, b) Effect of varying EDTA conc. on pH, Biomass of *Aspergillus tubingensis*(At), *Aspergillus niger*(An)

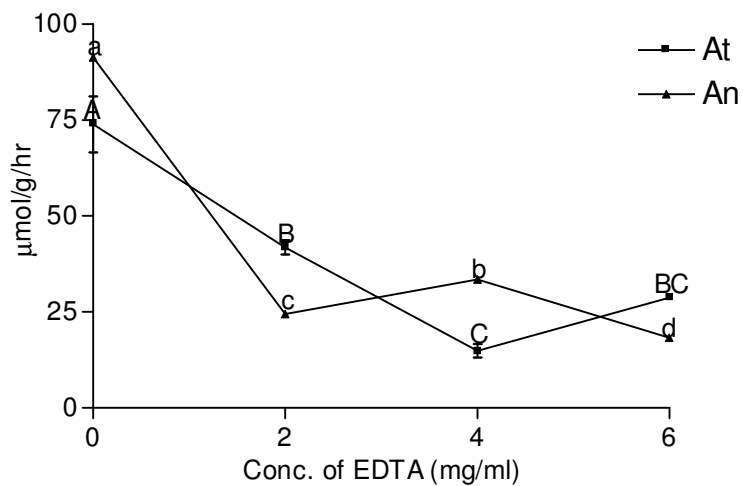
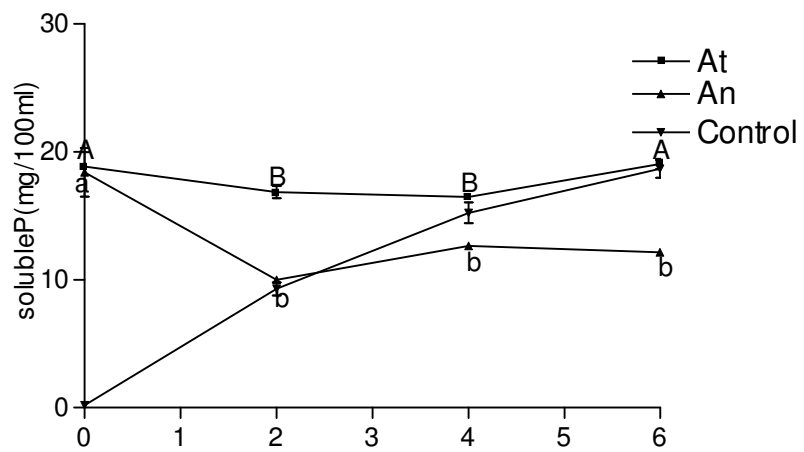


Fig.7(a, b) Effect of chelator conc. on soluble P level, acid phosphatase activity of *Aspergillus tubingensis*(At), *Aspergillus niger*

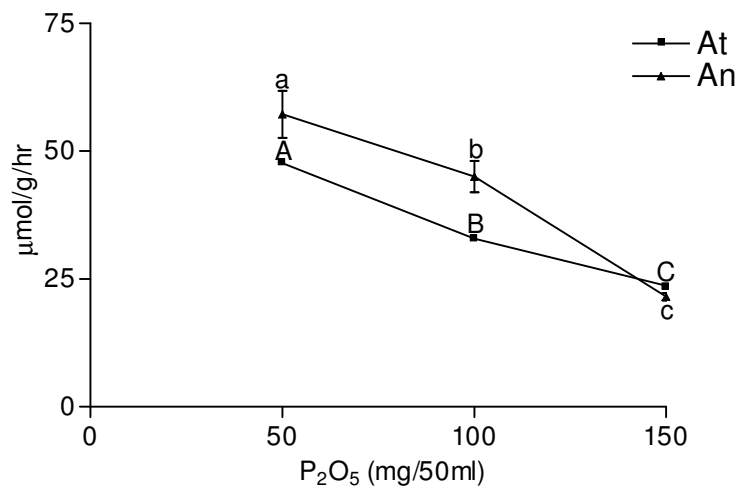
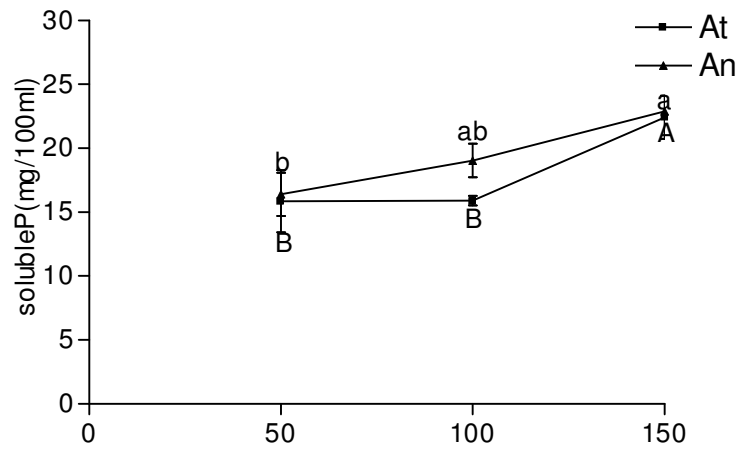


Fig.5(a, b) Effect of varying rock phosphate on soluble P and acid phosphatase activity of At, An.

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