Enhancement of Fertilizer Value of Phosphate Rocks by Phosphate Solubilizing Microbes

A Thesis

Submitted in fulfillment of

the requirements for the award of degree of

DOCTOR OF PHIOLSOPHY

IN

BIOTECHNOLOGY

Himani Singh

Roll. No. 9051003

Department of Biotechnology and Environmental Sciences

Thapar University

PATIALA - 147 004, INDIA

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CERTIFICATE

Certified that the thesis **"Enhancement of fertilizer value of phosphate rocks through phosphate solubilizing microbes"** which is submitted by Miss Himani Singh, in fulfillment of the requirement for the award of the degree of **DOCTOR OF PHILOSOPHY** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is a record of the candidate's own independent and original research work carried out by him under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or institute for the award of any degree.

M. Sudhakar

(Dr. M. Sudhakara Reddy)
Supervisor
Professor
DBTES, Thapar University,
Patiala- 147004

M. Sudhakar

(Dr. M. Sudhakara Reddy)
Head,
DBTES, Thapar University
Patiala- 147004

DECLARATION

I hereby declare that the work which is being presented in this thesis **“Enhancement of fertilizer value of phosphate rocks through phosphate solubilizing microbes”** submitted by me for the award of the degree of **DOCTOR OF PHILOSOPHY** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of Prof. M. Sudhakara Reddy, Head, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or abroad.

Date:

Place: **(Himani Singh)**

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Dedicated to my mother

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Conferences:

- **Himani Singh** and M. Sudhakara Reddy (2010). Solubilization of metal phosphates by fungi isolated from mine landfills of rock phosphate. National conference on "**Emerging Trends in Biopharmaceuticals: Relevance to Human Health**" and 4th Annual Convention of Biotechnology and Pharmacy. Department of Biotechnology and Environmental sciences, TIFAC-CORE, Thapar University

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Abbreviations

bp	Base pair
BLAST	Basic local alignment search tool
CTAB	Cetyltrimethyl ammonium bromide
CFU	Colony forming Units
DNA	Deoxyribonucleic acid
dNTP	2’-deoxynucleoside-5’-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
g	Gram
h	Hours
ha	Hectare
IPTG	Isopropyl-β-thiogalactoside
kb	Kilo base
l	Litre
mg	Milligram
mg/kg (ppm)	Milligram per kilogram (parts per million)
ml	Milliliter
NCBI	National centre for biotechnology and information
P	Phosphate
PCR	Polymerase chain reaction
PSMs	Phosphate solubilizing microorganisms
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid
RRP	Rajasthan rock phosphate
TCP	Tricalcium phosphate
Tris	Tris-(hydroxymethyl-) aminomethane
µg	Microgram
µl	Microlitre
mS cm^{-1}	Millisiemens per cm
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

Chapter 1

Introduction

INTRODUCTION

Phosphorus

Phosphorus (P) is one of the major nutrients, plants require to thrive. It functions as one of the major players in the process of photosynthesis, nutrient transport, energy transfer, early growth and root formation, cell division, DNA and RNA formation, flower blooms, seed development, improvement in plant strength and to tolerance for unfavorable environmental conditions. Phosphorus also affects the plant's structure at a cellular level. A plant with the proper amount of phosphorus available to it will grow more vigorously and mature earlier than plants with inadequate phosphorus. A plant with phosphors deficiency will exhibit stunted growth, lack of fruit or flowers, wilting and leaves may be greener or have a purple cast to them due to the photosynthetic process being affected (CFAITC 2009).

Phosphate is found to be present in form of phosphatic compounds like: Mineral forms- (oxyapatite, hydroxyapatite, apatite [Gen. formula $Ca_{10}(PO_4,CO_3)_6(F,OH,Cl)_2$]); Organic forms- (phytins, phospholipids, nucleic acids, inositol phosphate, soil phytate, etc.); Phosphatic rock - ($Ca_{10}(PO_4)_6F_2$). Natural rock mined from P rich deposits contains 14-37% P_2O_5 and are rich in minor elements such as B, Zn, Ni, I; Soluble phosphorus - sometimes called available inorganic phosphorus. It can include small amounts of organic phosphorus, as well as orthophosphate, the form taken up by plants (ibm.nic.in/apatiteandrockphosphate.pdf).

Despite its wide distribution in nature it is a deficient nutrient in most soils and is not found by itself in elemental form. In most of the soil its content is about 0.05 % of which only 0.1% is plant available rest of which is fixed in insoluble forms (Vassilev and Vassileva 2003) (Fig. 1.1).

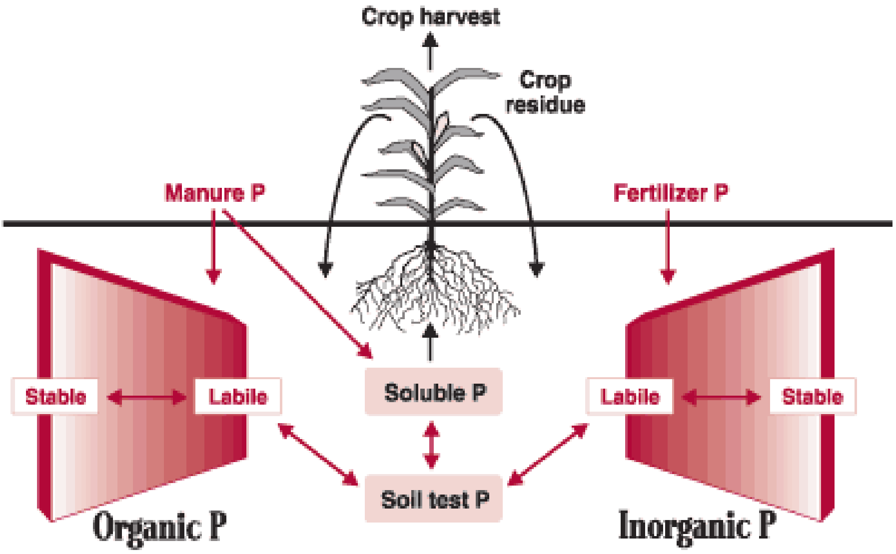


Figure 1.1: The phosphorus cycle. (Source: Livestock and Poultry Environmental Stewardship Curriculum)

Elemental P is extremely reactive and combines with oxygen when exposed to the air. In natural systems like soil and water, P exists as phosphate, a chemical form in which each P atom is surrounded by 4 oxygen (O) atoms. Orthophosphate, the simplest phosphate, has the chemical formula PO_4^{-3}. In water, orthophosphate mostly exists as $H_2PO_4^-$ in acidic conditions or as HPO_4^{2-} in alkaline conditions and plants most often absorb phosphorus in this form only (Rehm et al. 1998). Phosphorus is symbolized as P_2O_5 on fertilizer labels. Organic fertilizers, such as manure, contain phosphorus in limited quantities (CFAITC 2009). Many soils are defined as high P-fixation capacity (Fig. 1.2) since a substantial amount of any P fertilizer is rendered unavailable and frequent application of soluble forms of inorganic phosphate needed to maintain adequate P levels for plant growth (Vassilev et al. 2001).

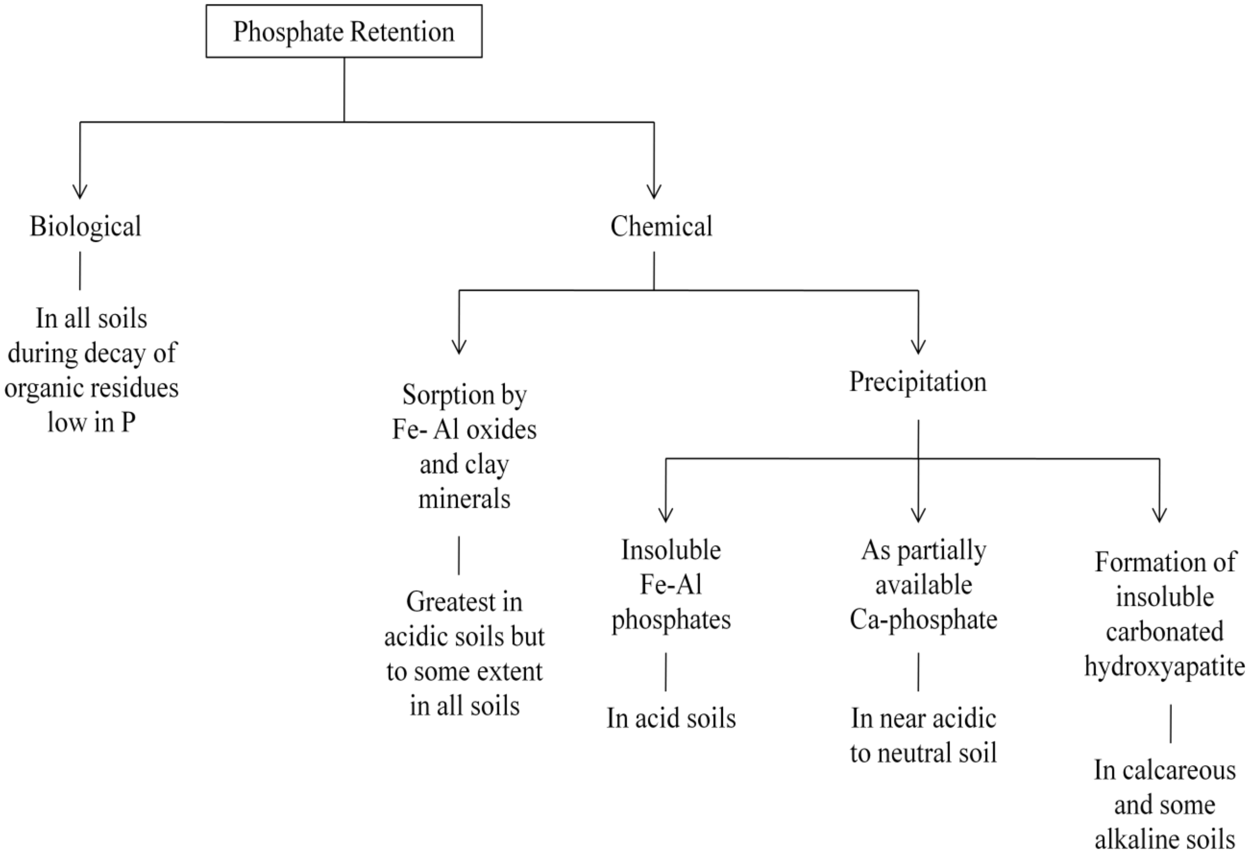


Figure 1.2: Phosphate fixation reaction in soil (Source Sauchelli 1951)

Acidic soil with pH below 5 or alkaline soil with pH higher than 7.3, both have negative effects on phosphorus. In acidic soils, P forms iron/aluminium (Fe/Al) phosphates and gets adsorbed to Fe/Al oxides or humic substances (Fig. 1.3). In alkaline or calcareous soils, P is often precipitated as calcium (Ca)-P. Organic P (mainly phytate) may represent more than 50% of total P in many soils (Osborne and Rengel 2002).

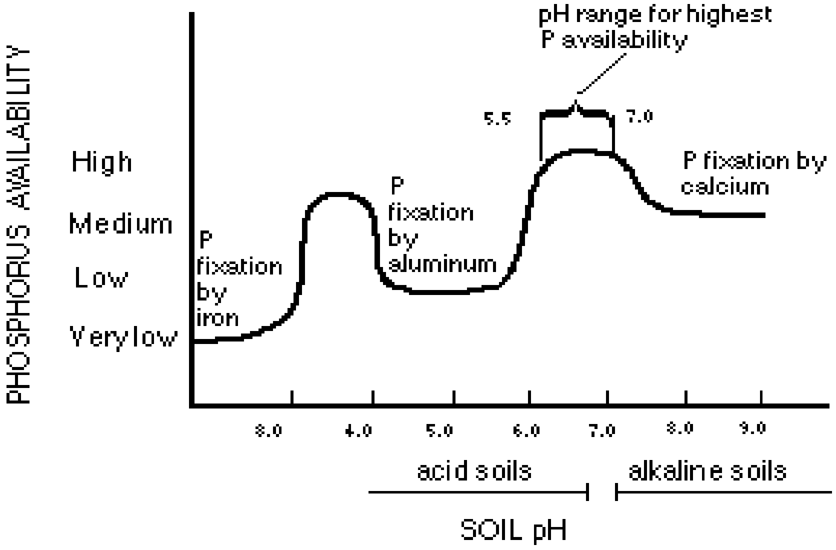


Figure 1.3: The availability of phosphorus is affected by soil pH (Source Busman et al. 2009)

Rock phosphate (Phosphorite)

Phosphate rocks should provide a cheap source of phosphorus fertilizer for agricultural use. India alone has about 260 million tons of rock phosphate (RP) deposits and this material should provide a cheap source of phosphate fertilizer for crop production (FAI 2002). Out of total resources, 35% are in Jharkhand, 31% in Rajasthan, 17% in Madhya Pradesh, 9% in Uttar Pradesh and 8% in Uttarakhand.

Unfortunately, rock phosphate is not plant available in soils with a pH greater than 5.5 - 6.0. Most RPs have low reactivity in alkaline and neutral soils and therefore its direct application is not always effective without previous treatment (Vassilev e al. 2001). Due to low availability of P in this native material, high transportation costs, and small crop responses, very little rock phosphate is currently used in agriculture (Rehm et al. 2010)

Rock phosphate is the raw material used in the manufacture of most commercial phosphate fertilizers in the market. One traditional treatment is adding acid to ground or pulverized phosphate rock (Rehm 2010). The manufacture of most commercial phosphate fertilizers begins with the production of phosphoric acid. Phosphoric acid is prepared commercially by heating calcium phosphate rock with sulfuric acid; purer grades may be prepared by treating red phosphorus with nitric acid. If sulfuric acid is used, single or normal, phosphate (SSP) is produced, with a phosphorus content of 16–21% as phosphorus penta oxide (P_2O_5). If phosphoric acid is used to acidulate the phosphate rock, triple phosphate (TSP) is the result. TSP has a phosphorus content of 43–48% as P_2O_5 (Pollution Prevention and Abatement Handbook, World Bank Group). These fertilizers are of high cost and the production causes environmental pollution.

Phosphate solubilizing microorganisms

For a sustainable agriculture system, it is imperative to utilize renewable inputs, which can maximize the ecological benefits and minimize the environmental hazards. Microbial solubilization of rock phosphate and its use in agriculture is receiving greater attention. Phosphate solubilizing microorganisms (PSMs) can be useful to reverse the process of phosphate fixation. PSMs is a group of heterotrophic microorganisms capable of solubilizing inorganic P from insoluble sources through the process of acidification, chelation and exchange reaction (Gerke 1992).

Phosphate solubilizing microorganisms are ubiquitous and their numbers vary from soil to soil. In soil, P solubilizing bacteria constitute 1–50% and fungi 0.5–0.1% of the total respective population. In general, P solubilizing bacteria generally outnumber P solubilizing fungi by 2–150 folds (Banik and Dey 1982; Kucey 1983; Kucey et al. 1989).

These PSMs include the following bacteria, fungi and yeast. They are:

Bacteria: *Bacillus megaterium, B. circulans, B. subtilis, Pseudomonas straita, P. rathonis;*

Fungi: *Aspergillus awamori, Aspergillus niger, Aspergillus tubingensis Penicillium digitatum, Penicillium rugulosum, Trichoderma sp.*

Yeast: *Schwanniomyces occidentails*

Mainly the solubilization of P by these microorganisms is attributed to excretion of organic acids like citric acid, glutamic acid, succinic acid, lactic acid, oxalic acid, glyoxalic acid, malic acid, fumaric acid, tartaric acid and ketobutyric acid (Hegde 1998; Reyes et al. 2006). The role of organic acids in dissolving mineral phosphates and phosphorylated minerals can be attributed to the lowering of pH which helps in formation of stable complexes with such cations as Ca^{++}, Mg^{++}, Fe^{+++} and Al^{+++}. These complexes are more stable than original inorganic phosphate compounds (Struthers and Sieling 1950; Dalton et al. 1952, Bradley and Sieling 1953; Mortensen 1963). The reaction of organic acid with calcium phosphate is one of acid dissolution, the amount of phosphate released depends upon the strength of acid. These acids form unionized association compounds with Ca^{++}, thus removing it from solution and soluble phosphate concentration is increased. Ca^{++} is also chelated to small extent with alpha hydroxy aliphatic acid (most strongly with tribasic like citric acid) (Johnston and Miller1959).

Phosphate solubilization is a complex phenomenon, which depends on many factors such as the nutritional, physiological, and growth conditions of the cultures (Cunningham and Kuiack 1992). Since PSMs are heterotrophs and solubilize insoluble phosphates by secreting organic acids, the role of carbon in this context is very important.

Apart from carbon, nitrogen is also an important element needed by microorganisms for synthesis of amino acids, proteins, purines and pyrimidine nitrogenous bases. It may be taken in the form of ammonia, nitrate, and nitrite or in amino form (Nahas 1996; Reyes et al. 1999b; Whitelaw et al. 1999; Seshadri 2004).

There are two components of P in soil, organic and inorganic phosphates. Mineralization of phosphate from soil organic P by phosphatase enzymes is of particular significance, as organic P accounts for a major proportion (generally 40 to 80%) of the total P in most soils, occurring primarily as inositol phosphates (Turner et al. 2002). Phosphorus can be released from organic compounds in soil by three groups of enzymes: (1) Nonspecific phosphatases, which perform dephosphorylation of phospho-ester or phosphoanhydride bonds in organic matter, (2) Phytases, which specifically cause P release from phytic acid and (3) Phosphonatases and C–P Lyases, enzymes that perform C–P cleavage in organophosphonates. The main activity apparently corresponds to the work of acid phosphatases and phytases because of the predominant presence of their substrates in soil (Rodriguez et al. 2006). These include changes to root structure, association with mycorrhizal fungi and other soil microorganisms and biochemical processes at the root-soil interface (Jakobsen et al. 2005).

The P solubilizing microorganisms also produce fungistatic and growth-promoting substances, which influence plant growth. The performance of these microorganisms is affected by availability of a carbon source, P concentration, type of phosphate, particle size of phosphate and other factors like temperature and moisture (Hegde 1998).

Solubilization of metal phosphates

Another important aspect of P solubilizing fungi are able to convert insoluble metal containing minerals and other compounds, e.g. certain oxides and phosphates, into soluble forms resulting in release of phosphates, other nutrients and metal cations into biogeochemical cycles (Jacobs et al. 2002b; Burgstaller and Schinner 1993). Elevated concentrations of heavy metals in soil from anthropogenic sources (fertilizers and amendments) or mining activities (Shetty et al. 1995) pose long-term risk to environmental and sustainable production. The deteriorated physical and biological characteristics of contaminated soils need to be improved to establish a vegetation cover.

Heavy metal-contaminated soil can be treated by operations based on chemical, physical or biological techniques. It is generally accepted that these remediation techniques may be grouped into two main categories: (1) *ex situ* techniques which are based on mechanical removal of the contaminated soil and subsequent disposal to a landfill site for further treatment and (2) *in situ* methods, which avoid excavation, transportation, and land-filling costs by treating the metal contaminated soil in place. *In situ* techniques are easy to perform, favored over the *ex situ* techniques, and preferred due to their lower cost and reduced impact on the ecosystem (Khan et al. 2004; Kumpiene et al. 2008)

Phosphate solubilizing fungi respond to heavy metals stress via processes such as transport across the cell membrane, biosorption to cell walls, entrapment in extracellular capsules, as well as precipitation and transformation of metals (Malik 2004). Metals and their compounds interact with fungi in soil and in culture medium in a variety of ways depending on the metal species, organism and environmental conditions (Gadd 1993;

Sayer and Gadd 2001; Nahas et al. 1990). Many filamentous fungi, particularly black *Aspergilli* (Reddy et al. 2002) can produce organic acids which supply both H^+ and a metal complexing anion and mediate release of mobile phosphate and metal species from insoluble sources (Gadd 1999). In addition, the organic acid anion is usually capable of forming a complex with the metal cation, thus affecting its mobility and toxicity. Acid phosphatases and phytases secreted by these microorganisms also have an important role in phosphate solubilization (Richardson et al. 2001). Most of these enzymes require divalent metal ions to manifest activity but above an optimal concentration, inhibition is observed, which depends on the nature of the metal. Phytate chelates with the calcium, magnesium, iron, cobalt and copper very strongly to form insoluble metal-phytate complexes (Gifford and Clydesdale 1990). These phytates should be dephosphorylated by phytases/acid phosphatases before assimilation (Satyanarayana and Singh 2009).

Zinc deficiency is plant's most common crop micronutrient deficiency in high-pH soils. Zinc-deficient soil is cultivated in the cropland of about half of India (Broadley 2007). Cobalt phosphate (Sayer et al. 1995) has been found to be less recalcitrant, therefore suitable for use in solubilization experiments. Aluminum and iron, natural constituent of soil, mostly get fixed to aluminum and iron phosphates in acidic soils when phosphorus fertilizer is applied. Copper phosphate is of concern because copper is both, an essential cofactor and a toxic element for plants (Gadd 2001).

Mycotoxins

Mycotoxins are toxic compounds produced by certain fungi under specific conditions. Among various mycotoxins, aflatoxins have assumed significance due to their deleterious effects on human beings, poultry and livestock. Grain is susceptible to aflatoxins produced either while the crop is growing by fungal species or during storage. Among 18 different types of aflatoxins identified, major members are aflatoxin B1, B2, G1 and G2 (Reddy and Waliyar 2002). It is very important to check the fungi for its deleterious effects on crop before recommending it to use as a bio fertilizer for enhancement of plant growth and nutrition.

Rhizospheric soil of phosphate mines

The functions of soil biota are central to decomposition processes and nutrient cycling. Soil is considered a storehouse of microbial activity, though the space occupied by living microorganisms is estimated to be less than 5% of the total space. Therefore, major microbial activity is confined to the ‘hot-spot’, i.e. aggregates with accumulated organic matter, rhizosphere (Lynch 1990; Pinton et al. 2001). Diversity and community structure in the rhizosphere is however influenced by both, plant and soil type (Latour 1996). Considerable higher concentration of PSMs is commonly found in the rhizosphere as compared to non-rhizospheric soil (Rodriguez and Fraga 1999). The microorganisms present in the phosphate rock mine landfills are well adapted to the higher concentration of the rock phosphates as well as harsh climate conditions.

Rhizosphere soil from *Jatropha curcas* plants growing in mine landfills of rock phosphate of Rajasthan State Mines and Minerals Limited (RSMML), Udaipur, India was collected for isolation of the P solubilizing microorganisms (Fig. 1.4).

Figure 1.4: Plantation of *Jatropha curcas* in landfill area of Rajasthan State Mines and Minerals Limited, Udaipur, India

Inoculation of PSMs

The PSMs occur in soil, usually their number are not high enough to compete with other microorganisms commonly established in the rhizosphere. Thus the amount of P liberated by them is generally not sufficient for a substantial increase of *in situ* plant growth. Therefore inoculation of plants by a target microorganism at a much higher concentration than the normal found in soil is necessary to take advantage of the property of phosphate solubilization for plant yield enhancement (Rodriguez and Fraga 1999). Inoculation of P solubilizing microorganisms in the soil amended with rock phosphates is a promising technique because it can increase P availability (Reyes et al. 2002) and improves the physio-chemical, biochemical and biological properties of rock phosphate amended soil (Caravaca et al. 2004). Many reports support the present study and had shown the improvement in plant growth using P solubilizing fungi (Whitelaw et al. 1997; Richa et al. 2007). An increase in P availability to plants through the inoculation of phosphate solubilizing bacteria has been reported in pot experiments and under field conditions (Pal 1998; Zaida et al. 2003).

The majority of the PSMs solubilize Ca–P complexes and only a few can solubilize Fe–P and Al–P (Banik and Dey 1983; Kucey et al. 1989). Hence, these PSMs could be effective in calcareous soils in which Ca-P complexes are present, but not in other soils in which phosphates are complexed with Fe and Al ions. So the use of phosphate solubilizing microorganisms in agriculture can not only compensate for higher cost of manufacturing fertilizers in industries but also mobilizes the fertilizers added to soil. PSMs will be a boon for the farmers where the soil pH is high.

Crops

Maize and wheat together account for more than half of the cereals consumed in the developing world. Among the food crops, after rice, wheat and maize are the most important commodities both in terms of calorie consumption and value of production. They are significant staples in the diets of billions of poor in the developing world and variations in productivity can profoundly influence their well-being. Today wheat and maize account for two thirds of all developing country food imports (CIMMYT 1998).

Over the next 30 years, it is expected that overall wheat and maize demand will continue to grow at an annual rate of 2.2%. The demand for feed maize is expected to raise substantially as a result of the rapid increase in demand for livestock products. Projections estimate that by 2020, 67% of world wheat consumption and 55% of world maize consumption will occur in developing countries (CIMMYT 1998).

Therefore, there is a demand on studies on these two crops, in order to improve the functional knowledge of compatibility of phosphate solubilizing microorganisms aiming at their co-inoculation to increase the nutrition and growth of plant species.

Aim of present study

It is estimated that the world demand of phosphorus as plant nutrient is more than 70 million tons of P_2O_5 per annum. To meet this demand huge investments are being made. The fertilizers are not only of high cost but have an adverse effect on the environment. In India only, 260 million tons of rock phosphate (RP) deposits are present. Rock phosphate as such is not available for plants in soils with a pH greater than 5.5-6.0 and even when conditions are optimal, yields are lower than those obtained with soluble phosphate (Reyes 2002). Because of this, extension services are reluctant to be recommended and farmers hesitate to utilize RP directly. Acidic soil could somewhat get benefitted by direct application of RP. In India, alkaline soils are present mostly in the drier parts of Gujarat, Rajasthan, Punjab, Haryana, U.P and Maharashtra.

There is strong evidence that PSMs increase the agronomic effectiveness of RPs (Barea et al. 2002). PSMs are commercially available in some countries (e.g. India), where large scale cultivation of PSMs is underway (by growing cultures in large flasks on rotary shakers or in batch fermentors). However, it is not evident whether these micro-organisms can increase the effectiveness of RPs under standard field conditions to such a magnitude that RP can be used as an alternative fertilizer. Field experiments need to be set up at specific sites in order to provide rigorous scientific testing of the practical usefulness of PSMs in enhancing the effectiveness of RPs, and thereby promoting their increased use. The present work is thus mainly focused on the study of P solubilizing microorganisms and assessment of their effect on the growth of wheat and maize plants in the soil fertilized with rock phosphate. The phosphate solubilizing microorganisms from rhizospheric soil of rock phosphate mine landfills were isolated as considerable higher concentration of PSMs is commonly found in the rhizosphere as compared to non-rhizospheric soil (Rodriguez and Fraga 1999) and the microorganisms present in the phosphate rocks landfills are well adapted to the higher concentration of the rock phosphates. The physiological aspects of the isolates were also been studied and most efficient isolates were executed for field trials using rock phosphate amended agricultural soil. Also examined the ability of these fungi to solubilize a variety of metal phosphates and attempted to get insight into the mechanism of solubilization.

OBJECTIVES:

- To study the diversity of phosphate solubilizers in rhizospheric soils in rock phosphate mine landfills
- To study physiology and characterization of the efficient phosphate solubilizers
- Enhancement of fertilizer value of rock phosphate through phosphate solubilizers

Chapter 2

Review of literature

REVIEW OF LITERATURE

Phosphorus (P)

Phosphorus (P) is a major growth-limiting nutrient, and unlike the case for nitrogen, there is no large atmospheric source that can be made biologically available (Ezawa et al. 2002). Root development, stalk and stem strength, flower and seed formation, crop maturity and production, N-fixation in legumes, crop quality, and resistance to plant diseases are the attributes associated with phosphorus nutrition. It is present at levels of 400–1200 mg kg^{-1} of soil (Fernández et al. 1988). The concentration of soluble P in soil is usually very low, normally at levels of 1 ppm or less (10 M $H_2PO_4^{-2}$) (Goldstein 1994) Its cycle in the biosphere can be described as ‘open’ or ‘sedimentary’, because there is no interchange with the atmosphere (Begon et al. 1990).

Soil phosphorus

Phosphorus deficiencies are limiting crop production in many agricultural soils worldwide despite its wide distribution in nature. Of total soil P, only 1 to 5% is in a soluble, plant-available form (Molla and Chowdhury 1984). This deficiency is a result of low inherent P fertility due to weathering, in combination with intensive, nutrient-extracting agricultural practices (Sanchez et al. 1997; Ssali et al. 1986). Additionally, phosphate diffusion to plant roots may be too low to meet the needs of the crop if soils have low P solubility and/or a high P fixation capacity (Gerke 1992; Hoberg et al. 2005; Sale and Mokwunye 1993). In regions where soils are high in total P, including some temperate soils, deficiencies may also occur if soluble forms of P are not replenished following plant uptake of P from soil solution. For example, organically managed soils have been found to become deficient in plant-available P over the long term without external P inputs (Entz et al. 2001; Oehl et al. 2002).

The soil P cycle is a dynamic process involving the transformation of P by geochemical (sorption-desorption) and biological (immobilization-mineralization) processes. Plant-available P occurs in the soil solution as orthophosphate anions, predominantly $H_2PO_4^-$ and $H_2PO_4^{-2}$. Solid inorganic and organic forms of P are found in labile and poorly soluble forms in the soil and as a result, can replenish plant-available P with varying degrees of effectiveness. Plant-available P or solution P, is in equilibrium with a relatively labile fraction of P that is adsorbed to aluminum or ferric hydrous oxides, clays, calcium carbonates and organic matter (i.e. is associated with the solid phase of the soil) (Pierzynski et al. 1994; Whitelaw 2000). As a result, solution P is easily replenished in response to plant uptake through desorption of P from the labile solid fraction (Cole et al. 1977; Whitelaw 2000). However, only a small fraction of P in the solid phase remains in a labile form, as it can become strongly adsorbed to the soil or participate in precipitation reactions. P may become strongly fixed and eventually precipitate as $AlPO_4.2H_2O$ and $FePO_4.2H_2O$ in acidic soils or with Ca^{2+} in alkaline soils. The effects of P precipitation are significant in acidic soils, where twice the amount of added P per unit surface area is fixed compared to neutral or calcareous soils (Whitelaw 2000).

The organic P pool generally constitutes 30 to 80% of the total soil P (Oberson et al. 1996) and represents a labile P fraction that may supply P to plants through mineralization by the microbial biomass (Stewart and Tiessen 1987). The microbial biomass is a small fraction of the total soil organic P, containing anywhere between 3 to

24% depending on cultivation (Brookes et al. 1984). However, it is significant in its role as recycler of P and as a relatively labile P source (Kwabiah et al. 2003).

Applied phosphate

Phosphate loss through crop removal can significantly reduce the soluble and labile P in the soil and decrease total soil P without external inputs. This leads to the need of frequent application of phosphate fertilizers, but its use on a regular basis has become a costly affair and also environmentally undesirable. Plants utilize fewer amounts of phosphatic fertilizers that are applied and the rest (70-90%) is rapidly converted into insoluble complexes in the soil (Vassilev and Vassileva 2003; Mikanova and Novakova 2002). Large amount of P applied as fertilizer enters in to the immobile pools through precipitation reaction with highly reactive Al^{3+} and Fe^{3+} in acidic and Ca^{2+} in calcareous or normal soils (Gyaneshwar et al. 2002; Hao et al. 2002). Efficiency of P fertilizer throughout the world is around 10-25% (Isherword 1998) and concentration of bioavailable P in soil is very low reaching the level of 1.0 mg kg^{-1} soil (Goldstein 1994). The addition of RP will increase total soil P with the potential to replenish labile P and plant available P. By utilizing plant and microbial mechanisms that can effectively extract P from RP and release it into the soil solution or into the labile fraction of the soil, RP resources may provide a viable alternative for P fertilization.

Rock phosphate (RP)

The optimal development of crops demands a high and 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. Natural phosphate rocks have been recognized as a valuable alternative for P fertilizers (Reddy et al. 2002). The beneficial effect of rock phosphate has made this material an attractive component for management in agriculture (Rajan et al. 1996). Phosphate rocks are the biggest reserves of phosphorus. Their principal characteristic is their insolubility, under appropriate conditions; they can be solubilized and become available for plants and microorganisms (Rodriguez and Fraga 1999). Therefore natural rock phosphates have been recognized as a valuable alternative source for P fertilizer, especially for acidic soils. There is a growing interest in ways of manipulating such rock phosphates to obtain a more valuable product. Common efforts include the use of chemico-physical means, that is, partially acidulating RPs reacting with synthetic organic acids and/or natural organic acids and decreasing particle size (Goenadi et al. 2000).

The P released from directly applied ground RP is often too low to provide sufficient P for crop uptake (Vassilev et al. 2001). Low-technology alternatives to the energy intensive and costly methods of conventional P fertilizer production have been proposed including enhancing plant and microbiological mechanisms that promote RP solubilization (Gyaneshwar et al. 2002; Richardson 2001; Trolove et al. 2003).

Solubilization of rock phosphate

Rock phosphate may originate from igneous, sedimentary, metamorphic and biogenic sources, with sedimentary being the most widespread. Forms of apatite, the primary P bearing mineral in RP, include fluorapatite, hydroxyapatite, carbonate-hydroxyapatite, and francolite (van Straaten 2002). In general, high carbonate-substituted forms of apatite (francolite) will solubilize more readily than pure forms of fluorapatite, releasing more P for plant use (Anderson et al. 1985). In addition to RP source, the major influences on RP solubility are soil properties, crop species and management practices (Chien and Menon

1995). These factors have various influences on the equilibrium of the dissolution reaction of a given apatite mineral. A simplified dissolution equation is shown in Equation 1.

$$Ca_{10}F_2(PO_4)_6 + 12H^+ \rightarrow 10Ca^{2+} + 6H_2PO_4^- + 2F^- \qquad (1)$$

Engelstad et al. (1974) found that the lower the soil pH, the more available the P from RP becomes. Moreover, since apatite dissolution releases Ca^{2+}, soils high in calcium do not support RP dissolution, in accordance with the mass action law (Chien and Menon 1995). Similarly, the dissolution of RP will be favored if Ca^{2+} is removed from soil solution (Flach et al. 1987). 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 Marwaha 1993).

A biological approach for extracting phosphate from RP was proposed as a less expensive and lower energy technique compared with the conventional processes (Goldstein and Rogers 1999; Khan et al. 2007). Various techniques for RP solubilization have been proposed, with increasing emphasis on application of P solubilizing microorganisms (Rodriguez and Fraga 1999; Whitelaw 2000; Vassilev et al. 2001; Vassilev and Vassileva 2003) employing low-grade RP which is often considered as a waste material from the mining industry.

Phosphate solubilizing microorganisms

Phosphate solubilizing microorganisms (PSMs) are ubiquitous in soils and play an important role in supplying P to plants in a more environmentally and sustainable manner (Gyaneshwar et al. 2002; Richardson 2001). Phosphate solubilizing microorganisms can increase plant availability of soil P by mediating the transformation and distribution of different P pools. Evidence of naturally occurring rhizospheric PSMs dates back to 1903 (Khan et al. 2007). These microorganisms enhance the solubility of rock phosphates by excreting organic acids such as citric acid and oxalic acid, thereby enhancing dissolution of sparingly soluble P sources, including Fe and Al phosphates and apatite, by means of acidification and complexation processes (He et al. 2002; Kpomblekou and Tabatabai 1994). According to Filho and Vidor (2000), the application of PSMs as inoculum or the management of their populations in soil is alternatives to improve P availability for plants.

Filamentous fungi, mainly *Aspergillus* and *Penicillium* are widely used to solubilize insoluble phosphates (Reyes et al. 2006). This ability is generally associated to the release of organic acids and thereby decrease in the pH (Relwani et al. 2008). The increase in soil weathering and the enhancement of nutrients availability in soil are also associated with the production of organic acids. The production and release of organic acids (mainly citric acid, oxalic acid, malic acid and gluconic acid) attribute to ion chelation and solubilization of inorganic P sources (Cunningham and Kuiack 1992; Gadagi 2007; Reyes et al. 2006).

Narsian and Patel (2000) observed that rock phosphate solubilization depends upon the nature of rock phosphate, the organism and the quality of organic acid secreted in the medium. Richa et al. (2007) and Narsian and Patel (2000) reported increased levels of soluble P with increase in RP concentration in the media by P solubilizing fungus. Vassilev et al. (2006) found reduction in the soluble P with increasing amount of RP in the medium. Bioactivation of poorly soluble rock phosphate was achieved by phosphate solubilizing fungi, a technique potentially applicable to activation of rock phosphate intended as raw material in production of phosphate fertilizers (Goenadi et al. 2000).

There are considerable higher concentrations of phosphate solubilizing bacteria in plant rhizospheres in comparison with nonrhizospheric soil (Rodriguez and Fraga 1999). Among the whole microbial population in soil, PSB constitute 1 to 50%, while phosphorus solubilizing fungi (PSF) are only 0.1 to 0.5% in P solubilization potential (Chen et al. 2006). Microorganisms involved in phosphorus acquisition include mycorrhizal fungi and PSMs (Fankem et al. 2006). Among the soil bacterial communities, ectorhizospheric strains from *Pseudomonas* and *Bacilli* and endosymbiotic rhizobia have been described as effective phosphate solubilizers (Igual et al. 2001). Strains from bacterial genera *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter* along with *Penicillium* and *Aspergillus* fungi are the most powerful P solubilizers (Whitelaw 2000). *Penicillium* spp. have been reported to exhibit traits such as mineral solubilization, biological control and are known to produce a wide range of secondary metabolites (Steven et al. 2004). *A. niger* and *A. tubingensis* have been reported as efficient organic acid producers and phosphate solubilizers (Reddy et al 2002). *Bacillus megaterium*, *B. circulans, B. subtilis, B. polymyxa, B. sircalmous, Pseudomonas striata* and *Enterobacter* could be referred as the most important strains (Subbarao 1988; Kucey et al. 1989). A nematofungus *Arthrobotrys oligospora* also has the ability to solubilize the phosphate rocks (Duponnois et al. 2006).

Although microbial inoculants are in use for improving soil fertility during the last century, however, a meager work has been reported on P solubilization compared to nitrogen fixation. Green chemistry and biotechnology are accepted as important tools in achieving sustainability. Their implementation, and the design of chemical products and microbial processes that reduce or eliminate the use and generation of hazardous substances and abundant waste materials, is an essential part of the efforts aimed at minimizing their negative impact (Kirchhoff 2005).

Bacteria

Phosphate solubilizing bacteria (PSB) are being used as biofertilizer since 1950s (Kudashev 1956; Krasilinikov 1957). Release of P by PSB from insoluble and fixed/adsorbed forms is an import aspect regarding P availability in soils. There are strong evidences that soil bacteria are capable of transforming soil P to the forms available to plant. Rhizobacteria, from the genera *Pseudomonas, Bacillus* and *Rhizobium*, are among the most powerful phosphate solubilizing bacteria (Rodriguez and Fraga 1999). Microbial biomass assimilates soluble P and prevents it from adsorption or fixation (Khan and Joergesen 2009). These bacteria in the presence of labile carbon serve as a sink for P by rapidly immobilizing it even in low P soils (Bunemann et al. 2004). Subsequently, PSB become a source of P to plants upon its release from their cells. The PSB and plant growth promoting rhizobacteria (PGPR) together could reduce P fertilizer application by 50 % without any significant reduction of crop yield (Jilani et al. 2007; Yazdani et al. 2009). It infers that PSB inoculants/biofertilizers hold great prospects for sustaining crop production with optimized P fertilization.

Fungi

Phosphorus absorption by plants can be increased by the presence of symbiotic organisms such as mycorrhizal fungi (Azcon-Aguilar et al. 1986) or by the inoculation with the soil mineral phosphate solubilizing fungi, particularly black *Aspergilli* (Vassilev et al. 1997;

Narsian and Patel 2000; Goenadi et al. 2000; Reddy et al. 2002) and some species of *Penicillium* (Asea et al. 1988; Cunningham and Kuiack 1992; Whitelaw et al. 1999). It has commonly been observed that filamentous fungi, predominantly *Penicillium* and *Aspergillus* species, have greater phosphate-solubilizing ability than many bacteria in both liquid and solid media (Banik and Dey 1982; Gaur et al. 1973; Kucey 1983). Contrary to this Alam et al. (2002) reported that bacteria are more effective in phosphorus solubilization than fungi. The black *Aspergilli include A. tubingensis, A. niger, A. awamori* and *A. aculateus.*

Mechanisms of phosphorus solubilization

Phosphorus solubilizing activity is determined by the ability of microorganisms to release metabolites such as organic acids, which through their hydroxyl and carboxyl groups chelate the cation bound to phosphate, the latter being converted to soluble forms (Sagoe et al. 1998). Phosphate solubilization takes place through various microbial processes/mechanisms including organic acid production and proton extrusion (Surange et al. 1995; Dutton and Evans 1996; Nahas 1996). Some bacterial species have mineralization and solubilization potential for organic and inorganic phosphorus, respectively (Hilda and Fraga 2000; Khiari and Parent 2005). General sketch of P solubilization by bacteria in soil is shown in figure 2.1. A wide range of microbial P solubilization mechanisms exist in nature and much of the global cycling of insoluble organic and inorganic soil phosphates is attributed to bacteria and fungi (Banik and Dey 1982). Phosphorus solubilization is carried out by a large number of saprophytic bacteria and fungi acting on sparingly soluble soil phosphates, mainly by chelation-mediated mechanisms (Whitelaw 2000). Inorganic P is solubilized by the action of organic and inorganic acids secreted by PSMs in which hydroxyl and carboxyl groups of acids chelate cations (Al, Fe, Ca) and decrease the pH in basic soils (Kpomblekou and Tabatabai 1994; Stevenson 2005). The PSMs dissolve the soil P through production of low molecular weight organic acids mainly gluconic and keto gluconic acids (Goldstein 1995; Deubel et al. 2000), in addition to lowering the pH of rhizosphere. The pH of rhizosphere is lowered through biotical production of proton/bicarbonate release (anion /cation balance) and gaseous (O_2/CO_2) exchanges. Phosphorus solubilization ability of PSMs has direct correlation with pH of the medium.

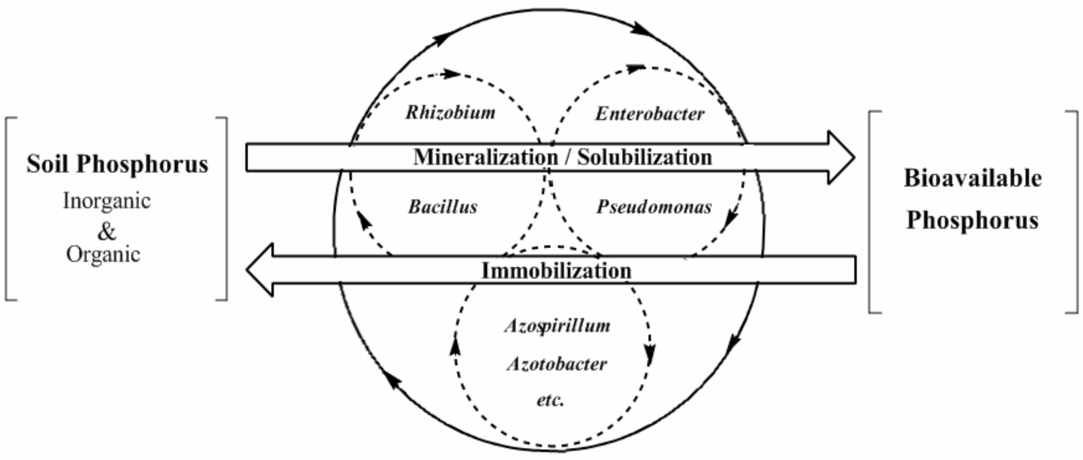


Figure 2.1: Schematic diagram of soil phosphorus mobilization and immobilization by bacteria (Khan et al. 2009).

Release of root exudates such as organic ligands can also alter the concentration of P in the soil solution (Hinsinger 2001). Organic acids produced by PSMs solubilize insoluble phosphates by lowering the pH, chelation of cations and competing with phosphate for adsorption sites in the soil (Nahas 1996). Inorganic acids e.g. hydrochloric acid can also solubilize phosphate but they are less effective compared to organic acids at the same pH

(Kim et al. 1997). In certain cases phosphate solubilization is induced by phosphate starvation (Gyaneshwar et al. 1999).

H^+ excretion and RP dissolution

Proton extrusion and organic acid production (Surange 1995; Lapeyrie et al. 1991; Nahas 1996; Dutton and Evans 1996) are the two important mechanisms for phosphate solubilization as it decreases the pH of the media which renders solubilization of inorganic phosphate. Microbial excretion of H^+ occurs in response to the assimilation of cations, primarily related to N source. It is a well-known fungal phenomenon that H^+ is excreted in exchange for NH_4^+ (Asea et al. 1988; Banik and Dey 1982; Beever and Burns 1980).

Organic acid production and RP dissolution

Microorganisms are known to produce organic acids in varying concentrations and types. Commonly reported organic acids produced by microorganisms include gluconic acid, citric acid and oxalic acids (Richardson 2001), while plants most commonly produce citric acid, oxalic acid and malic acid (Jones 1998; Ryan et al. 1995; Zheng et al. 2005). The ability of organic acids to solubilize phosphate rock is attributed to the following mechanisms: acidification, chelation and exchange reactions (Omar 1998).

Acidification

Organic acids contribute to the lowering of solution pH as they dissociate in a pH dependent equilibrium into their respective anion(s) and proton(s). As mentioned above, H^+ ions favor RP solubilization by shifting the equilibrium of the dissolution equation, subsequently releasing more P into solution. Organic acids buffer solution pH and will continue to dissociate as protons are consumed by the dissolution reaction (Welch et al.

2002). Although organic acids are present in soil, microorganisms often export organic acids as anions (Duro and Serrano 1981; Konings 1985; Netik et al. 1997), which must be actively transported across the plasma membrane (Stein 1967). The export of organic acid anions by fungi can occur by an H^+ symport transport system, causing acidification of the external solution (Netik et al. 1997). Hilda and Fraga (1999) reported that organic acid production, consequently the pH change and reduction potential are thought to be responsible for the dissolution of tricalcium phosphate in the culture medium. Akintokun et al. (2007) observed that a relationship seems to be established between the pH values, titratable acidity, the type of fungal species and solubilizing ability. This may imply that these parameters constitute the factors that are likely to affect solubilization of insoluble phosphates by soil fungi.

Chelation

In addition to pH reduction, organic acid anions can solubilize RP through chelation reactions. Chelation involves the formation of two or more coordinate bonds between an anionic or polar molecule and a cation, resulting in a ring structure complex (Whitelaw 2000). Organic acid anions, with oxygen containing hydroxyl and carboxyl groups, have the ability to form stable complexes with cations such as Ca^{2+}, Fe^{2+}, Fe^{3+}, and Al^{3+}, that are often bound with phosphate in poorly forms (Jones 1998; Kucey 1988). By complexing with cations on the mineral surface, organic acid anions weakens cation-oxygen bonds of the mineral structure and catalyze the release of cations to solution. Organic acid anions continue to alter the equilibrium of the dissolution reaction by complexing with cations in solution, effectively lowering the solution saturation point (Welch et al. 2002).

Results from a study by Kpomblekou and Tabatabai (1994) showed that the ability of organic acids to solubilize RP is affected by the chemical structure, as well as the type and position of functional groups of the organic ligand. These results are further supported by Narsian and Patel (2000), who tested the effects of known chelators including EDTA, DTPA, NTA, aluminon and oxine, on RP solubilization. This study concluded that RP solubilization was related to the functional groups of the chelators, particularly carboxylic and phenolic hydroxyls. The formation of complexes between chelator and cations such as Al^{3+} and Ca^{2+} depends on the number and kind of functional groups involved as well as the specific cation. It has been found that acids with an increased number of carboxyl groups are more effective at solubilizing RP (Xu et al. 2004; Kpomblekou and Tabatabai 1994). For example, Ca^{2+} was found to form complexes more readily with tricarboxylic acids such as citric acid, over dicarboxylic acids such as malic and tartaric acids (Whitelaw 2000). An increased number of hydroxyl (OH^-) groups has a positive effect on the ability of an organic acid anion to dissolve RP and a substitution seems to enhance this effect (Johnston 1954; Kpomblekou and Tabatabai 1994).

Citric acid, a tricarboxylic acid with one α and two β-substituted hydroxyl groups, has shown to be superior to other acids in its RP solubilizing ability (Kpomblekou and Tabatabai 1994; Xu et al. 2004). *Penicillium bilaii*, a significant citric acid producer, has shown to be an effective RP solubilizer (Cunningham and Kuiack 1992; Kucey 1988). In addition, *Aspergillus niger*, used in the industrial production of citric acid, has been recognized as one of the most effective organisms for RP solubilization (Abd-Alla and Omar 2001; Agnihotri 1970; Omar 1998; Sperber 1958). Tricarboxylic acids are not always superior to dicarboxylic acids in RP solubilizing ability. Sagoe et al. (1998) found oxalic and tartaric acids, which are dicarboxylic acids, release more P into solution than citric acid. This exception was attributed to the fact that oxalic and tartaric acids form poorly soluble precipitates with Ca^{2+}, effectively lowering the solution saturation point (Sagoe et al. 1998).

Ligand exchange reactions

Through ligand exchange reactions organic acid anions can mobilize P from the phosphate anions that are adsorbed to crystalline $Fe(OH)_3$ and $Al(OH)_3$ surfaces by chelating metal ions associated with bound P (Gahoonia et al. 1992; Jones 1998; Trolove et al. 2003). These ligand exchange reactions where hydroxide ions replace phosphate, causes decrease in the pH and P binding capacity of Fe and Al and consequently release P. In a study using four soils of varying pH, $CaCO_3$ and organic C contents, Gerke et al. (2000) found that in all soils more P was mobilized when citrate was added than when the soil, was subjected to a wide range of pH changes. This finding indicated that the P was mobilized due to ligand exchange between the citrate and the phosphate adsorbed to the Fe- and Al- sites rather than dissolution from Ca-P precipitates. In soil, citrate may mobilize phosphate when it is adsorbed at levels greater than 10 mol g^{-1} soil; however below this critical value, citrate will not out-compete phosphate for soil adsorption sites (Gerke et al. 2000).

Mineralization of organic P

Mineralization of soil organic P plays an imperative role in phosphorus cycling of a farming system. Organic P may constitute 4-90% of the total soil P. Almost half of the microorganisms in soil and plant roots possess P mineralization potential under the action of phosphatases (Cosgrove 1967; Tarafdar et al. 1988). The major portion of the P that is applied to soil rapidly becomes ‘fixed’ into inorganic and organic fractions which are poorly available to plants (Sanyal and De Datta 1991). Phosphatases (phytase and acid phosphatase) produced by soil microorganisms play a major role in mineralization of organic forms of soil P to release phosphate (Raghothama 1999). *Aspergillus* and *Penicillium* are major genera of phosphatases and phytase producing fungi (Aseri et al. 2009). High production of acid phosphatase and phytase enzyme by filamentous fungi in culture filtrate was reported by Relwani et al. (2008). Aseri et al (2009) reported that fungi execute extracellular phytase activity many times more than extracellular phosphatase activity. Richardson et al. (2005) reported that decrease in pH of soils makes phytase less effective in the soil environment. Pandey et al. (2007) showed higher acid phosphatase activity than alkaline phosphatase activity in all the treatments. Release of organic anions, and production of siderophores and acid phosphatase by plant roots/microorganisms (Yadav and Tarafdar 2003) or alkaline phosphatase (Tarafdar and Claasen 1988) enzymes hydrolyze the soil organic P or split P from organic residues. The largest portion of extracellular soil phosphatases is derived from the microbial population (Dodor and Tabatabai 2003). *Enterobacter agglomerans* solubilizes hydroxyapatite and hydrolyze the organic P (Kim et al. 1998). Mixed cultures of PSMs (*Bacillus*, *Streptomyces*, *Pseudomonas* etc.) are most effective in mineralizing organic phosphate (Molla et al. 1984).

Phytates account for a large component of the organic P, some 20-50 % of the total soil organic P (Anderson 1980; Dalal 1977); yet appear to be only poorly utilized by plants (Hayes et al. 2000; Richardson et al. 2000). Phytases have been reported in bacteria (Kim et al. 1998), yeasts (Lambrechts et al. 1992) and fungi (Shieh and Ware 1968). *Aspergillus* species, in particular, have been most extensively studied for commercial production of phytase (Ullah and Gibson 1987; Wyss et al. 1999) having significant role in phosphate solubilization and mineralization

Microbial diversity

The microbial diversity of soil containing insoluble forms of phosphate includes large number of phosphate solubilizing bacteria as well as fungi (Duponnois et al. 2005). According to Silva Filho and Vidor (2000), the community of P solubilizing microorganisms in soils is around 10^4 and 10^7 CFU (Colony Forming Units) g^{-1} of soil, depending on the area and the method of evaluation used. To isolate and identify the phosphate solubilizing microorganisms, visual detection and even semi quantitative estimation of the phosphate solubilization activity is possible using plate screening methods, which show clearing zones around the microbial colonies in the media containing insoluble mineral phosphate as single P source (Rodriguez and Fraga 1999; Chung et al. 2005).

Microbial diversity is measured by various techniques such as traditional plate counting and direct count as well as the molecular based procedures. Molecular based procedures can detect species, genera, families and even higher taxonomic groups (Nannipieri et al. 2003; Tilak et al. 2005). Typically 16S rRNA, 18S rRNA or ITS (Internal Transcribed Spacers) regions are targeted by primers for diversity studies of phosphate solubilizing microorganisms because these genes are present in all organisms and have well defined regions for taxonomic classification that have sequence database available to researchers. PCR, targeting the 16S rDNA, has been used extensively to study bacterial diversity and allow identification of bacteria as well as prediction of phylogenetic relationship, whereas 18S rDNA is increasingly used to study fungal community of phosphate solubilizing microorganisms (Kirk 2004; Tilak et al. 2005; Chung et al. 2005).

Fungal diversity

Eukaryotes possess four nuclear ribosomal RNA (rRNA) genes: 28-26S, 18S, 5.8S and 5S. These genes are repeated several hundred to a few thousand times in the genome and are nearly always arranged either in a single large tandem repeat array or in multiple tandem arrays found on one or a few chromosomes (Tilak et al 2005; Chung et al. 2005). The internal transcribed spacer (ITS) region of the 18S–5.8S–28S nuclear ribosomal cistron, now is extensively employed around the globe, having first been utilized scarcely a decade ago (Baldwin 1992, 1993). Brown et al. (1972) first proposed the idea that rRNA gene copies evolve ‘‘horizontally,’’ meaning that a mutation that arises in one copy spreads to all other copies, eventually homogenizing all of them. Later, this process was called ‘‘concerted evolution’’ (Zimmer et al. 1980). Because concerted evolution theory could explain the observed lack of genetic variability among rRNA gene copies in many different species, it eventually became dogma that it was the fundamental mode of rRNA multigene family evolution (Coen etal. 1982; Liao 1999, 2000). The ITS sequences can easily be amplified from minute amounts of DNA with PCR primers (White et al. 1990) and are conserved at the species level; however, there are enough differences between related fungal taxa, so that RFLPs generated with several endonucleases are a fairly good way to distinguish between different fungal species (Erland et al. 1994; Gardes and Bruns 1993; Karen et al. 1997; Timonen et al. 1997).

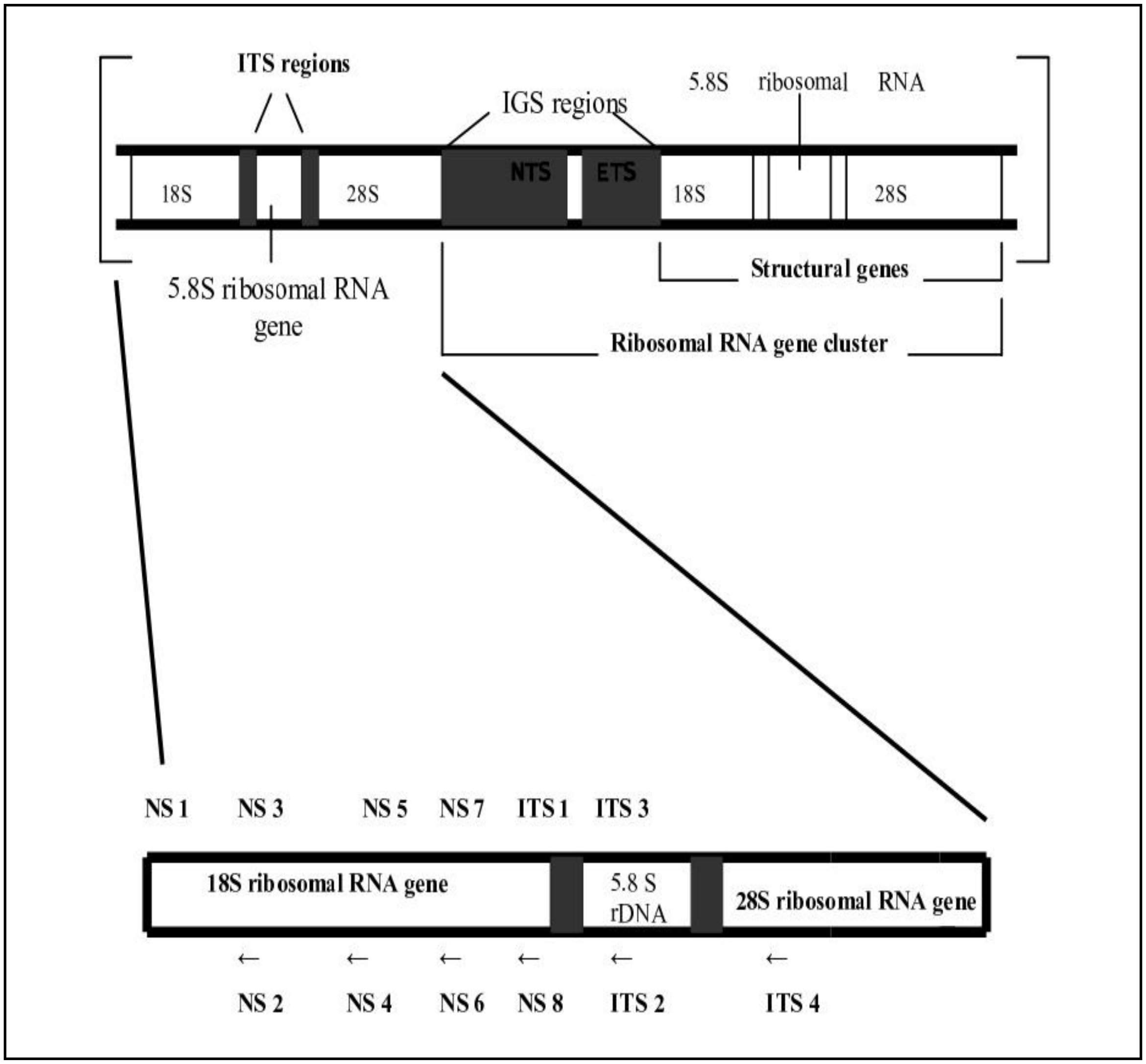


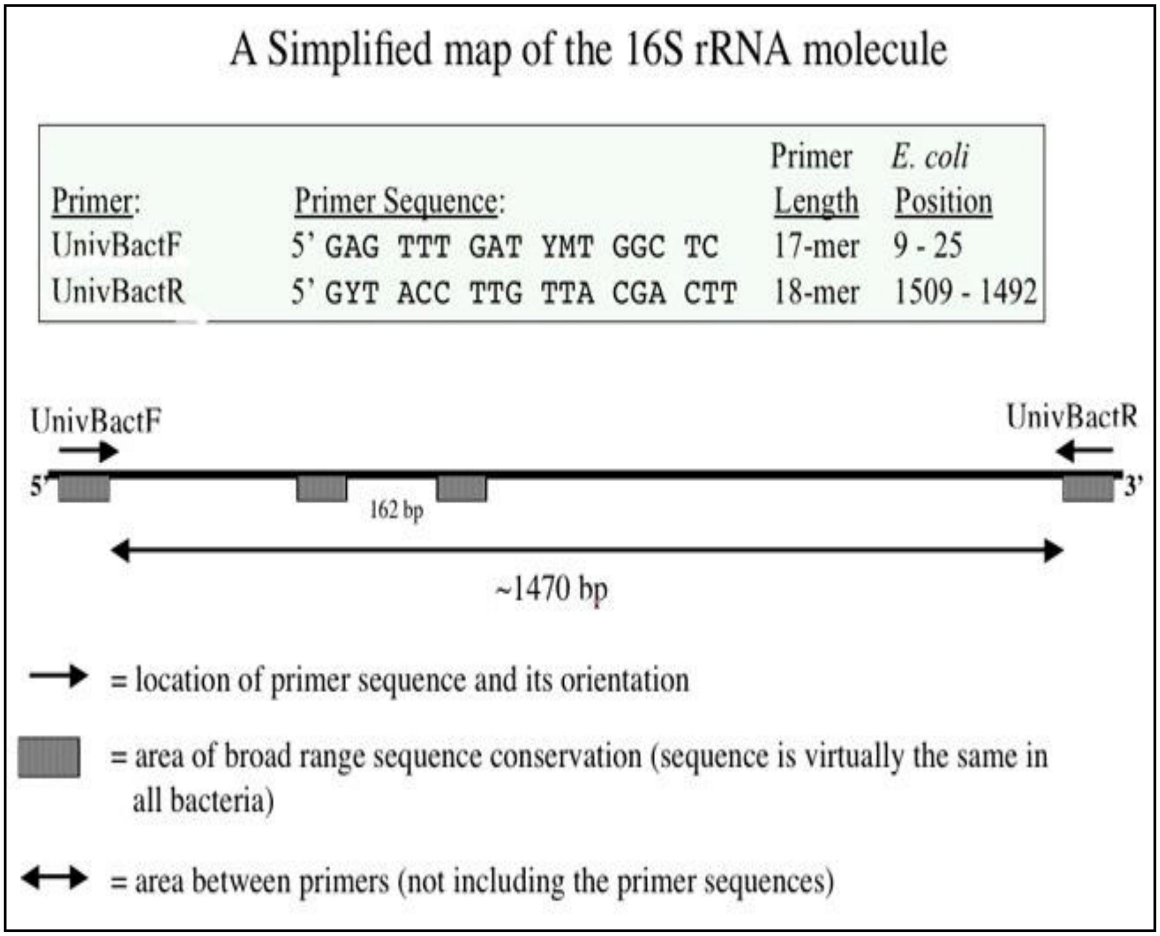
Figure 2.2: Structure of the ribosomal RNA gene cluster showing the positions of fungal PCR primers. The cluster is split into coding (18S, 5S and 28S genes) and non-coding (Inter-Genic Spacer or IGS and Internally Transcribed Spacer or ITS sequences) regions. Some of the non-coding areas are transcribed (Externally Transcribed Spacer or ETS and ITS) while others are not (Non-Transcribed Spacer or NTS sequence). The positions of the primers and their direction of replication are indicated by arrows. The IGS and ITS regions usually exhibit the most sequence variation while the coding areas are the most conserved (Source Mitchell 1995).

Aspergillus niger and *A. tubingensis* have been reported as efficient organic acid producers and phosphate solubilizers (Reddy et al 2002). Morphological discrimination of *A. tubingensis* from *A. niger* is quite difficult. These two species have a high morphological similarity and *A. tubingensis* was considered as a subspecies of *A.niger*. The utilization of molecular methods allowed a better distinction of the *A. niger* group until 1991 (Varga et al. 1993, 1994; Accensi et al. 1999, 2001) and the classification of *A. niger* and *A. tubingensis* as distinct species. The distinction between *A. niger* and *A. tubingensis* can be determined by RFLP analysis. This analysis is based on the presence of the restriction site for the endonuclease *Rsa*I (GT/AC) in ITS1 sequence from *A. niger* and its absence in *A. tubingensis* (Accensi et al. 1999).

Bacterial diversity

Since protein synthesis is a very ancient process and present in all living cells, rRNA molecules are excellent targets to estimate evolutionary relationships. In 1965, Zuckerkandl and Pauling proposed that macromolecules such as nucleic acids and proteins could serve as evolutionary chronometers. The sequence of the units should contain information about the evolutionary distances among the organisms, including those of bacteria (Akkermans et al. 1995)

The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny), but more recently it has also become important as a means to identify an unknown bacterium to the genus or species level. The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel 2001).



Primer:	Primer Sequence:	Primer Length	*E. coli* Position
UnivBactF	5' GAG TTT GAT YMT GGC TC	17-mer	9 - 25
UnivBactR	5' GYT ACC TTG TTA CGA CTT	18-mer	1509 - 1492

Figure 2.3: Structure of the ribosomal RNA gene cluster showing the positions of bacterial universal PCR primers
(source: http://serc.carleton.edu/microbelife/research_methods/genomics/modgen.html)

Ribosomal RNA is proposed as one of the best candidate and it has been used by Woese et al. (1990) for their studies on bacterial evolution. Major properties of rRNA are: 1) these are old molecules present in the ribosomes, 2) they are functionally constant, 3) have a wide distribution, 4) are well conserved over large phylogenetic distances, 5) they occur in large number in cells (10^4-10^5/cells), 6) in bacteria three types of rRNA molecules are present with different chain length and sedimentation rate(s): 5S rRNA (about 120 nucleotides), 16S rRNA (~ 1600 nucleotides) and 23S rRNA (~3000 nucleotides). The 5S molecule is too small and only suitable to distinguish major phylogenetic groups. 23S rRNA is excellent for phylogenetic studies, but so far few studies are available while 16S rRNA has been given most attention.

Effect of carbon and nitrogen sources on P solubilization

Phosphate solubilization is a complex phenomenon, which depends on many factors such as the nutritional, physiological and growth conditions of the cultures. Since PSMs are heterotrophs and solubilize insoluble phosphates by secreting organic acids, the role of carbon in this context is very important. Phosphate solubilizing activity is usually measured by using glucose (Whitelaw et al. 1999; Pradhan and Sukla 2005) or sucrose (Wenzel 1994; Cunningham and Kuiack 1992) as the sole carbon source. It was observed that PSMs utilize a variety of carbon compounds as energy sources, but the amount of soluble phosphate varies significantly with different sources of energy (Whitelaw et al. 1999). Apart from carbon, nitrogen is also an important element needed by microorganisms for synthesis of amino acids, proteins, purines and pyrimidine nitrogenous bases. It may be taken in the form of ammonia, nitrate, and nitrite or in amino form (Nahas 1996; Reyes et al. 1999b; Whitelaw et al. 1999; Seshadri et al. 2004). Furthermore, in most studies ammonium was found to be a better N source than nitrate (Nautiyal 1999; Narsain and Patel. 2000) followed by organic forms like urea and asparagine.

It was observed that rock phosphate solubilizing microorganisms utilize a variety of carbon compounds as energy sources, but the amount of soluble phosphate varied significantly with different sources of energy (Whitelaw et al. 1999). The nature and amount of organic acids excreted by fungi are mainly influenced by medium pH and buffering capacity, carbon source and the balance of nitrogen and phosphate (Mattey 1992; Reyes et al. 1999a). Reyes et al (1999a) reported that in case of glucose the total organic acid production was higher as compared to other C sources. Mannitol and glucose were also reported to be the best sources for *A. niger* to solubilize P (Seshadri et al 2004). Reyes et al. (1999a) showed that sucrose was the best carbon source for *P. rugulosum* for solubilization of hydroxyl apatite and $FeSO_4$. Sucrose and glucose significantly increased P solubilization by *A. tubingensis* compared to other carbon sources (Relwani et al. 2008). These studies suggest that different fungi use different carbon sources, and depending on the carbon source, the fungi use alternative metabolic pathways to produce organic acids. For the same C source, it is possible that the nature of the organic acid varies with the phosphate used in the culture medium influencing the solubilization mechanism. In addition, the nature and quantity of organic acid secreted by the fungus was influenced by the C source and the balance between N and P sources (Mattey 1992).

Using laboratory media, it has been observed that more RP is solubilized when using an NH_4^+ rather than a NO_3^- source of N (Whitelaw et al. 1999). In the same study, it was found that pH was generally lower and titratable acidity higher, when NH_4^+ was used. Similarly, Vora and Shelat (1998) tested a variety of N sources and found that ammonium sulphate promoted the most RP solubilization for bacterial species, *Bacillus* *circulans, B. brevis* and *B. coagulans*. For some microorganisms, the release of H^+ ions due to the assimilation of NH_4^+ seems to be the sole mechanism promoting RP dissolution. For example, in an experiment by Asea et al. (1988), the fungi *Penicillium bilaii* and *P. fuscum* were tested for their ability to solubilize RP on two different media: one containing NH_4^+ and another without N. In the NH_4^+ medium, both species were able to decrease pH and solubilize RP; however, in absence of N, only *P. bilaii* maintained the ability to lower the pH and solubilize RP. The latter results indicate that different mechanisms were utilized by different species; one mechanism required the presence of NH_4^+ in the medium and the other did not. The amount of P solubilized by *P. fuscum* was found to be directly related to pH, while *P. bilaii* solubilized more RP than could be accounted for by the pH change, indicating that another mechanism, in addition to H^+ production, was contributing to RP solubilization. Some studies show phosphate solubility increased when ammonium sulphate was used instead of KNO_3, $(NH_4)_2Fe(SO_4)$ and NH_4Cl (Nautiyal 1999; Narsain and Patel 2000). Other authors have found no correlation between the amount of RP solubilized and the pH, supporting the fact that an alternative mechanism of solubilization exists (Cerezine et al. 1988; Sperber 1958; Whitelaw et al. 1999; Sagoe et al. 1998).

Metal phosphates

Phosphate solubilization is effected by the type of metal present in rock phosphate. Metal phosphate solubilization by PSMs is an important biological process in the soil environment, making phosphates and metal cations available to plants. The majority of phosphate solubilizing microorganisms can solubilize phosphates of metal like cobalt, aluminium, manganese, strontium, zinc etc. Metal component of the phosphate may influence the availability of phosphate, biomass and growth rate depending upon the metal phosphate present (Jacobs et al. 2002b).

Phosphate solubilizing microorganisms produce organic acids. An increase in soil acidity entails an increase in mobile Al^{3+}, Ca^{2+}, Mg^{2+} and Fe^{3+}, here organic acids work as chelators and form metallo-organic complexes (Gadd 1999). Solubilization of metals occurs via proton release by PSMs by decreasing the negative charge of adsorbing surfaces to facilitate the sorption of negatively charged P ions. Proton release can also decrease P sorption upon acidification which increases $H_2PO_4^{\,-}$ in relation to HPO_4^{2-} having higher affinity to reactive soil surfaces (Whitelaw 2000). Carboxylic acids mainly solubilize metal phosphates (Henri et al. 2008; Khan et al. 2007) through direct dissolution of mineral phosphate as a result of anion exchange of PO_4^{3-} by acid anion, or by chelation of metal ions associated with phosphate (Omar 1998). Moreover, carboxylic anions replace phosphate from sorption complexes by ligand exchange (Otani et al. 1996; Whitelaw 2000) and chelate both metal ions associated with phosphate, releasing phosphate available for plant uptake after transformation. Ability of organic acids to chelate metal cations is greatly influenced by its molecular structure, particularly by the number of carboxyl and hydroxyl groups. Type and position of the ligand in addition to acid strength determine its effectiveness in the solubilization process (Kpomblekou and Tabatabai 1994). Phosphorus desorption potential of different carboxylic anions lowers with decrease in stability constants of Fe - or Al - organic acid complexes (log KAl or log KFe) in the order: citrate > oxalate > malonate / malate > tartrate > lactate > gluconate > acetate > formate (Ryan et al. 2001).

The leaching of metal compounds by fungi can occur by several mechanisms but organic acids occupy a central position in the overall process, supplying both protons and a metal complexing organic acid anion. The production of metal complexing organic acids assists both essential metal and anionic nutrition of fungi and plants via the solubilization of phosphate and sulphate from insoluble metal-containing substances, including salts and minerals (Gadd 2001). Solubilization of inorganic P by fungi has been linked to the production of various organic acids, including citric acid (Illmer et al. 1995), gluconic acid and oxalic acid (Gharieb 2000). Others have also observed P solubilization with decrease in pH values (Cerezine et al. 1988; Puente et al. 2004). The increased proton content responsible for the pH decrease can arise from the production of organic acid. Carboxylic acids and amino acids such as citric, malic and histidine are potential ligands for heavy metals and so could play a role in tolerance and detoxification (Rauser 1999; Clemens 2001).

Role of PSMs in crop productivity

Inoculation of plants by target PSMs at a much higher concentration than that normally found in soil is necessary to take advantage of property of phosphate solubilization for plant yield enhancement. P availability is a limiting step in plant nutrition therefore the main contribution of PSMs is in improvement of plant growth (Rodriguez and Fraga 1999). Considerable success was acclaimed in increased yield and quality of crops due to increased phosphate uptake by inoculating seeds with efficient phosphate solubilizers. Statistically significant increase in yield of the order of 5 to 10% has been recorded in about 30% of large number of inoculation experiments with phosphate solubilizing microorganisms (Rodriguez and Fraga 1999; Gyaneshwar et al. 2002). According to Silva Filho and Vidor (2000), the application of P solubilizing microorganisms as inocula or the management of their populations in soil are alternatives to improve P availability for plants. These microorganisms have the ability to solubilize and mineralize P from inorganic and organic pools of total soil P, making the element available for plants. Gyaneshwar et al. (2002) suggested that P solubilizing microorganisms are ubiquitous in soils and could play an important role in supplying P to plants in a more environmentally and sustainable manner.

Microbial community influences soil fertility through soil processes viz. decomposition, mineralization, and storage/release of nutrients. Microorganisms enhance the P availability to plants by mineralizing organic P in soil and by solubilizing precipitated phosphates (Chen et al. 2006; Kang et al. 2002; Pradhan and Sukla 2005). Thien and Myers (1992) indicated that by increasing soil microbial activities, bioavailability of P in a bioactive soil was remarkably enhanced. Such a phenomenon inspires the application of a similar principle on the bioactivation of relatively nonreactive rock phosphate. The fact that certain soil microorganisms are capable of solubilizing relatively insoluble phosphatic compounds has opened the possibility for inducing microbial solubilization of phosphates in soil. Many investigators believed that the phenomenon was closely related with the ability of the microorganisms in producing selected organic acids, and/or extracellular polysaccharides (Geonadi et al. 2000).

Doubts have also been raised on the ability of PSMs to liberate P under soil conditions (Brown 1972; Tinker 1980). Many reasons were proposed to account for the variations in the effectiveness of PSMs inoculations on plant growth enhancement and crop yields (Kucey et al. 1989): 1. Survival and colonization of inoculated PSMs in the rhizosphere; 2. Competition with native micro-organisms; 3. Nature and properties of soils and plant varieties; 4. Insufficient nutrients in the rhizosphere to produce enough organic acids to solubilize soil phosphates; and 5. Inability of PSMs to solubilize soil phosphates.

Field inoculation in combination with direct application of RP

Combined direct application of rock phosphate and PSMs has produced mixed results on plant growth responses, which were perhaps attributed to differences in microbial strains and/or soils being treated. Traditionally, most experiments using PSMs have employed two major strategies for increasing P availability from RP: the management of existing soil microbial populations to optimize their capacity to mobilize P and the development of specific microbial inoculants (Richardson 2001). Although there have been some successful results from soil inoculations of PSMs (Kucey 1987; Kucey and Leggett 1989; Omar 1998; Asea et al. 1988), in general, the results in terms of plant growth and crop yields, have been highly variable (Gyaneshwar et al. 2002). Organisms that can effectively solubilize RP in the laboratory, under controlled conditions, may be unable to do so in the field (Richardson 2001). These variations in effectiveness may be due to the reduced ability of inoculated PSMs to survive and colonize the rhizosphere, coupled with competition for resources with native soil microorganisms (Gyaneshwar et al. 2002).

Inoculation of the phosphate solubilizing microorganisms onto rock phosphate or reacting the rock phosphate with a liquid culture supernatant may be considered a better means to overcome the low solubility problems of rock phosphate (Goenadi 1996). Such an approach may eliminate factors inhibiting a successful interaction between phosphate solubilizing microorganisms and rock phosphate under field conditions. This approach will also make the production of single soluble phosphate possible without the use of chemical acidulation.

The competitiveness of P solubilizing microorganism in natural environments depend upon its ability to survive and multiply in soil. In general, the population sizes of the introduced microorganisms decline rapidly upon the introduction in soils (Ho and Ko 1985). The survival of the inoculant strain depends upon various factors such as soil composition (Bashan et al. 1995; Heijnen et al. 1994), temperature and the presence of the recombinant plasmids (Van Veen et al. 1997). The biotic factors that affect the survival of the inoculated microorganisms include competition, predation and root growth that provides the substrates to the microorganisms. The abiotic factors include texture, pH, temperature, moisture content and substrate availability in the soils (Paul and Clark 1988; Van Elsas et al. 1991). The biotic factors play a very important role in the survival of the inoculated strains as the decline observed in non sterile soils can often be abolished in sterile soils (Heijnen et al. 1988; Heijnen and Van Veen 1991). Additionally, an increase in the population of the introduced microbe can also be observed (Postma et al. 1988). The effectiveness of the introduced microorganisms also depends upon their physiological status. In a recent study, the survival of inoculant strains of various bacteria were shown to be not dependent on the availability of carbon or on selective predation but only on the initial inoculum density (Jjemba and Alexander 1999).

Effect of inoculation of PSMs along with RP on crop production

Vassilev et al. (2006) and Richa et al. (2007) reported that RP can be used as a crude phosphate fertilizer for direct application to the fields along with microbial inoculums. Field trials in India have shown that the use of PSMs can increase crop yields by up to 70 percent (Verma 1993; Wani and Lee 1992; Subba Rao 1982). The crops include oats, mustard, sugar beets, cabbage, tomato, barley, Egyptian clover, maize, potato, red gram, rice, chickpea, soybean and groundnut. *In vitro* studies have demonstrated the dissolution of RP by PSMs (Barea et al. 1983). Results from greenhouse trials have indicated a greater response of wheat and onion to RP application when seeds or seedlings are inoculated with PSMs. The increase in growth is greater with vesicular arbusular mycorrhiza (VAM) fungi and PSMs in combination than when these organisms are used singly (Young 1990; Toro et al. 1997; Singh and Kapoor 1999).

There are several reports regarding plant growth promotion due to inoculation of phosphate solubilizing microorganisms under greenhouse as well as field conditions (Wahid and Mehana 2000; Reyes et al. 2002). Though inoculation of PSMs showed improvement in growth and yield of different crops like wheat and mungbean (Whitelaw et al. 1997; Omar 1998; Saber et al. 2009) but the reports indicating the utilization of RP as fertilizer along with P solubilizing microorganisms are scarce, especially in field conditions. Higher available P, soil C levels, enzyme activities, lower soil pH (Caravaca et al. 2004) and organic acid secretion (Yadav and Dadarwal 1997) were also reported due to inoculation of P solubilizing fungi. The increase in soil weathering and the enhancement of nutrient availability in soil are frequently associated with the production of organic acids. The production and release of organic acids attribute to ion chelation and solubilization of inorganic P sources (Illmer and Schinner 1995). Kucey et al. (1989) reported that P solubilizing microorganisms in addition to solubilizing inorganic P also release growth promoting substances, which improve the germination and growth of plants and stimulate microbial activity in the rhizosphere.

In a study by Dwivedi et al. (2004), it was found that the inoculation of *Aspergillus awamori* to wheat and rice seedlings, increased the crop response to Musoorie RP over the non inoculated treatments, but was not statistically significant. It was suggested that possible reasons for this outcome could be that soil organic carbon levels were very low and that temperature fluctuations were not ideal for enhanced PSM activity (Dwivedi et al. 2004). Richa et al. (2007) and Saber et al. (2009) reported that inoculation with P solubilizing fungal strain affects both dry matter yield and P uptake, in RP amended soil. The soil properties like available P, organic carbon and pH were also improved after inoculation of the P solubilizing fungi along with RP (Richa et al. 2007). Vassilev et al. (1996) reported that application of *P. oxalicum* along with RP fertilization, reported a drop in pH values of the treatment compared to the control soils.

Use of PSMs can increase crop yields up to 70 percent (Verma 1993). Combined inoculation of arbuscular mycorrhizae and P solubilizing bacteria (PSB) give better uptake of both native P from the soil and P coming from the phosphatic rock (Goenadi et al. 2000; Cabello et al. 2005). Higher crop yields result from solubilization of fixed soil P and applied phosphates by PSB (Zaidi 1999). Microorganisms with phosphate solubilizing potential increase the availability of soluble phosphate and enhance the plant growth by improving biological nitrogen fixation (Kucey et al. 1989; Ponmurugan and Gopi 2006). *Pseudomonas* spp. enhanced the number of nodules, dry weight of nodules, yield components, grain yield, nutrient availability and uptake in soybean crop (Son et al. 2006). Phosphate solubilizing bacteria enhanced the seedling length of *Cicer arietinum* (Sharma et al. 2007), while co-inoculation of PSMs and PGPR reduced P application by 50 % without affecting corn yield (Yazdani et al. 2009).

Inoculation with PSB increased sugarcane yield by 12.6% (Sundara et al. 2002). Sole application of bacteria increased the biological yield, while application of the same bacteria along with mycorrhizae achieved the maximum grain weight (Mehrvarz et al. 2008). Single and dual inoculation along with P fertilizer was 30-40% better than P fertilizer alone for improving grain yield of wheat. Dual inoculation without P fertilizer improved grain yield up to 20% against sole P fertilization (Afzal and Bano 2008).

Since not many reports are available on the use of RP with PSMs as fertilizer, which is a cheap, consistent and environment friendly source of phosphate fertilizer, this is an attempt to isolate efficient phosphate solubilizers and inoculating them in the field with RP amended soils to increase the P levels.

Chapter 3

Materials and methods

MATERIAL AND METHODS

Isolation of phosphate solubilizing microorganisms

The rhizosphere soil from *Jatropha curcas* plants growing in mine landfills of rock phosphate of Rajasthan State Mines and Minerals Limited (RSMML), Jhamarkotra (24.58° N, 73.72° E), Udaipur, India were collected for isolation of P solubilizing microorganisms. The rock phosphate used was high grade Rajasthan rock phosphate (RRP) obtained from RSMML, Udaipur. Mineralogically the material was predominantly fluorapatite followed by some carbonate fluorapatite and chlorapatite. Minor minerals were oligoclase, sillimanite, quartz, etc. The size of the most of the material was less than 74 microns. Other properties of rock phosphate are given in table 3.1.

Table 3.1: Properties of Rock Phosphate

pH	7.3
EC (mS cm^{-1})	2.73
TDS (ppm)	1.75
P_2O_5 (%)	31.5
Al_2O_3 (%)	8.4
MgO (%)	3.37
CaO (%)	45.36
Organic Carbon (%)	0.044
Available P (%)	0.003

EC: Electric Conductivity, TDS: Total Dissolved Salts,

Isolation of phosphate solubilizing microorganisms was done by serially diluting the soil samples and inoculating them on Pikovskaya agar (Appendix I) plates.

Bacteria

One gram of soil sample was transferred in 10 ml of sterilized saline (0.85%) and mixed thoroughly. A serial dilution were made upto 10-8 and from each dilution 100 µl sample was spread on Pikovskaya agar plates (PKV) amended with RRP equivalent to 500 mg P_2O_5 l^{-1} media. The plates were incubated at 37 ºC. Distinct colonies observed on the plates showing halo zones were selected, purified by repeated culturing. These bacterial isolates were stored in 40% glycerol at -70 °C.

Fungi

The soil was serially diluted upto 10^{-6} dilution in the sterilized saline (0.85%). Since spores tend to cling together in water, a small amount of detergent (0.1% Tween 20) was applied to the dilution to disunite spores. Pour plating technique was adapted using Pikovskaya's agar amended with RRP equivalent to 500 mg l^{-1} media, having 0.003% w/v of rose bengal powder to selectively inhibit bacterial growth and restrict the size and height of colonies of more rapidly growing molds. The plates were incubated at 30 ºC. Distinct colonies showing hallo zones were selected, purified by repeated culturing and maintained on PKV agar slants at 4ºC.

These bacterial as well as fungal isolates were further tested for solubilization of tricalcium phosphate (TCP) and Rajasthan rock phosphate (RRP) in quantitative terms.

Quantitative estimation of bacterial solubilization of tricalcium phosphate

Pikovskaya's broth (100 ml) was dispensed in 250 ml conical flasks and TCP (109 mg equivalent 50 mg P_2O_5) was added as a phosphorus source. The flasks were inoculated with bacterial isolates and allowed to grow for different periods of time at 37 ºC. The soluble phosphorus was determined by method given by Bray and Kurtz (1945). The pH of culture filtrates was also measured.

Determining Soluble P (Bray and Kurtz 1945)

Reagents

1. *Boric acid (1%):* Dissolved 1 g of H_3BO_3 in 100 ml water.
2. *Sulphuric acid (2.5 M):* Carefully added 35 ml of H_2SO_4 (96%) to 200 ml water. Allowed to cool and made the volume upto 250 ml with water.
3. *Ammonium molybdate (4%):* 4 g of ammonium molybdate was dissolved in 100 ml water and was stored in dark.
4. *Potassium antimony tartrate (0.275%):* 275 mg of potassium antimony tartrate was dissolved in 100 ml water.
5. *Ascorbic acid (1.75%):* 1.75 g of ascorbic acid was dissolved in 100 ml water (prepared fresh).
6. *Mixed Reagent:* Added, using a measuring cylinder, successively the following reagents to a 500 ml bottle; 50 ml 2.5 M Sulphuric acid, 15 ml 4% Ammonium molybdate, 30 ml 1.75% Ascorbic acid, 5 ml 0.275% Potassium antimony tartrate and 200 ml water. Mixed well after each addition and prepared fresh reagent daily.
7. *Standard series of Phosphorus:* Weighed 4.394 g KH_2PO_4 (dried at 105°C) into a 250 ml beaker; about 200 ml water was added and allowed to dissolve. The volume was made up to one liter to get 1000 ppm standard P solution.

Ten ml of the 1000 ppm stock solution was pipetted in a 100 ml volumetric flask. The volume was made with water and homogenized. This makes a 100 ppm P solution. Then 30 ml of this 100 ppm P solution was pipetted into a 250 ml volumetric flask. Made to volume with water and homogenized. This makes a 12 ppm P solution. Took 0, 5, 10, 15, 20, and 25 ml of 12 ppm P solution to a 50 ml volumetric flask and made up to the volume with water to get a series of standards of, 0.0, 1.2, 2.4, 3.6, 4.8 and 6.0 ppm P.

Procedure

Pipetted successively in a test tube, 1 ml of the standard series, sample or blank, added 2 ml of boric acid and 3 ml of mixed reagent. Mixed well and allowed for one hour for the blue colour development. Measured the concentration of the solution at 720 nm, using spectrophotometer (Hitachi U-2001).

Screening of P solubilizing bacteria (PSB)

The isolates which were found to be highly efficient solubilizers of TCP were then tested for solubilization of RRP (158.7 mg equivalent 50 mg P_2O_5) in Pikovskaya's broth (100 ml) at 37ºC after different time intervals.

Four bacterial isolates were selected on basis of higher solubilizing efficiency of RP and were further tested for higher concentration of TCP and RRP i.e. equivalent to 0.05% P_2O_5, 0.5% P_2O_5, 1% P_2O_5 and 2% P_2O_5.

Screening of P solubilizing fungi (PSF)

Pikovskaya's broth (100 ml) was dispensed in 250 ml conical flasks and TCP (2.19 gm equivalent to 1% P_2O_5) was added. Inoculated with fungal isolates and allowed to grow at 30ºC for nine days. The soluble phosphorus (Bray and Kurtz, 1945 method), pH, acid phosphatase (Dorn and Rivera 1966) and phytase activity (Kim and Lei 2005) were determined in culture filtrates. The same experiment was done with Pikovskaya's broth (100 ml) amended with RRP (3.18 g equivalent to 1% P_2O_5).

Acid Phosphatase activity (Dorn and Rivera 1966)

Reagents

1. 0.6 M acetate buffer, pH 4.8
2. p- nitro phenyl phosphate solution (1.25 mg/ ml acetate buffer)
3. p- nitrophenol (PNP) : (1 mg/ml) solution in acetate buffer.
4. NaOH (1N): 40 g NaOH was dissolved in dist.water and volume was made to 1litre.

Procedure

a) For acid phosphatase activity, the p-nitrophenol liberated from p-nitrophenylphosphate at 30 ºC was measured at 410 nm in a spectrophotometer.

b) The substrate p-nitrophenylphosphate (1.25 mg/ ml) was dissolved in 0.6 M acetate buffer, pH 4.8 (acid phosphatases).

c) To 0.4 ml of the above said reaction mixture a 0.1 ml sample of the culture filtrate was added and incubated at 30 ºC for 1 hour.

d) The reaction was stopped with 1 ml of 1 N NaOH. Enzyme activity was expressed as micromoles of p-nitrophenol released per hour per milliliter of culture filtrate.

Phytase activity (Kim and Lei 2005)

Reagents

1. Citrate buffer (0.2M*)*, pH 5.5
2. Sodium phytate 1% (9 mM) in 0.2 M citrate buffer, pH 5.5
3. Trichloroacetic acid (TCA) 15% (w/v) (room temperature)
4. Color reagent (prepared fresh daily): mixed three volumes of 1 M sulfuric acid and one volume of 2.5% ammonium molybdate, one volume of 10% ascorbic acid (w/v) and mixed well.

5. KH_2PO_4 solution 9 mM was prepared. Then made a series of dilutions: 1:100, 1:200, 1:400, 1:800 and 1:1600 (corresponding to 90, 45, 22.5, 11.25, and 5.625 µM phosphate, respectively).

Procedure

a) Three aliquots (0.2 ml each) of samples were taken in 10 ml test tubes and incubated the test tubes in 37 °C water bath for 5 min.
b) 0.2 ml of 1% (w/v) sodium phytate in the citrate buffer was added to start the enzymatic hydrolysis of phytate. Incubated for 15 min at 37 °C.
c) The reaction was stopped by adding 0.4 ml of 15% TCA (room temperature).
d) Centrifuged the mixture at 3,000 rpm for 10 min and transferred the supernatant fraction to a new tube.
e) Mixed 0.2 ml of supernatant fraction with 1.8 ml of MiliQ water (Millipore).
f) Added 2.0 ml of fresh color reagent to each tube and mixed well. Incubated the mixture at 50 °C for 15 min and took the tubes to room temperature.
g) Read the absorbance of each sample solution at 820 nm, using water as the blank and the series diluted potassium phosphate solutions as standards.
h) Calculated the phytase activity per ml of sample. Enzyme activity was expressed as micromoles of inorganic P released per hour per milliliter of culture material from sodium phytate at 37 °C.

Characterization of P solubilizing microorganisms

Morphological and biochemical studies of P solubilizing bacteria

The bacterial isolates solubilizing TCP as well as rock phosphate (RRP) to greater extent were further characterized.

Gram staining

a) Bacterial smear of actively growing cells was spread on a glass slide and heat fixed.

b) The crystal violet was flooded for 10 sec.

c) Briefly, washed in water to remove excess crystal violet.

d) Gram's iodine was flooded for 10 sec and washed briefly in water.

e) Further, it was decolourised with acetone until the moving dye front has passed the lower edge of the section and washed immediately in tap water.

f) Safranine was used to counter stain for 15 sec and washed with water to remove the excessive stain. Visualized under microscope at different magnifications.

Catalase test

a) Small amount of bacterial cells were placed onto a clean microscope slide.

b) A few drops of H_2O_2 (3%) was added onto the smear.

c) A positive result was the rapid evolution of O_2 as evidenced by bubbling.

d) A negative result was no bubbles or only a few scattered bubbles.

Oxidase test

a) Small amount of organism from an agar slant/ plate was obtained with a sterile swab.

b) One drop of reagent (N,N,N',N'-tetramethyl phenylenediamine dihydrochloride) was placed onto the culture on the swab.

c) Positive reactions turned the bacteria violet to purple immediately or within 10 to 30 seconds. Delayed reactions were ignored.

Nitrate reduction test

Nitrate media (Appendix I) was used to determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) using the enzyme nitrate reductase. It also tests the ability of organisms to perform nitrification on nitrate and nitrite to produce molecular nitrogen. Nitrate broth contained nutrients and potassium nitrate as a source of nitrate. After incubating the nitrate broth, 2-3 drops of sulfanilic acid and α-naphthylamine were added. If the organism had reduced nitrate to nitrite, the nitrites in the medium will form nitrous acid. Sulfanilic acid was added; which reacted with the nitrous acid to produce diazotized sulfanilic acid. This reacts with the α-naphthylamine to form a red colored compound. Therefore, if the medium turns red after the addition of the nitrate reagents, it was considered a positive result for nitrate reduction.

Biolog test

To study the functional diversity of the bacterial isolates and to characterize them on basis of individual physiological profiles, the Biolog GN/GP plates (Biolog Inc., USA) were used. The isolated strains were characterized by determining their substrate utilization pattern in the Biolog GN/GP plates. For determination; the bacterial cells were grown on PKV agar in four quadrants. Bacterial cells were harvested from fourth quadrant by using sterile swab and dissolved in inoculation fluid provided by the manufacturer. The Biolog GN/GP plates were inoculated by 150 µl of the cell suspension that was adjusted to a density of approximately 3.0 x 10^6 cells/ ml by comparison with the turbidity standard supplied by the manufacturer. These plates were inoculated at 37 °C for 24 h, omitting the 4 h measurement. The color development in the micro plate wells was interpreted as positive, negative and borderline, in case it is not possible to differentiate positive from negative. The reading was also entered in the Biolog microlog GN/GP release 14.01/b databases to provide identification.

Fermentation of carbon substrates and biochemical tests

Test for the ability to produce acid by the fermentation of sugars was done. For this total of 35-carbohydrate fermentation tests were performed with isolated bacterial species according to the manufacturer's direction (Himedia Lab., Bombay, India). Inoculum was prepared by growing the cells in PKV broth at 37 °C in shaking condition until the inoculum turbidity was ≥0.5 OD at 620nm. Citrate utilization, LDC (lysine decarboxylase), ODC (ornithine decarboxylase), ONPG (Ortho-NitroPhenyl-β-D-Galactopyranoside test for lactose metabolism), TDA (tryptophan deaminase) hydrolysis and H_2S production tests were performed with all bacterial isolates by standard methods.

Antibiotic profiling of bacterial isolate

Bacterial isolates were grown in nutrient broth until the absorbance reached to 1.0. The grown bacterial cells were spread on nutrient agar and antibiotic discs were kept on it. These plates were incubated at 37 °C and the inhibition zones were noted. Ready precoated twenty antibiotic discs (Himedia Lab., India) were used to test the sensitivity of the bacterial isolates. These were: Norfloxacin (10 μg), Gentamicin (10 μg), Chloramphenicol (30 μg), Cefuroxime (30 μg), Ciprofloxacin (5 μg), Cefaperazone (75 μg), Ceftazidime (30 μg), Roxithromycin (30 μg), Calaritromycin (15 μg), Co-Trimoxazole (25 μg), Netillin (30 μg), Cefaclor (30 μg), Cephotaxime (30 μg), Cephadroxil (30 μg), Azithromycin (15 μg), Ampicillin/Cloxacillin (10/10 μg), Penicillin (10 units), Amikacin (30 μg), Sparfloxacin (5 μg) and Ampicillin/sublactam (10/10 μg).

Morphological studies of P solubilizing fungi

To study the morphology, the fungal isolates were grown on PKV agar media at 30 °C for three days. The fungal spores were placed on the slides and stained with lactophenol cotton blue. Identification was done through visual inspection of morphology by bright field microscopy under 40x magnifications (Aneja 1998) and further confirmed by ITS region sequence analysis of the ribosomal RNA gene.

Molecular methods for identification of P solubilizing microorganisms

Extraction of DNA

Several protocols have been developed and described for the preparation of genomic DNA from microorganisms. Most based upon the methods of Kirby (1957) and Marmur (1961), which generally comprise:

a) Chemical cell disruption by enzymatic digestion and detergent lysis

b) Extractions with organic solvents

c) Selective recovery of the DNA

Isolation of genomic DNA from P solubilizing bacteria (Jones and Barlett 1990)

a) A single colony of P solubilizing bacterial isolate was picked from a freshly grown plate and transferred into 20 ml of Nutrient Broth in a 250 ml conical flask. The culture was incubated for 16-24 h at 37°C with vigorous shaking (120 rpm).

b) Cells were harvested in 2.0 ml sterile microfuge tube after centrifuging at 13000 rpm for 5 minutes at 4 °C. Media was decanted from the microfuge tubes and the tubes were kept in an inverted position for 1 min to allow the last traces of media to drain away. The cell pellet was washed by using 10 mM Tris-HCl pH 8.0.

c) Each cell pellet was resuspended in 0.5 ml TE buffer thoroughly. 30 μl of 10% SDS solution and 3 μl of Proteinase K (20 mg/ml) was added, mixed well and incubated at 37 °C for 1 hour.

d) Equal volume (530 µl) of phenol: chloroform (1:1) was added, mixed well by inversion, and centrifuged at 13000 rpm at 4 °C for 10 min.

e) Upper aqueous phase was transferred to the sterile microfuge tube. Equal volume of phenol: chloroform (1:1) was added, mixed well by inversion and centrifuged at 13000 rpm at 4 °C for 10 min.

f) Upper aqueous phase was transferred to the sterile microfuge tube. One tenth volume of sodium acetate (3 M, pH 5.2) and 0.6 volume of ice cold isopropanol was added and gently mixed to precipitate DNA.

g) After centrifugation at 13000 rpm at 4 °C for 10 min., the supernatant was discarded and the pellet was washed twice with 70% ethanol to remove salts.

h) The pellet was air dried and dissolved in 50 µl Tris-EDTA (pH 8.0) buffer. Stored at 4 °C (or -20 °C) for further use.

Isolation of genomic DNA from P solubilizing fungi (Moller et al. 1992)

a) All the fungal isolates were grown on PKV agar media at 30 °C.

b) After 7 days of growth, the mycelia were scrapped off from the plates and 0.5 g harvested mycelia was placed in liquid nitrogen and ground into a fine powder using a sterile pestle and mortar.

c) Divided the mycelial powder into two eppendorf tubes.

d) To each tube, 1 ml of extraction buffer (100 mM Tris HCl, pH 8; 10 mM of Na EDTA and 2% SDS) preheated to 60 °C was added.

e) Mixed it well and allowed it to stand for 15-20 minutes.

f) Added 10 µl of Proteinase K equivalent to 100 µg (stock 10 mg/ml).

g) Incubated the tubes for 30- 60 minutes at 55-60 °C with occasional mixing.

h) Added 280 µl of 5M NaCl (final concentration 1.4M) and 130 µl of 10% CTAB ((hexadecyl trimethyl ammonium bromide) (1/10 of the volume) to each tube and incubated for 10 minutes at 65 °C.

i) Transfer half the volume (~750 µl) from each tube to two fresh tubes.

j) Added one volume of chloroform: isoamylalcohol mixture (24:1) mixed well and left for 30 minutes on ice and centrifuged for 10 minutes at 14,000 rpm at 4 ºC.

k) Removed the upper aqueous phase carefully without taking any of the interphase material and transferred it into a fresh eppendorf tube.

l) Added RNaseA (1mg/ml) to final concentration of 20µg/ml and incubated at 37 ºC for 30 minutes.

m) Added 225 µl of ammonium acetate (5 M) mixed gently and placed the tubes on ice for 30 minutes or longer (better to leave at 4 ºC for overnight).

n) Centrifuged at 14000 rpm at 4 ºC for 10 minutes and transferred the supernatant to fresh eppendorf.

o) Added 0.55 volume of isopropanol to precipitate the DNA. Immediately centrifuged at 14,000 rpm for 5minutes at 4 ºC.

p) Decanted the supernatant and washed the pallet twice with ice cold 70% ethanol to remove salts.

i) Air dried the pellet and dissolved in 50 µl of Tris-EDTA (pH 8.0) buffer. Stored at 4°C (or -20 °C) for further use.

Electrophoresis of DNA on agarose gels

DNA was loaded on 0.7 % (w/v) agarose gel prepared in 0.5x TBE, pH 8.0 (Appendix I) using a 6x loading dye (Appendix I). Ethidium bromide (0.5 µg/ml) was added to stain the gel prior to pouring. The nucleic acids were then electrophoresed at 50 volts (3 volts/cm) for 45-60 min and visualized on a U.V. transilluminator.

DNA purification

The DNA was purified by elution through the Wizard DNA Clean up system (Promega) according to manufacturer's instructions in order to remove contaminants, which can hamper in amplification of DNA.

Ethidium bromide fluorescent quantification

DNA was migrated electrophoretically in an agarose gel containing ethidium bromide (0.5 µg/ml). The quantity of DNA was visually determined with reference to a known λ standard phage (MBI fermentas, USA) DNA quantity by comparing the intensity of fluorescence. The phage is isolated from heat inducible lysogenic *E. coli* W3110 strain. The molecule is linear double stranded undigested DNA. DNA concentration is 0.3 µg/µl.

Spectrophotometric quantification of DNA

The concentration of extracted DNA in suspension was estimated by spectrophotometric measurement at A_{260}. For double-stranded DNA suspensions, an OD of 1.0 at a wavelength of 260 nm and using a cuvette with 1cm light path is equal to a concentration of 50 µg/ml (Sambrook and Russell 2001). The quality of the DNA was evaluated by measurement of the A_{260} and A_{280} and the A_{230}/A_{260} ratios. Ideally, the A_{260}/A_{280} ratio should be 1.8-2.0. Ratios less than 1.8 indicate protein or phenol contamination, while ratios greater than 2.0 indicate the presence of RNA.

Amplification of 16S rDNA

For amplification of 1.5 kb gene of 16S rRNA, the primers used were: Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-ACGGGCGGTGTGTTC-3' (Weisberg et al. 1991). DNA amplification was performed with Genamp PCR system (Applied Biosystem, USA). Reaction mixture for the PCR contained 1x PCR buffer, each dNTPs at a concentration of 200 μM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.1 μM and 2.5 U of Taq DNA polymerase (Fermentas, USA) in a final volume of 100 μl. PCR conditions for amplification of 1.5 kb fragment of 16S rDNA were as follows: Preheating at 94 °C for 3 min, 36 cycles of 94 °C for 2 min, 54 °C for 1 min and 72 °C for 2 min and final extension 72 °C for 7 min. Amplified DNA was verified by electrophoresis of aliquots of PCR product (5 μl) on a 1.2% agarose gel in 0.5% TBE buffer.

Amplification of ITS region

Fragments of the ITS1-5.8S-ITS2 of the rDNA from genomic DNA were amplified by the PCR using the primers ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3' as described by White et al. (1990). The 50 μl reaction mixture for PCR amplification contained the following: 10 ng DNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.5 μM of each primer and 2.5 units of Taq polymerase (Fermentas, USA). Amplifications were performed in Genamp PCR system (Applied Biosystem, USA) with an initial denaturation step of 94 ºC for 5 minutes followed by 35 cycles of 94 ºC for 1 minute, 50 ºC for 1 minute and 72 ºC for 1:30 minutes. Final extension at 72 ºC for 8 minutes was performed to ensure completion of all reactions. Controls containing no DNA template were included in every of amplification to test for the presence of contamination of reagents and reaction buffer. Aliquots (5µl) of amplification products were electrophoresed in 1.2% agarose gel and visualized on a UV transilluminator.

Restriction analysis of DNA samples by agarose gel electrophoresis

a) Sterile water was added in a sterile microfuge tube containing DNA solution and made up a volume of 17 µl (500 ng).
b) The appropriate 10x restriction enzyme assay buffer was added and mixed thoroughly by tapping the tube.
c) 1 µl (2-5 units) of the restriction enzyme was added, mixed by tapping the tube.
d) The mixture was incubated at the appropriate temperature for 1-2 h.
e) To stop the reaction, 4-5 µl gel-loading buffer was added, mixed by vortexing briefly (as the DNA samples need to be analyzed directly on agarose gel).
f) ITS-PCR products were digested with the restriction enzyme *RsaI* and 16S rDNA products were digested with the restriction enzymes *HinfI* and *BamHI* as per manufacturer's instructions.
g) The digested DNA was run through 2.5 % (w/v) agarose gels containing ethidium bromide and visualized and photographed using Gel Doc system.

Purification of PCR products

The amplicons were gel eluted using QIAquick columns (Qiagen Inc., USA). PCR products were purified by agarose gel (0.8%) electrophoresis prior to cloning. After staining with ethidium bromide, a defined band was visualized under UV irradiation and excised. Besides removing surplus primers, nucleotides, and salts, this method possessed the advantage that incomplete (shorter) amplification fragments are also removed prior to cloning. Subsequently, the DNA was excised from the gel, using the QIAquick gel extraction kit (Qiagen Inc., USA) as per manufacturer's direction. Purified PCR products were eluted with 40 µl TE buffer (pH 8.0). This purified product now can be used directly for the cloning in T-vector.

Ligation in T-vectors

The amplicons were ligated into pTZ57R/T vector. The final reaction volume for ligation was 30 µl (Appendix I). The reaction mixture was kept overnight at 22 °C and after that analyzed on 0.8% agarose gel.

Genetic transformation of *E. coli*

a) A single colony of *E. coli* DH5α was picked from a freshly grown plate and transferred into 20 ml of LB broth in a 250 ml flask. The culture was incubated for 16-20 h at 37 °C with vigorous shaking (200-250 cycles/min in a rotary shaker).

b) Aseptically transferred 200 µl of the above-saturated culture into 20 ml of fresh LB broth in a 250 ml flask. The culture was incubated with vigorous shaking at 37 °C for 2-3 h. To monitor the growth of the culture, determined the OD_{590} every one-hour (The OD_{590} should be ~ 0.5).

c) The above culture was transferred to sterile, disposable, ice-cold 50 ml polypropylene tubes. Cooled the cultures to 0 °C by storing the tubes on ice for 10 min.

d) The cells were recovered by centrifugation at 5000 rpm for 10 min at 4 °C. Decanted the media from the cell pellets. The tubes were kept in an inverted position for 1 min to allow the last traces of media to drain away. Pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice for 10-15 minutes. Cells were recovered by centrifugation at 5000 rpm for 10 min at 4 °C. Decanted the fluid from the cell pellets, the tube was kept in an inverted position for 1 min to allow the last traces of fluid to drain away.

e) Cell pellet was resuspended the in 1 ml of ice-cold 0.1 M $CaCl_2$. $CaCl_2$ treatment for 2 h induces considerably a transient state of "competence" in the *E. coli* cells.

f) Transferred 100 μl of the suspension of competent cells to a sterile and prechilled microfuge tube (1.5 ml capacity). Plasmid DNA sample (~100 ng in a volume of 5 μl or less) was added to each tube. (In control experiment, competent bacteria could receive no plasmid DNA at all.) Mixed the content of the tubes gently. Stored the tubes on ice for 30 minutes.

g) Transferred the tubes to a floater and were placed in a circulating water bath that was preheated to 42 °C. The tubes were left for exactly 2-3 min without shaking. Rapidly transferred the tubes to an ice bath and cells were chilled for 2 minutes.

h) Now added 1 ml of LB broth to each tube and incubated the cultures for 45 minutes in water bath set at 37 °C. This will allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.

i) Transferred the appropriate volume (40-90 μl) of transformed competent cells onto LB supplemented ampicillin (50 μg/ml) agar plates, which were spread with 100 μl of 100 mM IPTG (isopropyl beta-D-thiogalactopyranoside) and 20 μl of 50 mg/ml X-gal (5-bromo-4-chloro-3-indoyl β-D galactoside). Plates were incubated at 37 ºC. Transformed colonies were appeared in 12-16 h.

Blue/white screening for recombinant plasmids

After transformation of the ligated product, the *E. coli* DH5α (LacZ) bacterial host cells were spreaded on Luria Agar (Appendix I) medium containing 50 μg/ml ampicillin, for selection of transformants. X-Gal and IPTG were used to screen for colonies containing a recombinant plasmid. The cloning site in the pTZ57R/T easy vector is located in the multiple cloning site (MCS) of the plasmid's lacZα gene; if insert was present, non-functional β-galactosidase is produced, and the transformed bacterial colony is white. White colonies were picked and grown in 2 ml LB containing ampicillin (50 μg/ml) and simultaneously patching of these cultures was done on LA containing ampicillin. Plasmid was isolated (described in the proceeding section) and re amplification of the insert were done using vector's promoter specific sequences.

Isolation and purification of plasmid DNA from bacteria by alkaline lysis method

a) A single transformed *E. coli* colony was transferred into 2 ml of LB medium containing appropriate antibiotic (ampicillin used in a final concentration of 50 μg/ml) in a capped 15-ml tube. The culture was incubated overnight at 37 °C with vigorous shaking.

b) 1.5-2.0 ml of the above-saturated culture were transferred into a microfuge tube. Centrifuged at 8000 rpm for 5 min in a microfuge.

c) The medium was removed, leaving the bacterial pellet as dry as possible.

d) The bacterial pellet was resuspended in 200 μl of ice-cold Solution I (Appendix I) by vortexing to ensure that the bacterial pellet was completely dispersed in this solution. Place as such at room temperature for 3 min.

e) 200 μl of freshly prepared Solution II (Appendix I) was added. Closed the tube tightly and mixed the contents by gently inverting the tubes ten to twenty times for 30 seconds. Stored the tubes on ice for 3min.

f) Added 300 μl of ice-cold Solution III (Appendix I). Closed the tube, mixed by inversion to disperse Solution III through the viscous bacterial lysate.

g) The tube were stored on ice for 10-15 min and centrifuged at 12,000 rpm for 10 min at 4 °C in a microfuge. Carefully transferred the supernatant to a fresh tube.

h) Added 400 μl chilled phenol/choroform/isoamylalcohol mixture (25:24:1) for protein denaturation and centrifuged it for 10 minutes at 10000 rpm.

i) Took the supernatant in another tube and DNA was precipitated by adding equal volume of isopropanol.

j) Mixed well and allowed the mixture to stand at room temperature for 5-10 min. Centrifuged at 10,000 rpm for 10 min at 4 °C in a microfuge.

k) Supernatant was removed. The tube was kept in an inverted position on a paper towel to allow all of the fluid to drain away. Any adhering drops of fluid on the walls of the tube were removed.

l) The pellet was washed with 70% ethanol, centrifuged at 8000 rpm for 5 min

m) Air-dried the pellet and dissolved in 50 μl of TE buffer (pH 8.0). The DNA was stored at -20 °C for further use.

A final PCR reaction using ITS and 16S primers and restriction digest was performed to verify that the extracted plasmid DNA contained the insert.

Sequencing

The fragments of 16S rRNA and ITS1-5.8S-ITS2 amplified by PCR were sequenced according Sanger et al. (1977) using an automated DNA sequencer (DNA Sequencing Facility, Department of Biochemistry, South Campus, Delhi University, New Delhi, India).

DNA sequence analysis

The sequences were compared against the available DNA sequences in GeneBank (http://www.ncbi.nlm.nih.gov/) using BLASTN (Altschul et al. 1997) tool. The sequences were aligned using MultAlin (Corpet 1988) program and the alignments were manually corrected.

In case of bacteria, the phylogenetic tree (Neighbor Joining) was constructed using the MEGA 4.0 (Tamura et al. 2007) software and the bootstrap values were inferred from 1000 replicates (Felsenstein, 1985).

In fungi, the phylogenetic analysis was based on parsimony using PAUP v4.0 b10 (Phylogenetic Analysis Using Parsimony* and other Methods; Swofford 2002). Trees were inferred using the branch-and-bound search option. MaxTrees was equal to 5000, branches of zero length were collapsed and all multiple parsimonious trees were saved.

The Consistency (CI), Retention (RI), Related consistency (RC) and Homoplasy (HI) indices were determined for all the data sets. Clade stability was assessed in bootstrap analyses with 1000 replicates. Trees were figured in Treeview (Page 1996). Bayesian analyses utilized the Metropolis-coupled Markov Chains Monte Carlo search algorithm as implemented in the program MrBayes v 3.1.2 (Ronquist and Huelsenbeck 2003). Two simultaneous independent replicates of *Aspergilli* were run for 5 million generations and of *Penicillium* for 3 million generations with sampling at every 100th generation, and the convergence of the runs visualized using Tracer ver. 1.4 (Drummond and Rambaut 2007). The first 20% of the trees were considered as burn-in (burn in = 2500) and the remaining 40,000 trees were summarized. This Bayesian approach of phylogenetic analysis was repeated five times to test the independence of the results from topological priors (Huelsenbeck et al. 2002). Only Bayesian posterior probabilities (PP) greater than or equal to 85 are considered significant. Optimal models of DNA substitution was inferred using the Akaike information criterion (AIC) (Akaike 1981) as implemented in MrModelTest ver. 2.3 (Nylander 2004).

Effect of carbon and nitrogen source on solubilization of TCP

To study the effect of different carbon sources on the growth and phosphate solubilizing activity of bacterial as well as fungal isolates, glucose was replaced with an equal amount (10 g l^{-1}) of mannitol or xylose or fructose or sucrose or arabinose sterilized separately and added to the Pikovskaya's broth [C source, 10.0 g; $(NH)_2SO_4$, 0.5 g; NaCl, 0.2 g; MgSO4.7H2O, 0.1 g; KCl, 0.2 g; Yeast extract, 0.5 g; $MnSO_4$, 0.1 mg; $FeSO_4.7H_2O$, 0.1 mg; tricalcium phosphate (TCP), 5.0 g (equivalent to 2.3 g P_2O_5); water, 1000 ml; pH 7.0±0.2]. Nitrogen sources were evaluated similarly by replacing ammonium sulfate with 0.5 gl^{-1} ammonium nitrate or sodium nitrate or potassium nitrate or urea or asparagine. The flasks were incubated at 30 °C under shaking for fifteen days for bacteria and for seven days for fungi. The media was analyzed for soluble P and pH reduction. In case of fungi the mycelium was washed repeatedly with distilled water and dried at 70 °C for 48 h. The fungal growth was expressed as biomass produced per flask containing 50 ml of medium. Acid phosphatase, phytase activity and organic acid were also estimated in the culture filtrate of fungi.

The combination of carbon and nitrogen source in which the organism showed best solubilization was then used to test the solubilization of 5.0 g RP/l PKV (equivalent to 1.6 g P_2O_5/l) by that particular organism.

Organic acid estimation

Organic acids produced by P solubilizing microorganisms during P solubilization were determined using the HPLC method. The HPLC equipment was series 200 of Perkin Elmer, USA, equipped with Polypore-H column (Brownlee Column, Perkin Elmer, USA) and a Micro-Guard column (Perkin Elmer, USA). After 7 days of incubation, the culture filtrates were passed through a 0.22 µm filter and subjected to HPLC with a polypore H column (Perkin Elmer, USA). The mobile phase consisted of 0.008 N H_2SO_4 at a flow rate of 0.3 ml min^{-1}. Detection was performed by a UV/VIS detector at 210 nm (Relwani et al.2008). HPLC profiles of the culture filtrates were analyzed by comparison with the elution profiles of pure organic acids (Bio-Rad Standard containing oxalic acid, succinic acid, acetic acid, citric acid, malic acid, and formic acid whereas gluconic acid (Sigma, USA) was injected separately).

Solubilization of metal phosphates

To study metal phosphate solubilization activity, inoculum of P solubilizing isolates were added to each 250 ml conical flask containing 50 ml of Pikovskaya's broth amended with different metal phosphates (copper phosphate, ferric phosphate, zinc phosphate, aluminium phosphate and cobalt phosphate) equivalent to 5 mM P_2O_5. PKV medium having TCP [$Ca_3(PO_4)_2$] equivalent to 5 mM P_2O_5 served as control for each set. The flasks were incubated at 30°C under shaking condition. The culture media was analyzed for soluble P, enzyme activity, organic acid exudation and dry biomass.

To study the effect of different carbon sources on the solubilization of metal phosphates by fungal isolates, glucose was replaced with an equal amount (10 g l^{-1}) of mannitol or xylose or fructose or sucrose or arabinose sterilized separately and added to the Pikovskaya's broth (50 ml) having different metal phosphates equivalent to 5 mM P_2O_5. Nitrogen sources were evaluated similarly by replacing ammonium sulfate with 0.5 gl^{-1} ammonium nitrate or sodium nitrate or potassium nitrate or urea or asparagine. The flasks were incubated at 30 °C under shaking for fifteen for seven days. The media was analyzed for soluble P and pH reduction. The mycelium was washed repeatedly with distilled water and dried at 70 °C for 48 h. The fungal growth was expressed as biomass produced per flask containing 50 ml of medium. Acid phosphatase and phytase activity were also estimated in the culture filtrate of fungi.

Enhancement of fertilizer value of rock phosphate

Inoculation of plants by target phosphate solubilizing microorganisms at a much higher concentration than that normally found in soil was necessary to take an advantage of property of phosphate solubilization for plant yield enhancement.

Fungal inoculum for fields was prepared using seven days old fungal spores of each of the fungi. The spores of *Aspergillus tubingensis* (AT6), *A. tubingensis* (AT1), *A. niger* and *P. oxalicum* were scraped and mixed separately with sterile (autoclaved at 121°C for 30 min) vermiculite (2-4 mm). For fungal consortium (FC) the scraped spores of all the four fungi which were of equal quantity, were mixed with the 100 g of sterile vermiculite. To prepare bacterial consortium (BC) the four isolates C19, 2C9, SC18 and 4G2 were grown separately to an OD 1.0 (A_{600}), centrifuged at 15000 rpm for 15 minutes and peletted cells were mixed with 100 g of sterile vermiculite. An agricultural field was used for the experiment having soil with sand: 66%; silt: 15.45%; clay: 18.75% (Sandy loam); pH of 8.1; available P: 1.6 mg kg^{-1}; EC: 0.3 mS/cm; CEC: 12.7 meq/100 g; Total N: 0.10% and organic carbon: 0.09%. Forty two field plots measuring 2x2 m^2 each were prepared. The plots with different treatments were arranged in a randomized complete block design with three replicates per treatment. Seven plots were amended with 35 g each of RRP (20 mg P_2O_5 kg^{-1} soil) before seeding, while non-fertilized controls were maintained for each microbial treatment. The treatments consisted of:

Soil;
Soil + *A.tubingensis* (AT6);
Soil + *A.niger;*
Soil + *A.tubingensis* (AT1)*;*
Soil + *P.oxalicum;*
Soil + fungal consortium (FC);
Soil+ bacterial consortium (BC);
Soil + RRP;
Soil + RRP + *A.tubingensis* (AT6)*;*
Soil + RRP + *A.niger;*
Soil + RRP + *A.tubingensis* (AT1)*;*
Soil + RRP + *P.oxalicum*;
Soil + RRP + fungal consortium (FC);
Soil + RRP + bacterial consortium (BC);

Seeds of wheat (variety HD2733) were sown (120 kg/ha) in December, 2008. At the time of seeding, 100 g vermiculite containing inoculum of each fungal treatment (4.8-5.4 x 10^7 spores) and of bacterial inoculum (4.8 x 10^7 g^{-1}) was added to treatment plots. Irrigation was done once before wheat was sown to have adequate soil water storage for seedling establishment. The plants were irrigated regularly. No fertilizer was added. The crop was harvested after five months and studied for various parameters such as yield of wheat and P content in the plant parts. Phosphorus (P) content was measured using the molybdovando method described by Reuter et al. (1997). From each plot, ten randomly selected plants were uprooted and checked for shoot height. The plants were oven dried at 65 °C for 24 hours and measured for shoot weight and root weight and total P (Kitson and Mellon 1944). The soil was analyzed for its pH, EC, organic carbon (Walkley and Black 1934), total P (Kitson and Mellon 1944), available P (Olsen et al. 1954), total N content and phosphatase (Tabatabai and Bremner 1969) and phytase activity (Kim and Lie 2005). One week after planting and one week before harvesting, the viability of inoculated microorganisms in the soil was determined by isolating both fungi and bacteria using serial dilution technique.

Two months after wheat harvesting, the field was tilled and irrigated without disturbing the experimental design. Maize cultivar Kanchan 25 was sown (30 kg/ha) and at the same time, 25 g vermiculite having inoculum of each fungal treatment (8.2 – 9.0 x 10^6) and bacterial inoculum (8.6 x 10^6) was added to the treatment plots, irrigation was done conventionally and urea was added at 25 g N $plot^{-1}$ at knee length stage and at emergence of flag leaf. The crop was harvested after three months and various growth and yield parameters were measured. From each plot, ten randomly selected plants were uprooted and checked for shoot height, shoot weight, root weight and total P (Kitson and Mellon 1944). The soil was analyzed for different parameters as described for wheat crop. The viability of inoculated cells in the soil were observed one week after planting and one week before harvesting as mentioned previously.

Plant and soil analytical procedures

Determination of soil reaction (pH)

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Reagents

Standard buffer solutions of 4, 7 and 9.2 pH

Procedure

a) Weighed 20 g of air dried soil passed through 2mm sieve into a 100 ml beaker.

b) 50 ml of distilled water was added to it.

c) Thoroughly stirred for 2-3 minutes using a glass rod.

d) Further, kept the suspension on shaker at 37 °C for overnight.

e) Next day allowed suspension to settle down for 30 minutes.

b) In the mean while, switched on the pH meter and after 10 minutes of warming up period, adjusted the pH meter reading to the pH of the buffer solution with the help of standardization knob.

c) Checked the instrument with two buffer solutions of known pH viz. one acidic and other alkaline.

d) Rinsed the electrode with distilled water and carefully wiped with filter paper.

e) Measured the pH of sample by immersing the electrode in supernatant solution.

f) Recorded pH value when the reading had stabilized (usually after 1 minute).

Determination of soil electrical conductivity (EC) and total dissolved salts (TDS)

Reagent

KCl solution: Dissolve 0.5232 g dry KCl in distilled water and make up the final volume 1 liter. This solution has an electrical conductivity of 1.0 mS cm^{-1} at 25 °C. (mS cm^{-1}, which is equal to mmho cm^{-1} (1 mho = 1 Siemen). The TDS of this solution is 650 ppm.

Procedure

a) Weighed 20 g soil and transferred it to a 100 ml beaker.

b) Added 50 ml distilled water to it.

c) Shook intermittently with glass rod for one hour and allowed to stand. (Alternatively, the clear extract after pH determination can also be used for EC measurement).

d) In the meanwhile, switch on the EC meter and allowed it to warm for 20 minutes.

e) KCl solution (EC=1mS cm^{-1}) was used to calibrate the meter.

f) The electrode was dipped in the supernatant solution and recorded the reading displayed.

Available P in soil (Olsen et al. 1954)

Reagents

1. *Sodium hydroxide* (1M)*:* Dissolved 4 g of NaOH in 100 ml distilled water.
2. *Sodium bicarbonate* (0.5 M): Dissolved 42 g $NaHCO_3$ in one liter water and adjusted pH to 8.5 with 1M NaOH.
3. *Sulphuric acid* (4M): Carefully added 56 ml conc. H_2SO_4 to 150 ml water and allowed to cool and made up to 250 ml with water.
4. *Ammonium molybdate* (4%): Dissolved 4 g of ammonium molybdate in 100 ml water and stored it in a polythene bottle in dark.

5. *Potassium antimony tartrate* (0.275%): Dissolved 275 mg of potassium antimony tartrate in 100 ml water.
6. *Ascorbic acid* (1.75%): Dissolved 1.75 g of ascorbic acid in 100 ml water (Prepared fresh).
7. *Mixed Reagent:* Added successively, using a measuring cylinder, the following reagents to a 500 ml bottle; 50 ml 4 M Sulphuric acid, 15 ml 4% Ammonium molybdate, 30 ml 1.75% Ascorbic acid, 5 ml 0.275% Potassium antimony tartrate, 200 ml water. Mixed well after each addition and prepared fresh daily.
8. *Standard series of Phosphorus:* Weighed 4.3943 g KH_2PO_4 (dried at 105ºC) into a 250 ml beaker, added about 200 ml of water and allowed to dissolve. Made the volume up to one liter to get 1000 ppm standard P solution. Pipetted 10 ml of the 1000 ppm stock solution in a 100 ml volumetric flask. Made up to volume with water and homogenize. This makes a 100 ppm P solution. Pipetted 10 ml of this 100 ppm P solution to a 250 ml volumetric flask. Made up to volume with extracting solution and homogenized. This makes a 4 ppm P solution. Pipetted out of this 4 ppm P solution respectively 0, 5, 10, 15, 20, and 25 ml in to a 50 ml volumetric flask and made each flask up to volume with extracting solution to get a series of standards of; 0.0, 0.4, 0.8, 1.2, 1.6 and 2.0 ppm P.

Procedure

a) Weighed 5 g of sample into a 250 ml shaking bottle and 100 ml of sodium bicarbonate extractant was added, shook for 30 minutes and filtered through Whatman No. 42 filter paper.

b) Pipetted 5 ml of the standard series, sample or blank to a test tube and added 5 ml of mixed reagent. Shook and kept for one hour for the blue colour development.

c) Measured the concentration of the solution at 882 nm or 720 nm, using spectrophotometer.

Total organic carbon in soil (Walkley and Black 1934)

Procedure

a) Weighed 1 g of dried soil sample and transferred it into a 150 ml conical flask.

b) 10 ml of 1N $K_2Cr_2O_7$ was added and mixed well.

c) The blank was prepared in which all reagents except soil were added.

d) The conical flask was kept on teflon /asbestos sheet and added 20 ml of concentrated H_2SO_4 from the sides of the flask. Swirled the flask during addition.

e) The flask was allowed to stand for 30 min and there after 70 ml of water was added.

f) These flasks were swirled thoroughly and allowed to settle the soil particles overnight.

g) The supernatant was decanted and read the color intensity using red filter at 660 nm.

h) For standard, weighed 0, 10, 20, 30, 40 and 50 mg of anhydrous sucrose crystals into a 100 ml volumetric flask and repeated the same procedure.

Calculation

The quantities of sucrose (0, 10, 20, 30, 40 and 50 mg) were multiplied by 0.4207 and found the quantities (%) of carbon present. Because 10 mg sucrose contained 4.207 mg C and if suppose 4.207 mg C was found in 1000 mg of soil, it meant:

100 mg soil contained = (4.207 x 100)/ 1000 mg carbon;

Organic carbon (%) = Colorimetric reading x 0.0042

Because organic matter contains 58% carbon so,

Organic matter = Organic carbon (%) x 100/ 58 or

Organic matter = Organic carbon (%) x 1.724 (Van Bemmelen factor)

Sample preparation for elemental analysis

For the release of mineral elements from soil and sediments, di-acid (HNO_3- $HClO_4$) oxidation of sample was carried out.

HNO_3/$HClO_4$ Digestion

a) Weighed 0.5-1.0 g sample of air dried soil in digestion tube and added 15 ml conc. HNO_3 digest on electrical heaters for 1 h at 145 °C in acid-proof digestion chamber having fume exhaust system.

b) Then added 5 ml of $HClO_4$ and heated at about 100 °C for first one hour and then raised the temperature to about 200 °C.

c) Continued the digestion until the contents become colorless and only white dense fumes appeared.

d) Reduced the acid contents to about 2-3 ml by continuing heating at the same temperature.

e) Removed from the hot plate, cooled and added diluted HCl.

f) The solution was filtered through Whatman No. 42 filter paper and the final volume made was 50 ml with diluted HCl.

g) This was used to determine total phosphate.

Total phosphorus in soil and plant samples (Kitson and Mellon 1944)

(Vanadomolybdophosphoric Yellow color Method)

Ammonium molybdate reacts under acidic conditions to form a heteropoly acid and molybdophosphoric acid. In presence of vanadium, yellow vanadomolybdophosphoric acid is formed. The intensity of the yellow colour is proportional to phosphate concentration. Concentration ranges for different wavelengths are:

P Range (mg/l)	Wavelength (nm)
1.0 – 5.0	400
2.0 – 10	420
4.0 – 18	470

Reagents

1. Vanadomolybdate solution.

   Solution A**:** dissolved 25 g ammonium molybdate [$(NH_4)_6Mo_7O_{24}.4H_2O$] in 300 ml water in a 500 ml beaker.

   Solution B: dissolved 1.25 g ammonium (meta) vanadate [NH_4VO_3] in 300 ml boiling water. Cooled, added 250 ml concentrated HNO_3 and cooled again. Added solution A to solution B and made up to 1000ml in a volumetric flask.

2. Phosphorus stock standard solution (50 mg l^{-1} P):

   Dissolved 0.2195 g of dried KH_2PO_4 in distilled water and mixed thoroughly. Acidified with 25 ml of 7 N H_2SO_4 and made the volume up to one litre to get 50 mg/l P solution. Add four to five drops of toluene to prevent microbial activity. (KH_2PO_4 is dried at 100 °C for 1 h and cooled in a desiccator before weighing)

Procedure

a. Placed 10 ml of acid digests of soil sample in a 50 ml volumetric flask, added 10 ml of the vanadate-molybdate reagent and dilute to 50 ml.

b) Mixed well and read the P concentration after 10 minutes using spectrophotometer at 420 nm.

c) Took 0, 1, 2, 3, 4 and 5 ml of the 100 mg/l P solution in 50 ml volumetric flasks and developed the colour as mentioned above.

d) Calibrated the spectrophotometer with known P concentration and read the concentration of sample.

Calculation:

$$\mathrm{P(mg/kg)} = \frac{\begin{array}{c}\text{Volume make up}\\ \text{after acid digestion (ml)}\end{array}}{\text{Weight of sample (g)}} \times \frac{50}{\begin{array}{c}\text{Volume of digest used}\\ \text{to develop color (ml)}\end{array}} \times \mathrm{P\ (mg/l)\ in\ 50ml\ solution}$$

Total nitrogen estimation in soil

(Modified Kjeldahl’s method in Soil chemical analysis by Jackson 1962)

Digestion of soil samples:

The method employs a digestion to convert nitrogen present in soil to ammonium sulphate. The ammonium nitrogen was subsequently determined by distillation and titration.

Reagents

1. Conc. H_2SO_4
2. Kjeldahl catalyst Tablets: B.D.H. cat No. 33064 (Each tablet contains 1 g sodium sulphate and 0.1 g copper sulphate)

Procedure

a) Weighed and transferred 1g of finely ground (air dry < 0.25 mm) soil into a kjeldahl flask. Added 10 ml conc. H_2SO_4 and mixed by swirling.

b) Heated at 200 ºC in a digestion block until white vapors were released during digestion.

c) Added 1 Kjeldahl catalyst tablet and heated for 3-4 hours until tablet dissolves (200 ºC) to get the precipitation.

d) Removed the digestion tubes from the block and allowed to cool for 5 minutes.

e) Transferred the whole sample into volumetric flask and made the volume to 50 ml.

ii) Determination: (Distillation- Titration method)

Reagents

1. NaOH (40%): Dissolved 400 g NaOH pellets in distilled water and made the volume to one liter.
2. Boric acid (4%): Dissolved 40 g H_3BO_3 in a liter of distilled water.
3. Mixed indicator: Dissolved 0.066 g methyl red and 0.099 g bromocresol green in 100 ml of 95% alcohol.
4. HCl (0.01 N)

Procedure

a) Transferred 5 ml of sample digest to the distillation apparatus.

b) Added 10 ml of 40% NaOH solution, formation of brown precipitates of ferric hydroxide, when the liquids were mixed indicated the neutralization of acid.

c) 10 ml of 4% boric acid and 2-3 drops of mixed indicator were taken in a 100 ml Erlenmeyer flask and placed under the delivery tube of the condenser so that the tip was below the surface of the liquid.

d) Closed the sample inlet and drainage outlet and passed steam into the distillation flask. The liquid boiled and the indicator in the boric acid solution changed color from pink to green as soon as ammonia began to distill over.

e) After a minute or two, lowered the flask so that the tip of the delivery tube was clear of the liquid.

f) When about 20-25 ml of distillate had collected, rinsed the tip of the tube with little water and removed the flask.

g) Stopped the entry of steam. The distillation flask will empty automatically and the vacuum generated can be used to rinse the apparatus by immersing the tip of the delivery tube in distilled water.

h) Titrated the distillate against 0.01 N HCl to the pink color of the indicator.

i) Carried out titration for the reagent blank also.

Calculation:

$$\%\ \text{Nitrogen in soil} = \frac{\text{volume of 0.01 N HCl used (ml)} \times 0.014 \times\ 50}{\text{Volume of aliquots used (ml)}}$$

Phosphatase activity in soil (Tabatabai and Bremner 1969)

Reagents

1. p- nitrophenyl phosphate solution (0.115M) : Dissolved 4.268 g of p-nitrophenyl phosphate disodium salt hexahydrate in 100 ml of buffer.

2. NaOH (0.5N): Dissolved 20 g NaOH pellets in distilled water and made the volume to one litre.
3. p- nitrophenol (PNP) : (1 mg/ml) solution in modified universal buffer.
4. Calcium chloride (0.5M): Dissolved 73.5 g in one liter distilled water.
5. Toluene: Concentrated.
6. 5X Modified Universal Buffer (MUB) (Skujins et al. 1962)

Tris (hydroxyl methyl) amino methane	12.10 g
Maleic acid	11.60 g
Citric acid	14.00 g
Boric acid	6.28 g
NaOH (1N)	488 ml
Distilled water up to	1000 ml

Filter sterilized the modified universal buffer and diluted to 1x to make working buffer solution. The pH was adjusted to 5.5-6.5 for acid phosphatase activity and 7.5-11.0 for alkaline phosphatase activity.

Procedure

a) Weighed 1 g soil sample (air dry, <0.25 mm) and transferred into a flask.
b) 4 ml modified universal buffer, 0.25 ml toluene and 1ml of filter sterilized 0.115 M disodium p- nitrophenyl phosphate solution was added in the flask
c) Swirled the flasks for few seconds to mix the contents.
d) The flasks were stoppered and incubated at 37 °C for one hour in the dark.
e) Added 1ml of 0.5M $CaCl_2$ and 0.5M NaOH to stop the reaction.
f) The mixture was swirled and filtered through Whatman No 12 filter paper.

g) Transferred the filtrate to glass cuvette and measured the yellow color at 420 nm.

h) Phosphatase activity was indicated as the amount of p- nitrophenol released in the filtrate from the p-nitrophenyl phosphate substrate per gram of the mycelium. The p-nitrophenol content was calculated with reference to a calibration graph plotted from the results obtained by standards containing 0, 10, 20, 30, 40 and 50 µg of p-nitrophenol.

i) To perform controls, followed the procedure described for the assay but made the addition of 1 ml p-nitro phenyl phosphate after the addition of 0.5N NaOH (i.e. immediately before filtration).

Calculation:

$$\text{Phosphatase activity } (\mu\text{M PNP/g mycelium/hour}) = \frac{\text{Concentration of PNP}(\mu\text{M})}{2\times\text{weightof mycelium}(\text{g})}$$

Phytase activity in soil (Kim and Lie method 2005)

Extraction

a) Weighed 5g of sample into a 250 ml shaking bottle and 10 ml of sodium bicarbonate (0.5M) extractant was added.

b) Shook for 30 minutes and centrifuged for 5 minutes at 12000 rpm.

c) Took supernatant in aliquots of 0.2 ml and determined the phytase activity by method given by Kim and Lie (2005) as described previously.

Analysis of wheat for aflatoxins

Monitoring grain for the presence of aflatoxins is important to ensuring consumer safety. Wheat samples from the plots inoculated with P-solubilizing fungi were analyzed for B1, B2, G1 and G2 aflatoxins. Extraction method was based on AOAC-990.33 (2005).

1. 50 gm of wheat sample was mixed with 200 ml methanol and 50 ml 0.1N HCl and homogenized. After homogenization 125 ml the sample was filtered through filter paper in separatory funnel.
2. 50 ml of 10% NaCl and 50 ml of Hexane was added to the filtrate and shaken for 1 min. After layer separation lower layer was collected in another separatory funnel (Upper layer was discarded).
3. 50 ml of dichloromethane (DCM) was added and shaken for 1 minute. After separation lower layer of DCM was collected in a beaker. The extraction was repeated for two more times with 25 ml DCM and all the extracts were collected. The extracts were filtered through sodium sulphate to remove water. The filtered extract was evaporated to 2 ml on rotavapour.
4. Slurry of 2 gm of silica gel was made with 10 ml of ether-hexane (3+1) and transferred to a cleanup column.
5. After silica gel was settled column was washed with 5 ml ether-hexane (3+1). Stopcock acs opened and 1gm of sodium sulphate was added on top.
6. The DCM extract was added on top. 25 ml of benzene-acetic acid (9+1) was added to column with open stopcock. 30ml of ether-hexane (3+1) was added. Drained each wash on top of sodium sulphate. The washes were discarded.
7. Aflatoxin was eluted with 100 ml of DCM-acetone (90+10). The elute was evaporated to 5 ml on Rotavapour. The elute was transferred to test tube and was evaporated to dryness on water bath in presence of nitrogen stream.

8. 200 μl of hexane was added to dried extract followed by 50 μl of triflouroaceticacid (TFA). Mixed on vortex for 30 seconds. After 5 minutes 0.950 ml of water-acetonitrile (9+1). Mixed on vortex for 30 seconds and kept for 10 minutes.
9. After the layers were separated the lower layer was kept for HPLC.
10. HPLC was used for separating these potentially carcinogenic aflatoxins using Zorbax-ODS (Agilent). The mobile phase consisted of solvent mixture of deionized water, methanol, and acetonitrile at a flow rate of 0.8 ml min^{-1}.
11. The concentration of aflatoxins B1, B2, G1 and G2 were calculated by comparing the peaks obtained in case of reference standards.

Statistical analysis

Three replicates were used for each experiment. The data were analyzed by analysis of variance (ANOVA) and the means were compared with Tukey's test at $P < 0.05$. In the field experiment the plots with different treatments were arranged in a randomized complete block design with three replicates per treatment. Data was analyzed by ANOVA and the means were compared using Tukey's test at $P < 0.05$. All the analysis was performed by using GraphPad Prism 4.03 software.

Results

Chapter 4

Diversity of phosphate solubilizing microorganisms

4.1 Isolation of Phosphate Solubilizing Microorganisms

4.1.1 Isolation of phosphate solubilizing bacteria

Rhizosphere soil from plants growing in mine landfills of rock phosphate of Rajasthan State Mines and Minerals Limited (RSMML), Udaipur, India was collected. The bacteria isolated from these samples varied from 4 - 40x10^5 CFU/g to 1 - 12x10^{10} CFU/g of soil samples. Most of the bacterial colonies appeared at 10^{-3} and 10^{-4} dilutions. The growth was observed at even 10^{-8} dilution. Based on their morphology and growth, a total of 188 bacterial colonies were further patched on the Pikovskaya agar (PKV) plates having rock phosphate (RP) equivalent to 50 mg P_2O_5 100 ml^{-1} media out of which only 64 bacterial colonies showed solubilization zone (Fig. 4.1.). These 64 isolates were then tested for their solubilization efficiency in quantitative terms.

Figure 4.1: Bacterial colonies showing solubilization zone on Pikovskaya agar

4.1.2 Quantitative estimation of bacterial solubilization of tricalcium phosphate (TCP)

To see the solubilization efficiency of the isolates in quantitative terms, these isolates were allowed to grow for different time intervals in the PKV broth (100 ml) containing tricalcium phosphate (TCP 109 mg equivalent to 50 mg P_2O_5). It was observed that solubilization increased with the incubation period till the fifteenth day in most of the isolates after that it started decreasing. The maximum soluble P in some isolates was observed after twenty days. The pH reduction was observed in all the isolates with time period. The pH range was between 6.7 and 4.0 after twenty days. Out of 65 bacteria isolated, only 17 isolates were found to be highly efficient solubilizers of TCP which showed maximum solubilization in fifteen days. These 17 isolates are PS4F25 , C19 , PS4B7 , PS4D12 , PS4D7 , 2C9 , PS8H3 , PS4H7 , SC4 , 4G2 , SC36 , SC18 , SC6 , PS4B18 , PS4D6 , PS4F9 and PS4C9. These isolates showed solubilization between 158 to 278 µg/ml of P (Table 4.1)

Table 4.1: Soluble P and pH reduction of the Pikovskaya media (TCP equivalent to 50 mg P_2O_5) inoculated with 65 bacterial isolates after different time intervals.

Bacterial isolates	**5 Days**		**10 Days**		**15 Days**		**20 Days**	
	Soluble P (µg/ml)	**pH**	**Soluble P (µg/ml)**	**pH**	**Soluble P (µg/ml)**	**pH**	**Soluble P (µg/ml)**	**pH**
PSWS	105.72±6	5.32±0.4	83.81±5	4.44±0.5	62.00±4	4.95±0.5	62.80±1	4.91±0.2
PSWR	60.95±3	4.85±0.3	90.48±4	4.27±0.1	87.62±2	4.02±0.1	86.62±5	4.04±0.4
PS4F8	74.29±4	4.99±0.2	117.15±3	4.78±0.2	82.86±1	4.75±0.2	82.39±4	4.72±0.5
PS4F9	**54.29±3**	**4.02±0.3**	**150.10 ±2**	**3.99±0.4**	**158.48 ±3**	**3.65±0.4**	**144.48±2**	**3.61±0.1**
PS4B18	**85.72±4**	**4.89±0.4**	**142.00 ±1**	**4.45±0.3**	**194.00 ±5**	**4.33±0.3**	**143.00±3**	**4.30±0.3**
PS2D12	77.14±2	6.01±0.3	155.00 ±3	5.98±0.2	168.00 ±4	5.85±0.2	105.20±5	5.75±0.5
PS2D11	71.43±4	4.44±0.1	121.00 ±4	4.23±0.4	126.00±2	4.13±0.1	128.60±1	4.03±0.3
PS4B19	86.67±3	4.80±0.4	95.00±2	4.43±0.1	123.00±3	4.21±0.5	125.80±4	4.20±0.1
PS6F1	98.10±3	4.63±0.3	112.38±1	4.56±0.3	120.00±5	4.36±0.4	132.00±2	4.33±0.2
PS2H1	68.57±5	4.55±0.4	74.29±5	4.46±0.5	72.38±1	4.30±0.2	76.38±3	4.30±0.4

PS4F3	44.76±2	6.63±0.2	69.53±4	5.65±0.4	71.43±4	5.53±0.3	65.43±5	5.52±0.5
PS8D1	77.14±1	6.61±0.4	30.48±3	5.71±0.2	80.00±2	5.55±0.1	86.00±1	5.51±0.1
PS4H20	38.10±3	4.47±0.1	80.00±2	4.33±0.3	100.00±5	4.16±0.5	112.00±3	4.17±0.3
PS4H17	54.29±4	6.65±0.5	135.24±4	6.59±0.5	107.62±3	6.56±0.2	101.62±2	6.52±0.2
PS2D3	60.95±5	5.33±0.3	151.43±5	5.30±0.1	137.15±1	5.26±0.4	125.15±4	5.25±0.4
PS4B1	35.24±2	4.51±0.2	149.53±1	4.48±o.4	96.19±3	4.46±0.3	90.19±5	4.43±0.5
PS4F23	56.19±1	4.62±0.1	32.00±3	4.47±0.2	57.14±5	4.41±0.5	57.80±1	4.44±0.4
PS4F24	48.57±3	4.48±0.4	20.00±2	4.25±0.1	130.48±2	4.14±0.1	131.88±4	4.10±0.1
PS4F19	28.57±4	6.72±0.3	54.00±4	6.69±0.4	120.95±4	6.60±0.3	124.95±2	6.55±0.3
PS4F21	44.76±5	5.21±0.5	75.00±4	4.27±0.3	136.19±3	4.02±0.4	132.19±1	4.00±0.5
PS8H13	53.33±2	5.45±0.3	28.00±5	4.74±0.5	43.81±5	4.69±0.2	57.81±4	4.67±0.1
PS4H14	58.10±4	6.10±0.1	58.00±3	4.98±0.2	134.29±1	4.84±0.1	128.29±2	4.83±0.2
PS4H15	59.05±1	5.72±0.2	5.00±4	4.97±0.4	73.33±3	4.70±0.5	77.33±5	4.72±0.4
PS4M6	77.14±3	6.04±0.4	68.00±2	5.46±0.1	129.53±2	5.06±0.3	123.53±3	5.00±0.3
PS4M8	52.38±4	5.28±0.3	60.00±1	4.55±0.2	141.91±4	4.08±0.2	145.91±4	4.01±0.2

PS4E24	49.52±5	5.81±0.2	28.00±5	4.66±0.5	76.19±5	4.38±0.1	77.92±2	4.33±0.4
PS4E23	83.81±2	6.64±0.5	74.00±4	6.32±0.4	81.91±1	6.15±0.4	82.59±5	6.11±0.5
PSG	113.34±3	5.52±0.1	31.00±3	4.45±0.1	105.72±4	4.14±0.3	113.72±3	4.13±0.1
PS4H14	48.57±1	6.69±0.4	134.00±4	5.53±0.3	138.00±2	5.42±0.5	127.80±1	5.40±0.3
PS4H11	63.81±2	4.30±0.3	61.00±2	3.95±0.4	86.00±3	3.89±0.4	90.60±4	3.85±0.1
PS4H12	32.38±2	5.35±0.5	88.00±3	5.11±0.2	107.00±1	4.58±0.2	103.00±2	4.59±0.4
PS6F1	54.29±3	4.65±0.1	99.00±1	3.86±0.1	82.00±5	3.56±0.1	86.20±3	3.54±0.5
PSS	62.86±5	5.85±0.3	88.00±4	5.21±0.5	117.00±3	4.50±0.3	111.00±5	4.52±0.3
PS4F14	80.95±4	6.60±0.2	105.00±5	6.26±0.1	52.00±2	6.08±0.5	55.20±3	6.03±0.2
PS4H12	58.10±1	5.50±0.2	152.00±2	5.21±0.3	150.00±4	4.04±0.2	151.00±2	4.00±0.2
PS4H13	63.81±2	5.20±0.5	140.00±3	4.88±0.2	104.00±1	4.19±0.1	110.40±4	4.14±0.4
PS4H12	58.10±4	5.80±0.2	140.00±4	5.64±0.4	124.00±3	4.09±0.4	122.40±1	4.06±0.5
PS8H22	39.05±2	5.12±0.1	98.00±5	4.86±0.5	83.00±5	4.20±0.5	87.00±5	4.12±0.3
PS8H23	38.10±5	6.11±0.5	186.00±2	5.44±0.4	97.00±2	5.56±0.3	93.00±2	5.51±0.1
PS4H28	32.38±3	5.80±0.2	122.00±4	4.87±0.2	16.00±3	4.48±0.1	15.36±4	4.43±0.4

PS2H1	68.57±1	5.39±0.4	115.00±3	5.11±0.3	78.00±5	4.14±0.3	78.60±1	4.10±0.2
2C9	**56.19±4**	**6.25±0.1**	**181.00±5**	**6.78±0.1**	**229.00±1**	**4.40±0.2**	**209.00±3**	**4.42±0.3**
PS41	56.19±2	5.80±0.3	155.00±4	5.31±0.1	136.00±3	4.45±0.4	127.60±2	4.41±0.5
PS4H2	75.24±3	5.05±0.2	112.00±3	4.22±0.3	141.00±1	4.08±0.1	101.00±4	4.04±0.1
C19	**59.05±54**	**5.50±0.1**	**265.00±1**	**4.22±0.2**	**277.80±4**	**4.38±0.5**	**245.80±3**	**4.35±0.2**
PS4C9	**40.95±1**	**6.05±0.4**	**170.00±2**	**4.31±0.4**	**190.00±2**	**4.90±0.3**	**183.00±1**	**4.87±0.4**
PS4F25	**47.62±3**	**5.55±0.2**	**117.00±5**	**5.38±0.5**	**171.00±5**	**5.11±0.2**	**165.10±5**	**5.07±0.3**
PS4H17	74.29±2	6.71±0.4	101.00±4	6.02±0.4	143.00±3	6.10±0.3	141.00±2	6.08±0.5
4G2	**99.05±5**	**5.95±0.1**	**193.00±3**	**5.11 ±0.2**	**263.00±1**	**4.19±0.1**	**243.00±4**	**4.15±0.4**
PS4H27	**62.86±4**	**4.43±0.4**	**190.00±4**	**3.59±0.4**	**188.00±4**	**3.03±0.2**	**182.80±1**	**3.00±0.2**
PS4H29	**55.24±2**	**5.40±0.3**	**177.00±2**	**4.72±0.1**	**188.00±2**	**4.62±0.4**	**186.20±5**	**4.64±0.3**
PS8H7	43.81±3	5.65±0.2	89.00±1	4.62±0.3	113.00±4	4.55±0.3	109.00±2	4.52±0.1
PS4B27	**52.38±1**	**6.15±0.5**	**163.00±5**	**4.82±0.5**	**176.00±5**	**5.20±0.5**	**161.00±4**	**5.21±0.5**
PS4D12	**58.10±4**	**5.50±0.2**	**110.00±3**	**4.21±0.1**	**178.00±1**	**4.08±0.2**	**105.00±3**	**4.03±0.2**
PS4D17	**79.05±2**	**5.05±0.1**	**181.40±2**	**4.46±0.3**	**194.00±3**	**4.38±0.4**	**161.00±1**	**4.32±0.1**

PS4D16	**58.10±5**	**6.10±0.3**	**131.00 ±5**	**5.65±0.2**	**194.00 ±2**	**5.50±0.5**	**123.00±5**	**5.52±0.4**
PS4D15	73.33±3	5.68±0.4	163.00±3	4.82±0.4	137.00±4	4.14±0.3	129.00±2	4.11±0.3
P2E4	38.10±4	6.12±0.5	49.00±1	5.47±0.5	69.00±5	5.42±0.1	63.00±4	5.32±0.5
PS1C	80.00±1	4.83±0.1	115.00±4	4.34±0.1	137.00±2	4.10±0.4	129.00±3	4.00±0.4
E1	44.76±4	4.87±0.4	53.00±5	4.68±0.3	53.00±1	4.60±0.2	51.00±1	4.40±0.3
SC 4	**64.76±3**	**5.10±0.3**	**104.00±2**	**4.55±0.2**	**201.00±4**	**4.32±0.3**	**181.00±5**	**4.22±0.2**
SC 1	82.86±2	5.64±0.5	90.00±3	5.03±0.4	91.00±3	5.12±0.1	83.00±2	5.10±0.1
SC 18	**60.95±4**	**5.70±0.2**	**201.00±1**	**4.91±0.5**	**234.00±1**	**4.80±0.5**	**214.00±4**	**4.75±0.3**
SC 36	**45.72±3**	**5.32±0.4**	**174.00±4**	**4.04±0.2**	**194.00±5**	**4.13±0.2**	**184.00±1**	**4.10±0.5**
SC6	**32.38±5**	**5.230.3**	**185.00±5**	**4.22±0.1**	**219.00±3**	**4.33±0.4**	**203.00±5**	**4.29±0.2**

Values are Mean±SD, n=3. Efficient isolates are indicated in bold.

4.1.3 Quantitative estimation of bacterial solubilization of rock phosphate

The 17 isolates namely PS4F25, PS4C19, PS4B7, PS4D12, PS4D7, PS2C9, PS8H3, PS4H7, SC4, 4G2, SC36, SC18, SC6, PS4B18, PS4D6, PS4F9 and PS4C9 were tested for solubilization of Rajasthan rock phosphate (RRP) 158.7 mg equivalent 50 mg P_2O_5 in 100ml PKV broth (Table 4.2). The soluble P in case of RP was less than TCP in all the isolates. The maximum solubilization was observed after fifteen days and after that the soluble P decreased except in PS4F25, PS4D12, PS4H7 and SC4.

Table 4.2: Soluble P and pH reduction of the Pikovskaya media (RRP equivalent to 50 mg P_2O_5) inoculated with 17 bacterial isolates after different incubation period

Bacterial isolates	**5 Days**		**10 Days**		**15 Days**		**20 Days**	
	Soluble P(µg/ml)	pH	Soluble P(µg/ml)	pH	Soluble P(µg/ml)	pH	Soluble P(µg/ml)	pH
PS4F25	12.55±4	5.32±0.1	13.38±4	4.95±0.1	17.55±3	4.25±0.5	17.85±3	4.22±0.4
C19	**15.38±3**	**4.85±0.5**	**19.55±1**	**4.02±0.2**	**29.55±1**	**4.00±0.1**	**27.10±5**	**4.56±0.2**
PS4B7	5.38±5	4.99±0.2	11.22±2	4.75±0.4	14.00±4	3.95±0.2	13.99±4	4.50±0.1
PS4D12	9.55±2	4.02±0.4	10.05±3	3.65±0.3	13.77±2	4.35±0.3	14.43±2	4.38±0.3
PS4D7	1.55±1	4.89±0.3	5.72±5	4.33±0.5	15.33±5	4.42±0.4	15.32±1	4.29±0.5
2C9	**14.22±5**	**6.01±0.5**	**21.72±1**	**5.85±0.2**	**30.31±2**	**3.84±0.1**	**30.13±4**	**4.40±0.1**
PS8H3	12.55±3	4.44±0.1	16.55±3	4.13±0.1	18.44±4	4.03±0.3	17.65±2	3.95±0.3
PS4H7	4.38±2	4.2±0.3	11.88±5	4.21±0.3	17.66±1	3.53±0.5	17.74±5	3.53±0.4
SC4	8.72±5	4.63±0.4	12.72±4	4.36±0.4	22.42±3	4.44±0.1	22.90±1	4.30±0.2
4G2	**7.72±4**	**4.55±0.2**	**7.88±2**	**4.3±0.5**	**37.33±5**	**4.02±0.4**	**34.43±3**	**3.65±0.5**
SC36	8.22±1	6.63±0.4	24.22±1	6.53±0.1	15.11±1	4.4±0.2	14.87±5	4.80±0.1
SC18	**4.88±2**	**5.71±0.1**	**9.22±5**	**5.55±0.3**	**32.00±3**	**4.38±0.3**	**31.54±2**	**4.35±0.4**
SC6	8.72±4	4.27±0.5	14.72±2	4.16±0.2	17.55±2	4.5±0.5	15.54±4	4.73±0.3
PS4B18	16.05±3	6.65±0.3	18.72±4	6.56±0.5	22.29±4	4.14±0.4	22.00±1	4.63±0.4
PS4D6	8.22±5	5.33±0.4	10.88±3	5.26±0.2	18.89±5	4.42±0.2	16.65±4	4.43±0.1
PS4F9	6.72±2	4.51±0.2	14.38±1	4.46±0.4	17.8±2	4.02±0.1	16.80±3	3.65±0.3
PS4C9	8.22±4	4.62±0.5	15.22±5	4.41±0.3	18.6±1	5.02±0.3	18.00±2	5.00±0.5

Values are Mean±SD, n=3. Efficient bacterial strains are indicated in bold.

The pH reduction (3.5 to 6.7) with increase in incubation period was observed in all the isolates. Out of 17 only 4 isolates namely C19, 2C9, SC18 and 4G2 showed significantly high solubilization range of RP i.e. 30 to 37 µg/ml whereas other isolates showed solubilization between 13 to 22 µg/ml (Table 4.2). These four efficient isolates were used for further studies.

4.1.4 Effect of different concentrations of insoluble P on solubilization

The four bacterial isolates were tested for the solubilization of higher concentrations of TCP. The solubilization range varied from 200 to 250 µg/ml in all the four bacterial isolates. The solubilization increased with increase in P_2O_5 concentration but when TCP equivalent to 1% P_2O_5 or more was added there was slight decrease in soluble P. When the solubilization at higher concentrations of RRP was tested, the results showed that the solubilization range varied from 30 to 88 µg/ml, which was not significant when compared to TCP. The solubilization increased with increase in P_2O_5 concentration but after 1% P_2O_5 the soluble P decreased. This reduction was higher than that of TCP (Figure 4.2).

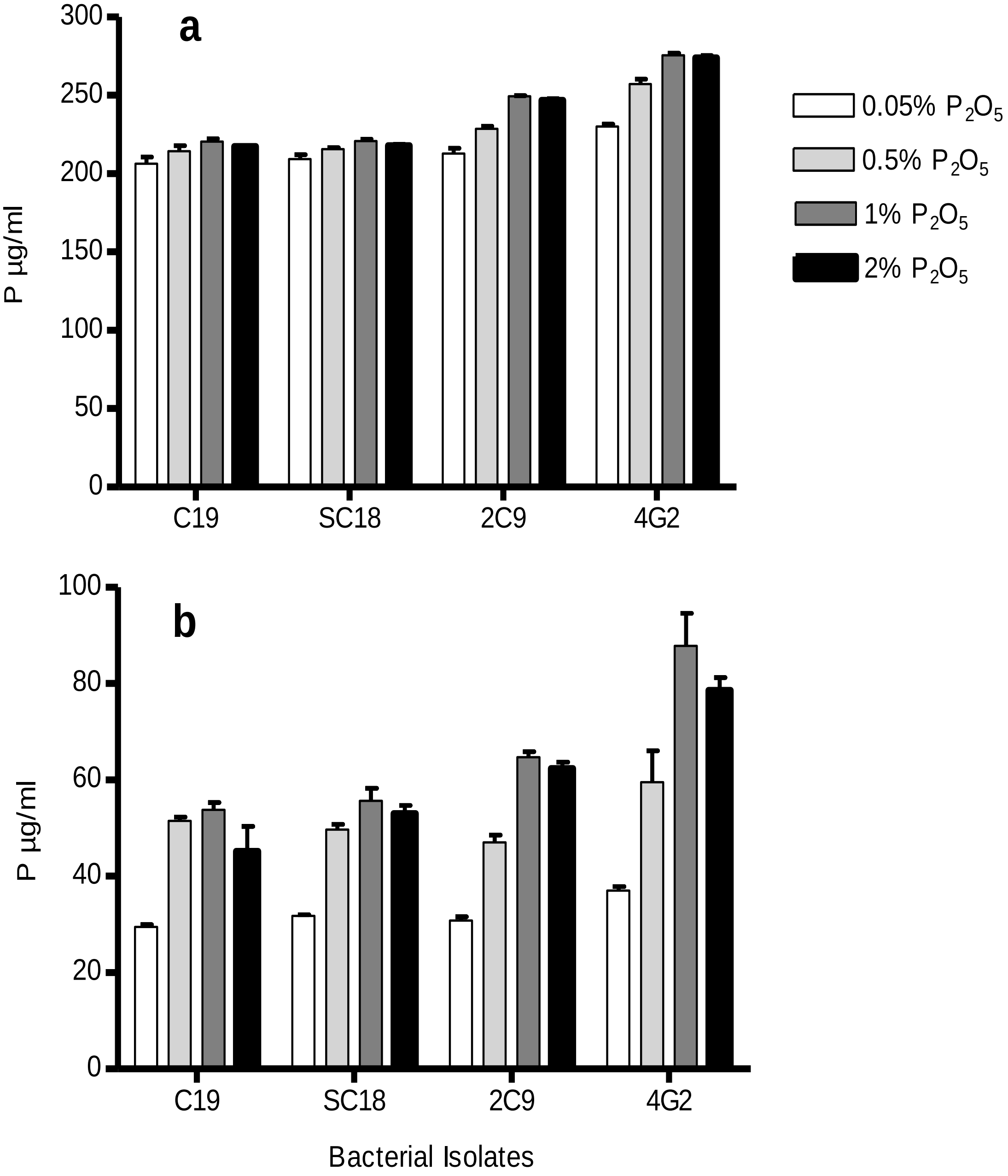


Figure 4.2: Solubilization of different concentrations of TCP (**a**) and RRP (**b**) by four bacterial isolates after fifteen days of incubation period.

4.1.5 Isolation of phosphate solubilizing fungi

The fungi isolated from rhizospheric soil samples of mine landfills of RRP varied from $1x10^4$ CFU/g to $3x10^6$ CFU/g of soil samples. Most of the fungal colonies appeared between 10^{-2} and 10^{-4} dilutions. No growth was observed at 10^{-6} to 10^{-8} dilution. Based on their morphology and growth, a total of 52 fungal colonies were further cultured on the PKV agar plates having RP equivalent to 50 mg P_2O_5 100 ml^{-1} media out of which 14 fungal colonies showed solubilization zone (Fig. 4.3).

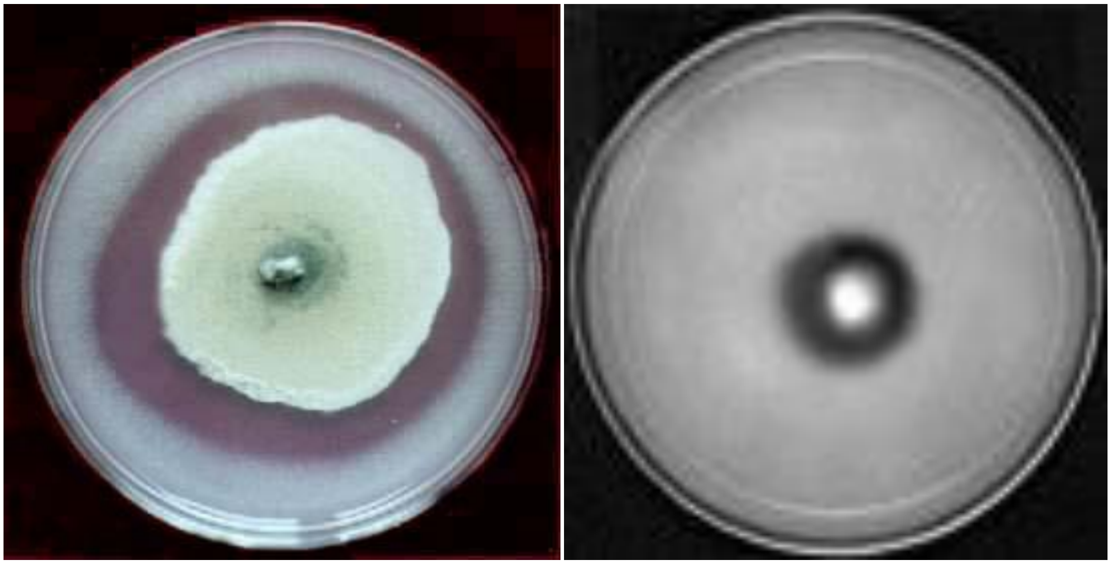


Figure 4.3: Solubilization zone in Pikovskaya agar by *Penicillium* (left) and *Aspergillus* (right).

4.1.6 Quantitative estimation of fungal solubilization of tricalcium phosphate

Solubilization of TCP (equivalent to 1% P_2O_5) by these 14 fungal isolates was tested in quantitative terms in different incubation periods (Table 4.3-4.6). The solubilization increased till the seventh day, therafter it started decreasing in all the isolates. The soluble P was more than 400 µg/ml in 2N, 5, 6, 7, 11N, 12N and 2D4 isolates. pH of the culture filtrate decreased up to the ninth day but after seven days, the reduction was very low.

The pH reduced to below 3.7 after seven days. The acid phosphatase and phytase enzyme activity was determined in the culture filtrate and it was found that there was no clear relationship between enzyme activity and amount of soluble P. However, the phytase activity was higher than acid phosphatase activity in all the isolates. When fungal dry biomass was observed after different incubation period it was found that the dry biomass of the fungi increased with increase in the incubation period.

Table 4.3: Different solubilization parameters of fungal isolates after one day in Pikovskaya media having TCP equivalent to 1% P_2O_5.

Fungal isolates	pH	Soluble P (µg/ml)	Acid Phosphatase (µM/ml/h)	Phytase activity (µM/ml/h)	Dry biomass (mg)
2N	5.38±0.1	202±10	3±0	12±0.6	163±8
6	4.57±0.2	250±12	10±0.5	420±22	150±7
5	4.64±0.2	261±13	7±0.3	288±14	156±8
3	5.2±0.4	243±12	2±0	4±0.2	146±7
7	5.23±0.3	263±13	4±0	10±0.5	158±8
3D1	4.44±0.0	216±10	14±0.5	594±30	130±6
2D1	4.93±0.2	234±11	3±0	19±0.9	140±7
2D2	4.23±0.2	**316±16**	11±0.5	463±23	**189±9**
2C2	4.41±0.2	201±10	**33±0.2**	**1469±73**	121±6
2C1	4.3±0,0	**283±14**	16±0.8	726±36	170±9
11N	4.02±0.0	256±12	26±1.3	1134±56	154±8
12N	4.85±0.3	221±11	9±0.4	390±20	132±7
10N	4.1±0.0	**327±17**	**26±1.3**	**1163±58**	**196±10**
2D4	4.6±0.2	214±11	10±0.5	434±21	129±6

Values are Mean±SD, n=3. Efficient isolates are shown in bold.

Table 4.4: Different solubilization parameters of fungal isolates after three days of incubation period in Pikovskaya media having TCP equivalent to 1% P_2O_5.

Fungal isolates	pH	Soluble P (µg/ml)	Acid Phosphatase (µM/ml/h)	Phytase activity (µM/ml/h)	Dry biomass (mg)
2N	2.56±0.1	254±13	19±0.9	842±42	196±10
6	**2.91±0.1**	**336±16**	25±1.2	1119±55	180±9
5	3.56±0.2	256±13	72±3.6	3188±160	188±9
3	3.03±0.1	236±12	34±1.7	1512±75	175±8
7	**3.03±0.1**	**399±19**	7±0.3	318±16	189±9
3D1	3.91±0.2	256±13	19±0.9	857±42	156±7
2D1	3.8±0.2	280±14	22±1.1	959±47	169±7
2D2	3.73±0.1	324±16	38±1.9	1673±85	227±11
2C2	3.81±0.2	289±14	20±1.0	900±45	145±7
2C1	3.16±0.0	280±14	42±2.1	1862±93	204±10
11N	3.72±0.1	262±18	26±1.3	1148±57	184±9
12N	4.07±0.2	320±16	47±2.3	2051±102	159±8
10N	3.48±0.2	**349±17**	26±1.3	1134±55	235±12
2D4	**2.73±0.1**	**391±19**	16±0.8	726±36	154±8

Values are Mean±SD, n=3. Efficient isolates are shown in bold.

Table 4.5: Different solubilization parameters of fungal isolates after seven days of incubation period in Pikovskaya media having TCP equivalent to 1% P_2O_5.

Fungal isolates	pH	Soluble P (µg/ml)	Acid Phosphatase (µM/ml/h)	Phytase Activity (µM/ml/h)	Dry biomass (mg)
2N	2.15±0.1	400±20	37±2	1629±81	235±12
6	**2.15±0.1**	**426±21**	13±1	580±29	216±10
5	3.08±0.2	405±20	150±8	6612±330	225±11
3	3.08±0.2	355±17	13±01	565±28	210±10
7	**3.02±0.2**	**463±23**	20±1	857±42	227±11
3D1	3.15±0.2	400±20	17±1	755±37	187±9
2D1	3.2±0.2	386±19	3±0	55±27	202±10
2D2	3.69±0.2	369±18	17±1	740±37	273±18
2C2	2.88±0.1	327±16	37±2	1629±81	174±9
2C1	3.01±0.1	318±6	16±1	711±35	244±12
11N	3.67±0.2	313±20	28±1	1235±61	221±11
12N	3.12±0.1	402±20	10±1	420±21	191±9
10N	3.61±0.2	393±20	28±1	1250±62	282±14
2D4	**3.55±0.2**	**430±21**	21±1	900±45	185±9

Values are Mean±SD, n=3. Efficient isolates are shown in bold.

Table 4.6: Different solubilization parameters of fungal isolates after nine days of incubation period in Pikovskaya media having TCP equivalent to 1% P_2O_5.

Fungal isolates	pH	Soluble P (µg/ml)	Acid Phosphatase (µM/ml/h)	Phytase Activity (µM/ml/h)	Dry biomass (mg)
2N	2.13±0.1	269±13	53±2	2328±116	243±12
6	**2.12±0.1**	**394±20**	32±1	1425±71	223±11
5	3.04±0.1	360±18	63±3	2765±138	233±11
3	3.45±0.1	373±19	72±4	3159±157	217±10
7	**3.01±0.1**	**380±2**	31±2	1367±68	235±11
3D1	3.16±0.1	276±13	18±2	784±39	194±9
2D1	3.44±0.2	347±17	69±3	3028±150	209±10
2D2	3.66±0.1	344±17	51±2	2256±112	282±14
2C2	2.11±0.0	371±18	56±3	2474±123	180±9
2C1	3.04±0.1	302±15	60±3	2634±131	253±12
11N	3.65±0.2	315±22	33±1	1469±123	229±11
12N	3.18±0.1	333±16	46±2	2037±101	198±10
10N	3.63±0.2	355±17	18±1	770±38	292±15
2D4	**3.14±0.1**	**377±19**	54±2	2387±119	192±10

Values are Mean±SD, n=3. Efficient isolates are shown in bold.

4.1.7 Quantitative estimation of fungal solubilization of rock phosphate

Pikovskaya's broth (100 ml) with RRP (3.18 gm equivalent to 1% P_2O_5) was inoculated with isolated fungal culture and after different incubation periods, the solubilization parameters were observed (Table 4.7 – 4.10). The solubilization of RRP was less than TCP. It was observed that the solubilization of RP increased with the time till the seventh days and after that the soluble P started decreasing. The pH reduced to below 5.3 after seven days. Four isolates namely 2N, 6, 7 and 2D4 showed more than 250 µg/ml of P in the culture filtrate. Extracellular phytase activity was more than extracellular acid phosphatase activity in all the isolates. Dry biomass of the fungi increased with increase in time.

Table 4.7: Solubilization parameters of fungal isolates after one day of incubation period in Pikovskaya media having RRP equivalent to 1% P_2O_5.

Fungal isolates	pH	Soluble P (µg/ml)	Acid Phosphatase (µM/ml/h)	Phytase activity (µM/ml/h)	Dry biomass (mg)
2N	4.65±0.2	2±0	286±14	8920±441	85±4
6	**4.31±0.2**	**70±3**	388±19	12482±624	117±6
5	4.76±0.3	1±0	979±48	14866±743	39±2
3	5.28±0.3	1±0	353±17	9919±495	55±3
7	**4.82±0.2**	**250±11**	2±0	15225±761	150±7
3D1	4.44±0.2	2±0	1708±85	10432±525	16±1
2D1	5.02±0.2	3±0	701±35	15456±772	156±7
2D2	**4.83±0.2**	**97±3**	133±6	11278±563	108±5
2C2	6.3±0.3	2±0	48±2	13123±656	89±4
2C1	4.4±0.2	7±0	13±1	8381±419	133±7
11N	5.09±0.2	3±0	649±32	9817±490	121±6
12N	5.85±0.3	2±0	200±10	2896±145	121±6
10N	4.43±0.2	2±0	341±17	9253±462	107±5
2D4	**4.08±0.1**	**101±5**	267±13	11816±592	139±6

Values are Mean±SD, n=3. Efficient isolates are shown in bold.

Table 4.8: Solubilization parameters of fungal isolates after three days of incubation period in Pikovskaya media having RRP equivalent to 1% P_2O_5.

Fungal isolates	pH	Soluble P (µg/ml)	Acid Phosphatase (µM/ml/h)	Phytase Activity (µM/ml/h)	Dry biomass (mg)
2N	3.95±0.2	256±12	1517±75	8324±416	154±7
6	**3.84±0.1**	**283±14**	3340±167	10580±529	**170±8**
5	4.73±0.2	201±10	3114±155	18386±919	120±6
3	5.25±0.2	231±11	632±31	8236±411	138±6
7	**4.78±0.2**	**458±22**	148±7	15105±755	**275±13**
3D1	3.88±0.1	207±10	3097±154	11585±579	124±6
2D1	4.26±0.2	223±11	437±21	6873±343	134±6
2D2	4.04±0.1	262±13	2715±135	17703±885	157±7
2C2	5.01±0.2	215±11	468±23	7006±350	129±6
2C1	4.22±0.2	245±12	367±13	10013±500	147±7
11N	4.32±0.2	234±12	2333±116	3657±182	141±7
12N	3.98±0.1	245±11	753±37	3452±172	147±7
10N	3.85±0.2	243±12	1638±81	7313±365	146±7
2D4	**4.04±0.2**	**398±19**	361±13	17703±885	**239±11**

Values are Mean±SD, n=3. Efficient isolates are shown in bold.

Table 4.9: Solubilization parameters of fungal isolates after nine days of incubation period in Pikovskaya media having RRP equivalent to 1% P_2O_5.

Fungal isolates	pH	Soluble P (µg/ml)	Acid Phosphatase (µM/ml/h)	Phytase Activity (µM/ml/h)	Dry biomass (mg)
2N	3.9±0.2	231±11	1547±77	15823±791	158±7
6	**3.8±0.1**	**273±13**	3407±170	9877±493	**175±8**
5	4.7±0.2	181±9	3176±158	16917±845	124±6
3	5.2±0.3	137±7	644±32	12816±640	143±3
7	**4.7±0.2**	**368±18**	151±7	17258±862	**283±14**
3D1	3.8±0.2	200±10	3159±157	7758±387	128±6
2D1	4.2±0.2	147±7	445±22	6937±346	138±7
2D2	4±0.2	259±12	2769±138	17361±868	162±8
2C2	5±0.3	173±8	478±23	112±2	133±6
2C1	4.2±0.2	148±7	374±18	15994±799	151±7
11N	4.3±0.2	216±10	2380±119	3247±162	145±4
12N	3.9±0.2	148±7	768±38	16130±806	152±5
10N	3.8±0.1	121±6	1671±83	1914±95	150±3
2D4	**4±0.2**	**395±19**	368±13	16746±837	**246±12**

Values are Mean±SD, n=3. Efficient isolates are shown in bold.

Table 4.10: Solubilization parameters of fungal isolates after twelve days of incubation period in Pikovskaya media having RRP equivalent to 1% P_2O_5.

Fungal isolates	pH	Soluble P (µg/ml)	Acid Phosphatase (µM/ml/h)	Phytase activity (µM/ml/h)	Dry biomass (mg)
2N	3.9±0.2	216±10	1570±78	16062±803	164±8
6	**3.8±0.1**	**248±12**	3458±172	15447±772	**181±9**
5	4.7±0.2	181±9	3224±161	17258±862	128±6
3	5.2±0.3	117±5	654±32	14593±729	148±7
7	**4.7±0.2**	**337±16**	153±7	**17361±868**	**293±14**
3D1	3.8±0.1	186±9	3206±160	16404±820	133±6
2D1	4.2±0.2	139±7	452±22	14456±722	143±7
2D2	4±0.2	205±10	2811±140	17429±871	167±8
2C2	4.9±0.2	123±6	485±24	6322±316	137±6
2C1	4.2±0.2	271±13	380±19	16882±844	157±7
11N	4.3±0.2	165±8	2415±120	6391±319	150±7
12N	3.9±0.2	137±6	780±39	16746±837	157±7
10N	3.8±0.1	169±8	1696±84	10765±538	155±7
2D4	**4±0.2**	**387±14**	373±18	16643±832	**254±12**

Values are Mean±SD, n=3. Efficient isolates are shown in bold.

4.2 Microbial diversity of phosphate mine landfills

4.2.1 Characterization of bacteria

The bacterial isolates were screened for solubilization in quantative terms which showed that only four bacterial isolates namely C19, 2C9, SC18 and 4G2 solubilized TCP as well as rock phosphate (RP) to greater extent as compared to other bacterial isolates. These bacterial isolates had diverse morphotypes. The diversity found either in cell or in colony morphology was rather discrete. 2C9 and SC18 were yellowish in color and other two isolates were whitish to cream whitish in color. All the four isolates showed gram positive reaction (Table 4.11). All isolates were catalase positive. 2C9, SC18 and 4G2 oxidase positive and C19 was oxidase negative (Table 4.11). The ability to reduce nitrate was a positive character of all isolates. All isolates were found to be negative for utilization of citrate. 2C9 and 4G2 showed negative response for urease production while C19 and SC18 showed small production of extracellular urease (Table 4.11). Extra-cellular acid phosphatase was positive for all the isolates. 2C9 and SC18 were found to hydrolyze xylan and carboxy methyl cellulose (CMC) extracellularly while C19 and 4G2 failed to produce xylanase and cellulase in presence of substrates xylan and CMC respectively (Table 4.11). For all other biochemical tests such as lysine, ornithine, ONPG, TDA hydrolysis and H_2S production, these isolates were found to be negative.

Table 4.11: Biochemical characterization of the bacterial isolates

Isolates	Gram Staining	Catalase	Oxidase	Nitrate Reduction	Citrate Utilization	Urease	Xylanase/ CMCase	Acid phosphatase
C19	+	+	-	+	-	±	-	+
SC18	+	+	+	+	-	±	+	+
2C9	+	+	+	+	-	-	+	+
4G2	+	+	+	+	-	-	-	+

+: positive reaction; -: negative reaction and ±: weak positive

Biolog Test

Different carbon substrate utilization patterns of bacterial isolates were observed and matched with the BIOLOG database. The isolates were able to utilize number of varied carbon substrates, which ranged from 76 to 82. Maximum number of 82-carbon substrates was utilized by 4G2. Different carbon substrates (76) were utilized by two isolates SC18 and 2C9 while C19 utilized 79 different carbon substrates. Homology searching of the Biolog database revealed that all the four isolates show similarity with genus *Bacillus.*

Carbohydrate fermentation and antibiotic profiling

The ability to ferment various carbohydrates by these bacteria was determined. Majority of the isolates were able to ferment different carbon substrates (Table 4.12). Bacterial isolates were tested for their sensitivity to different antibiotics. Three P solubilizing isolates were found resistant to some antibiotics, but rest all other isolates were sensitive (Table 4.12).

Table 4.12: Fermentation of carbon substrates and antibiotic profiling of the bacterial isolates

Isolates	**Carbon substrate fermentation***	**Antibiotic Resistance**
C19	Xylose, Maltose, Fructose, Dextrose, Galactose, L-Arabinose, Mannose, Sodium gluconate, Rhamnose	Norfloxacin, Netillin, Chloramphenicol, Ceftazidime
SC18	Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, L-Arabinose, Mannose, Sodium gluconate, Rhamnose, Mannitol	Norfloxacin, Netillin, Cefaclor, Chloramphenicol, Amikacin,
2C9	Xylose, Maltose, Fructose, Dextrose, Galactose, L-Arabinose, Mannose, Sodium gluconate, Rhamnose, Mannitol	Norfloxacin, Netillin, Cefaclor, Chloramphenicol, Amikacin, Ceftazidime
4G2	Xylose, Maltose, Fructose, Dextrose, Galactose, L-Arabinose, Mannose, Sodium gluconate, Rhamnose	Norfloxacin, Netillin, Cefaclor, Chloramphenicol, Cephadroxil Amikacin, Ceftazidime

[#] Total of 35-carbohydrate fermentation tests was performed with isolated bacterial species using HiCarbo Kit (Himedia Lab., Bombay, India).

4.2.2 Identification of bacterial isolates

The bacterial isolates were subjected to 16S rDNA amplification and about 1.5 kb amplicon was observed in all isolates (Fig 4.4). In RFLP (using *Hinf I*) analysis of these PCR products it was observed that bacterial isolate 2C9 (lane 6) and SC18 (lane 8) showed same banding pattern while other two, C19 and 4G2 (lane 7 and 8) showed entirely different restriction pattern as clear in Figure 4.4.

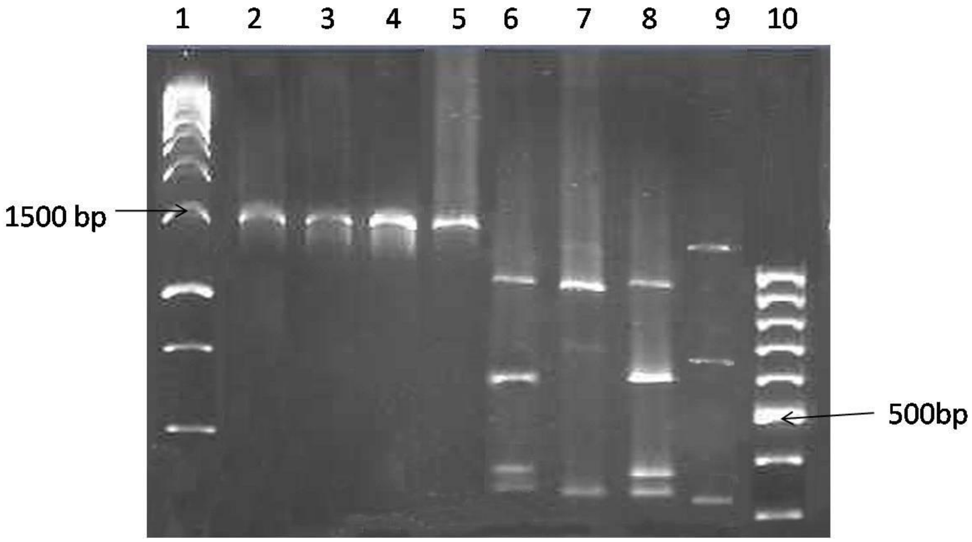


Figure 5.1: Lane1: 1Kb marker, Lane 2: amplified product of 2C9, Lane 3: amplified product of C19, Lane 4: amplified product of SC18, Lane 5: amplified product of 4G2; Amplified products digested with ***Hinf* I:** Lane 6: 2C9, Lane 7: C19, Lane 8: SC18, Lane 9: 4G2 and Lane 10: 100 bp Marker

Prior to sequencing, PCR products were purified and subcloned using Ins T/A clone PCR cloning kits (Fermentas, USA) according to the manufacturer's instructions and incorporated into transformed *E. coli DH5α* cells. Transformants were screened for the 16S rRNA inserts and sequenced (DNA sequencing facility, University of Delhi, Delhi, India). These gene sequences were submitted to the GenBank NCBI database under the accession numbers HQ156781 to HQ156784 and HQ439438 (Appendix II). Nucleotide sequence comparisons were performed using the BLAST database (Altschul et al. 1997). Sequence data from BLAST results showed that out of four bacterial isolates two sequences (2C9 and SC18) showed maximum similarity (99%) with *Bacillus pumilus*.The sequence of 4G2 showed close similarity (99%) with *B. cereus.* Sequence analysis of C19 showed similarity (99-98%) to sequence of unknown *Bacillus spp*.

The Phylogenetic tree (Fig. 4.5) clearly differentiated the sequences of bacterial isolates in three clades naming A (*B. pumilis*), B (*B. cereus*) and C (*Bacillus sp.*). The sequences of the 2C9 and SC18 grouped together with *B. pumilis* in clade A. The sequence of 4G2 clustered into *B. cereus* (clade B) and sequence of C19 grouped with *Bacillus sp.* (clade C).

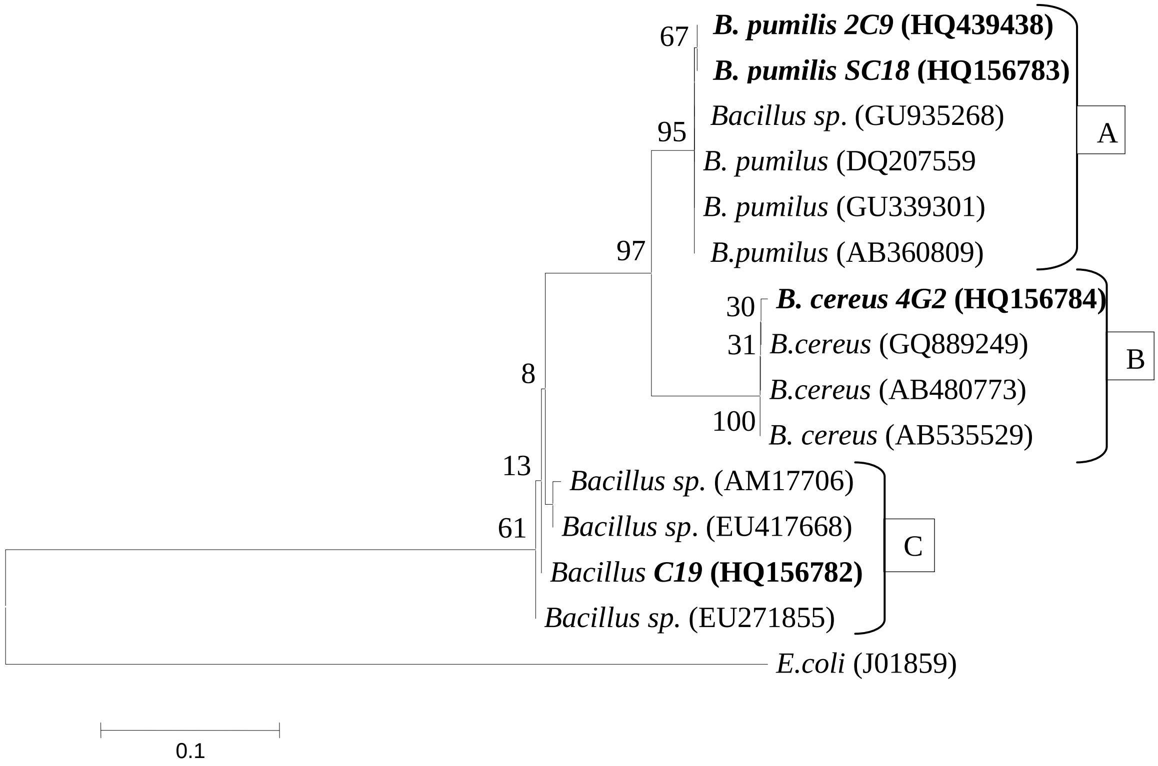


Figure 4.5: Neighbor-joining tree based on bacterial 16S rDNA sequence data from bacterial isolates, 2C9 (HQ439438), SC18 (HQ156783), 4G2 (HQ156784) and C19 (HQ156782) of current study along with sequences available in GenBank database. *E.coli* (J01859) was used as outgroup. The percentage of bootstrap analysis (1000 replications) appears at the nodes. The scale represents the number of base substitutions per site.

4.2.3 Characterization of fungi

The 14 fungal isolates were screened for solubilization efficiency. Morphological studies showed that out of 14 fungi 13 isolates (2N, 06, 05, 03, 3D1, 2D1, 2D2, 2C2, 2C1, 11N, 10N, 12N, 2D4) belonged to black mold i.e. genus *Aspergillus*, whereas one belonged to *Penicillium* (07). Out of 13 *Aspergilli* some showed characteristic morphology of *A.niger* while others showed morphological similarities with *A. tubingensis.*

Aspergillus tubingensis

The purified colonies had radial villus and white mycelia at the edge of each colony in early culture stages (3~5 d). After seven days, the colony became black at the head of mycelium and white in the reverse side in PKV agar plate, respectively.

The vesicle of *A. tubingensis* was round and radial. Diaphragms in hypha were visible under microscope. The value of metulae and conidial diameter were about 6 and 2.5 µm, respectively. Sporangia consisted of many spores (Fig. 4.6).

Figure 4.6: Microscopic view of *A. tubingensis* under 40x magnification.

Aspergillus niger

*A. niger o*n PKV agar, after 5 days at 28°C, the initial growth was white, which later on turned to black due to formation of black spores (Fig. 4.7) and reverse turning pale yellow. Good growth was seen at 37°C.

Microscopic morphology showed septate hyphae with smooth-walled, simple conidiophores measuring up to 1 mm in length. Conidiophores end in vesicle, which was globose and entirely covered (radiating) with two series of sterigmata (biseriate). Conidia produced from these sterigmata ranged between brown to black in color, round, rough walled and in chains measuring 4–5 µm in diameter (Fig. 4.7).

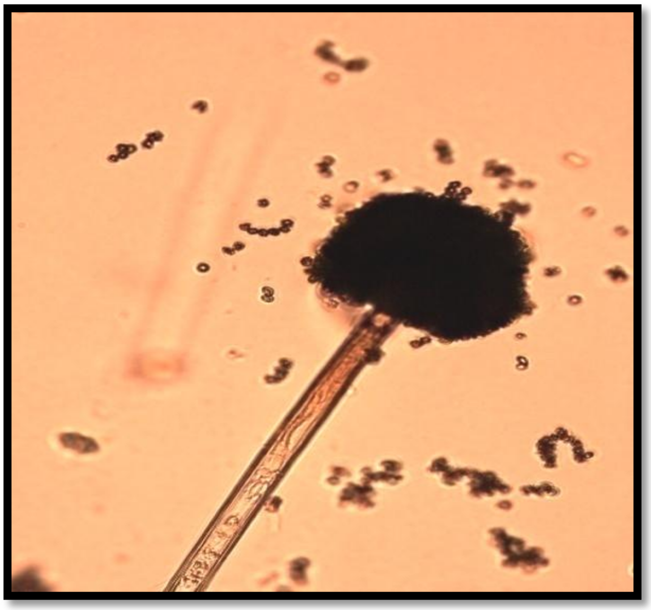


Figure 4.7: Microscopic view of *A. niger* under 40x magnification.

Differentiation in *Aspergillus niger* and *A. tubingensis*

A. niger was differentiated from *A. tubingensis* by its microscopically long conidiophores, larger conidial diameter, yellow at reverse in agar plate, whereas *A.*

tubingensis had small conidiophores, two times smaller conidial diameter, white at reverse in agar plate, shaggy hairy/villus look.

Penicillium oxalicum

Penicillium showed green colour colonies with white edges on PKV agar plates consisting of a dense felt of conidiophores. Microscopically, conidiophores showed branching and phialides produced in groups from branched metulae, giving brush-like appearance (Fig. 4.8). Conidia were globuse, greenish and smooth.

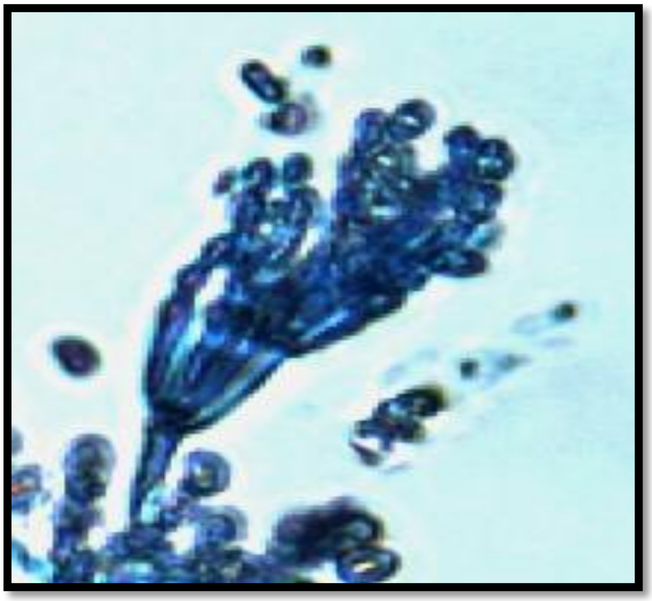


Figure 4.8: Microscopic view of *Penicillium* under 40x magnification

4.2.4 Identification of fungal isolates

Genomic DNA was isolated from the mycelia of the 14 fungal isolates. ITS region of the rDNA from genomic DNA was amplified by the PCR using ITS1 and ITS4 primers. Resultant PCR products were viewed after electrophoresis in agarose gel. The results showed that all isolates produced a single band of approximately 600 bp (Fig. 4.9).

The PCR products were cloned into pTZ57R/T Vector. The amplified products of 13 clones were subjected to restriction enzyme analysis with *RsaI* enzyme to see the variation between *Aspergillus niger* and *Aspergillus tubingensis*. The isolate 07 had already been grouped differently as *Penicillium,* on basis of its different morphology. RFLP analysis (Fig. 4.9) revealed that 06 and 03 show same banding pattern and can be grouped together as *Aspergillus tubingensis,* as these were *RsaI* negative isolates, whereas other isolates can be put together in second group i.e. *Aspergillus niger,* as it was *RsaI* positive. So a representative isolates from each group i.e. 06 and 2D4 and 07 were selected for sequencing and further studies.

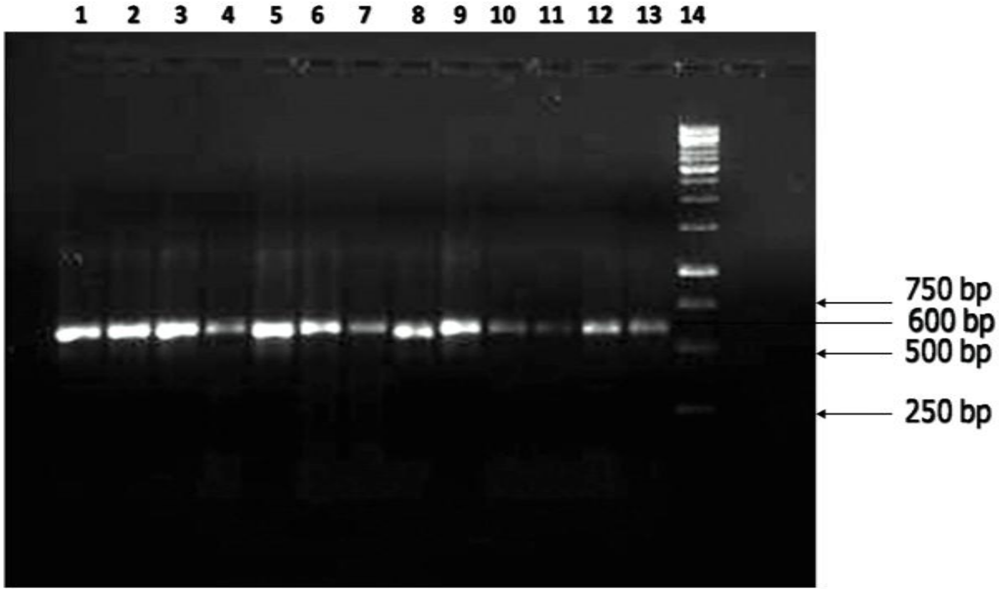


Figure 4.9: PCR products of 13 different fungal isolates: Lane1: 2N, Lane 2: 06, Lane 3: 05, Lane 4: 03, Lane 5: 2D1, Lane 6: 2D2, Lane 7: 2C2, Lane 8: 2C1, Lane 9: 11N, Lane 10: 12N, Lane 11: 10N, Lane12: 2D4, Lane 13:3D1, Lane 14: 1 kb Marker

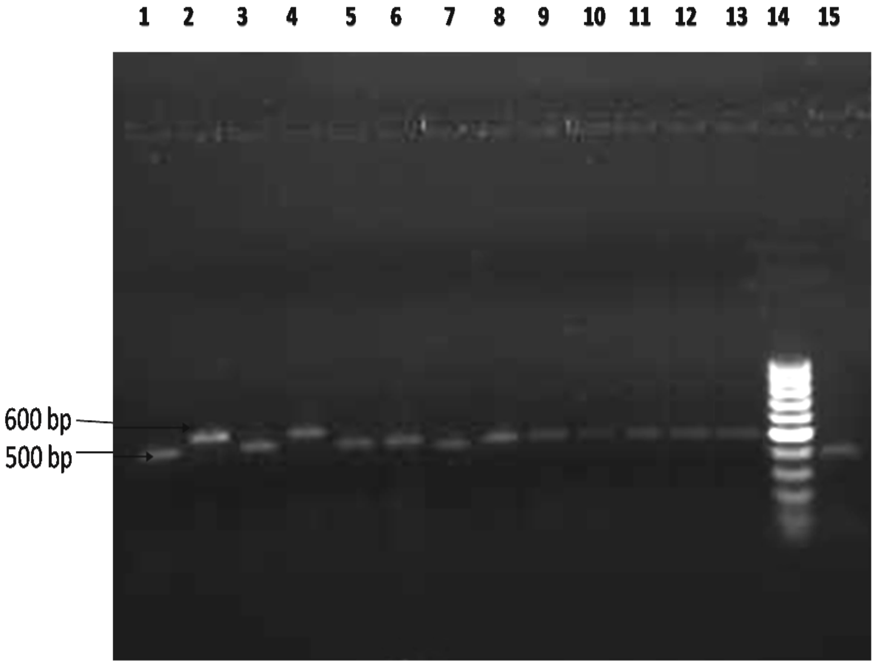


Figure 4.10: Amplified products of isolates digested with ***RsaI***: Lane1: 2N, Lane 2: 06, Lane 3: 05, Lane 4: 03, Lane 5: 2D1, Lane 6: 2D2, Lane 7: 2C2, Lane 8: 2C1, Lane 9: 11N, Lane 10: 12N, Lane 11: 10N, Lane12: 2D4, Lane 13:3D1, Lane 14: 100 bp Marker & Lane 15: 07

Amplification of ITS/5.8S rRNA region of fungi using ITS1 and ITS4 primers resulted in different size products i.e. 06 (639 bp), 2D4 (600 bp) and 07 (585 bp). These gene sequences were submitted to the GenBank NCBI database under the accession numbers HM801881 to HM801883. Sequence analysis using BLASTN revealed that 06 belonged to *Aspergillus tubingensis* and 2D4 belonged to *Aspergillus niger*. The sequence analysis of 07 showed 100% similarity with *Penicillium oxalicum*.

Phylogenetic analyses of the ITS region showed a reasonable degree of correlation with the morphological classification schemes of species within the genus. In case of *Aspergillus* (AT6 and 2D4) rooted tree was constructed using *Penicillium rugulosum* as out group. The aligned dataset contained 615 characters out of which 459 were constant, 38 were parsimony-uninformative and 118 were parsimony-informative characters. Gaps were treated as missing. The branch-and-bound search yielded parsimony tree had, Tree length (TL) = 246, Consistency index (CI) = 0.769, Retention index (RI) = 0.9135, Rescaled consistency index (RC) = 0.7018 and Homoplasy index (HI) = 0.2317. Maximum likelihood analysis recovered as single topology (-ln L = 1954.0760). In case of *Penicillium* (07), the aligned dataset contained 598 characters out of which 495 were constant, 46 were parsimony-uninformative and 57 were parsimony-informative characters. Gaps were treated as missing. The tree had, TL = 127, CI = 0.937, RI=0.966, RC = 0.905, HI=0.063. Maximum likelihood analysis recovered as single topology (-ln L = 1448.4465).

The phylogenetic trees clearly differentiated 06 (*Aspergillus tubingensis)* and 2D4 (*A. niger)* into two different clades (Fig. 4.11). *Penicillium rugulosum* (GU396584) was used as an outgroup taxa to root the tree. In *Aspergillus* tree, 06 is grouped into *Aspergillus tubingensis* and supported by high bootstrap value (BS) and posterior probability (PP) values. The sequence of 2D4 belonged to *A.niger* group. Other black *Aspergilli,* namely *A. ibericus*, *A. elipticus*, *A. fumigatus*, *A. aculeatus and A. tamarii* branched separately and were clustered into different seperate clades. In *Penicillium* tree, the sequence of 07 was grouped with *P. oxalicum* with high BS and PP values, whereas *Aspergillus japonica* (AJ876880) served as outgroup in the tree (Fig. 4.12).

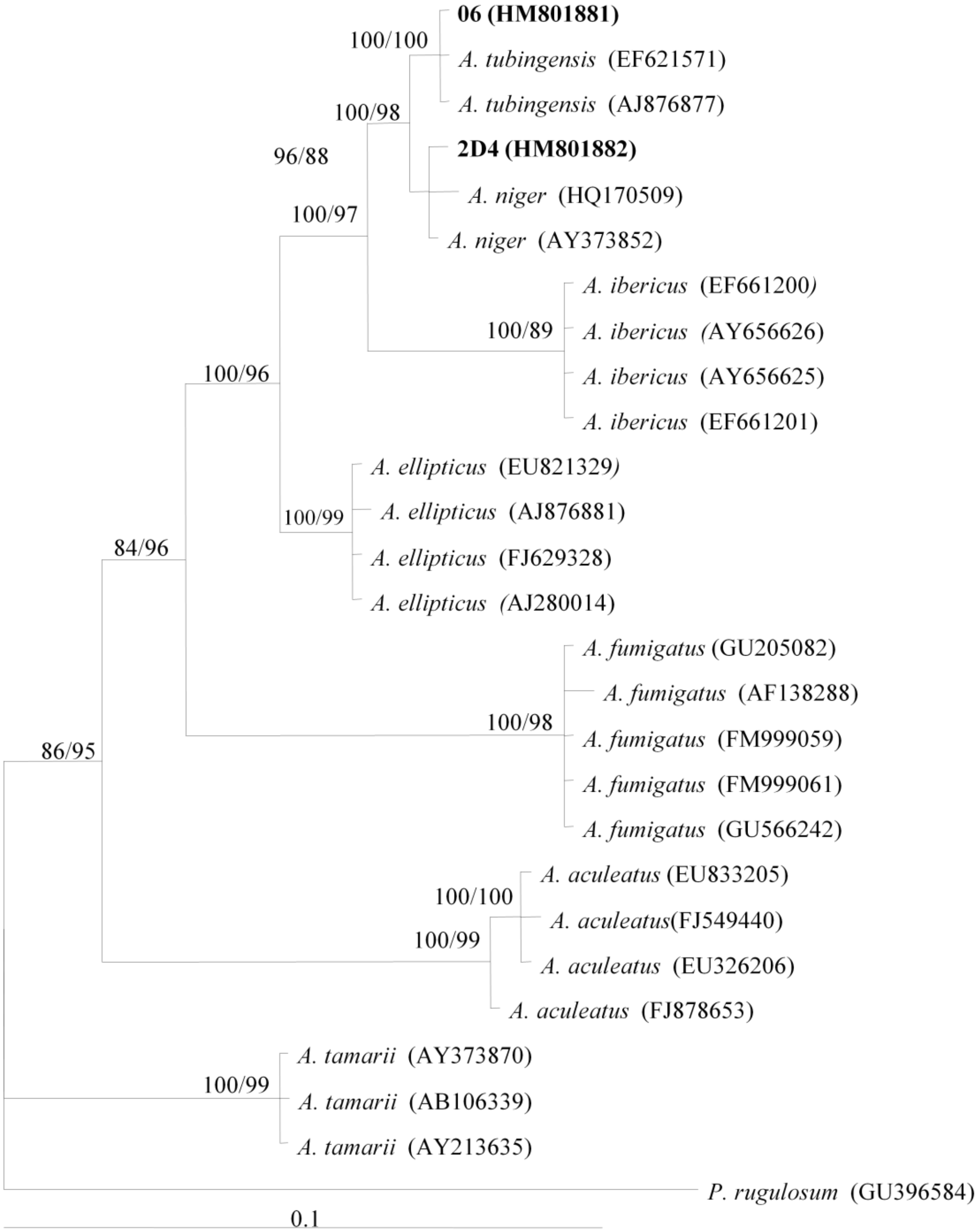


Figure 5.9: Phylogeny of 06 (HM801882) and 2D4 (HM801882) generated from Maximum Likelihood analysis of ITS sequences, rooted with *Penicillium rugulosum* (GU396584) as outgroup. Parsimony bootstrap support (BS) and Bayesian posterior probability (PP) values >85% are given at the internodes (BS/PP). The scale gives the posterior probability rate.

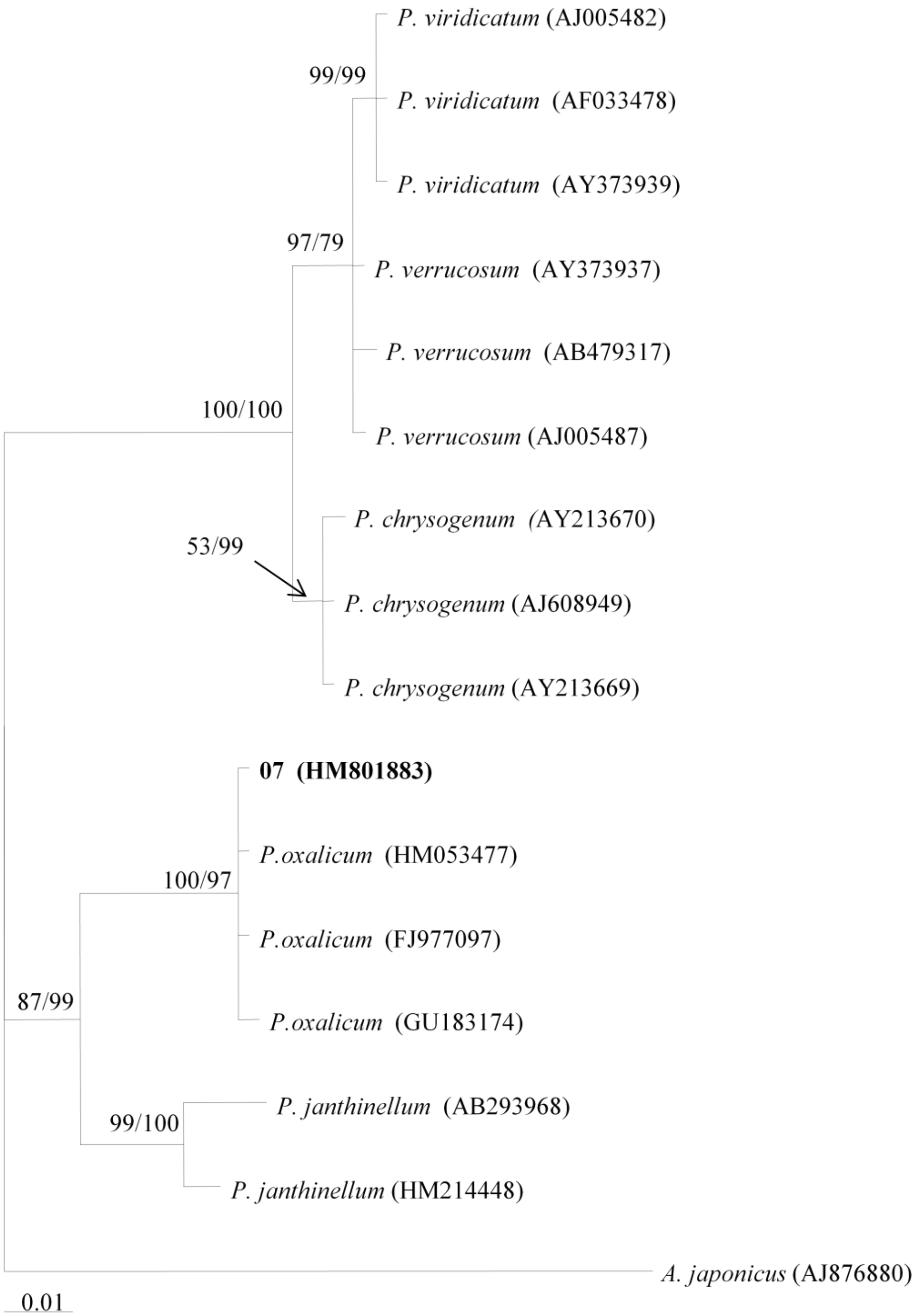


Figure 5.10: Phylogeny of 07 (HM801883) generated from Maximum Likelihood analysis of ITS sequences, rooted with *Aspergillus japonicas* (AJ876880) as outgroup. Parsimony bootstrap support (BS) and Bayesian posterior probability (PP) values >85% are given at the internodes (BS/PP). The scale gives the posterior probability rate.

Chapter 5

Physiological characterization

5.1 Effect of carbon and nitrogen sources on solubilization of TCP by bacterial isolates

To study the effect of different carbon and nitrogen sources on phosphate solubilizing activity of bacterial isolates, the four isolates (*Bacillus C19, B. pumilis 2C9, B. pumilis SC18* and *B. cereus 4G2*) were grown in presence of mannitol or xylose or fructose or sucrose or arabinose as C sources and ammonium nitrate or sodium nitrate or potassium nitrate or urea or asparagine as nitrogen sources.

5.1.1 Carbon sources

The bacterial isolates showed different solubilization patterns in different carbon sources. The results showed that *Bacillus C19* showed highest solubilization in presence of sucrose followed by mannitol as C source and correspondingly the pH reduction was also higher. However, in presence of xylose the amount of soluble P and pH reduction was found to be minimum (Fig. 5.1). The maximum solubilization as well as pH reduction in case of *B. pumilus SC18* was in the medium having glucose as C source and minimum in arabinose. In other carbon sources, the soluble P was almost same. *B. pumilus 2C9* showed best solubilization and maximum pH reduction in presence of glucose followed by sucrose amongst C sources. *B. cereus 4G2* showed highest solubilization and pH reduction in media having glucose. In all isolates, the extracellular phytase activity was much higher than extracellular acid phosphatase activity (Table 5.1).

5.1.2 Nitrogen sources

When solubilization by bacterial isolates with different N sources was tested, *Bacillus* C19 showed maximum solubilization in media having urea; however, the pH reduction in other N sources was more than in urea (Fig. 5.2). In this case the phytase activity was also highest among other N sources. Same was in case of *B. pumilus SC18,* the maximum solubilization was observed in asparagine with maximum phytase activity, but pH reduction was not highest as compared to other N sources. In case of *B. pumilus 2C9* and *B. cereus 4G2* the highest solubilization as well as pH reduction was observed in the media having ammonium sulphate as N source (Table 5.2).

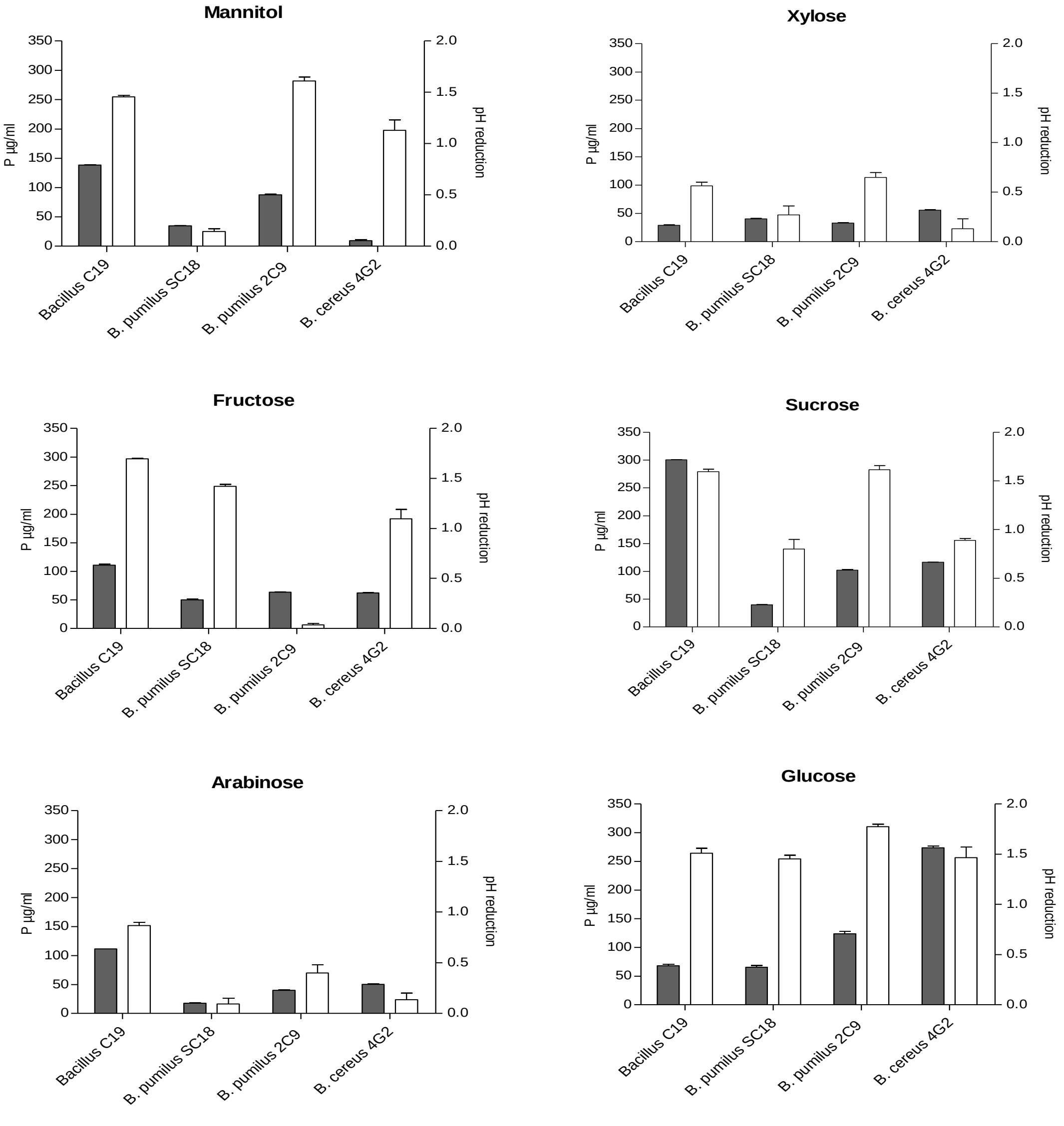


Figure 5.1: Influence of carbon sources on P solubilization (filled bars) and pH reduction (empty bars) by different bacterial isolates in presence of different C sources in PKV media having TCP equivalent to 2.3g P_2O_5/l grown for 15 days

Table 5.1: Influence of carbon sources on acid phosphatase and phytase activity by different bacterial isolates in presence of different C sources in PKV media having TCP equivalent to 2.3g P_2O_5/l grown for 15 days

Bacterial isolates	**Acid Phosphatase activity (µM/ml/h)**	**Phytase activity (µM/ml/h)**
Mannitol		
Bacillus C19	**140.8±0.6 a**	9871±68b
B. pumilus SC18	**131.3±1.4b**	9504±59c
B. pumilus 2C 9	**257.0±0.6c**	10255±59a
B. cereus 4G2	48.2±1.2d	9751±17cb
Xylose		
Bacillus C19	8.1±0.3a	10384±68a
B. pumilus SC18	9.9±0.1a	10127±34ab
B. pumilus 2C 9	1.6±0.4c	10093±34ab
B. cereus 4G2	5.7±0.3b	10008±68b
Fructose		
Bacillus C19	123.2±1.4a	10264±102ab
B. pumilus SC18	50.7±1.4b	10196±68b
B. pumilus 2C 9	44.2±0.8c	9760±59c
B. cereus 4G2	89.5±0.3d	**10272±59b**
Sucrose		
Bacillus C19	5.7±1.9d	**11067±85a**
B. pumilus SC18	112.1±0.9b	10196±85b
B. pumilus 2C 9	51.5±0.9c	10127±68ab
B. cereus 4G2	164.1±1.2a	9862±93b
Arabinose		
Bacillus C19	1.6±0.4c	10589±51a
B. pumilus SC18	9.1±0.6a	9700±102b
B. pumilus 2C 9	8.8±0.3ab	**10503±68a**
B. cereus 4G2	6.2±0.6b	9726±59b
Glucose		
Bacillus C19	50.5±1.2c	10777±68a
B. pumilus SC18	23.1±2.2d	**10409±93b**
B. pumilus 2C 9	247.3±2.5a	10435±51b
B. cereus 4G2	**176.3±1.6b**	9461±22c

Values sharing a common letter within the column and within the C source are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

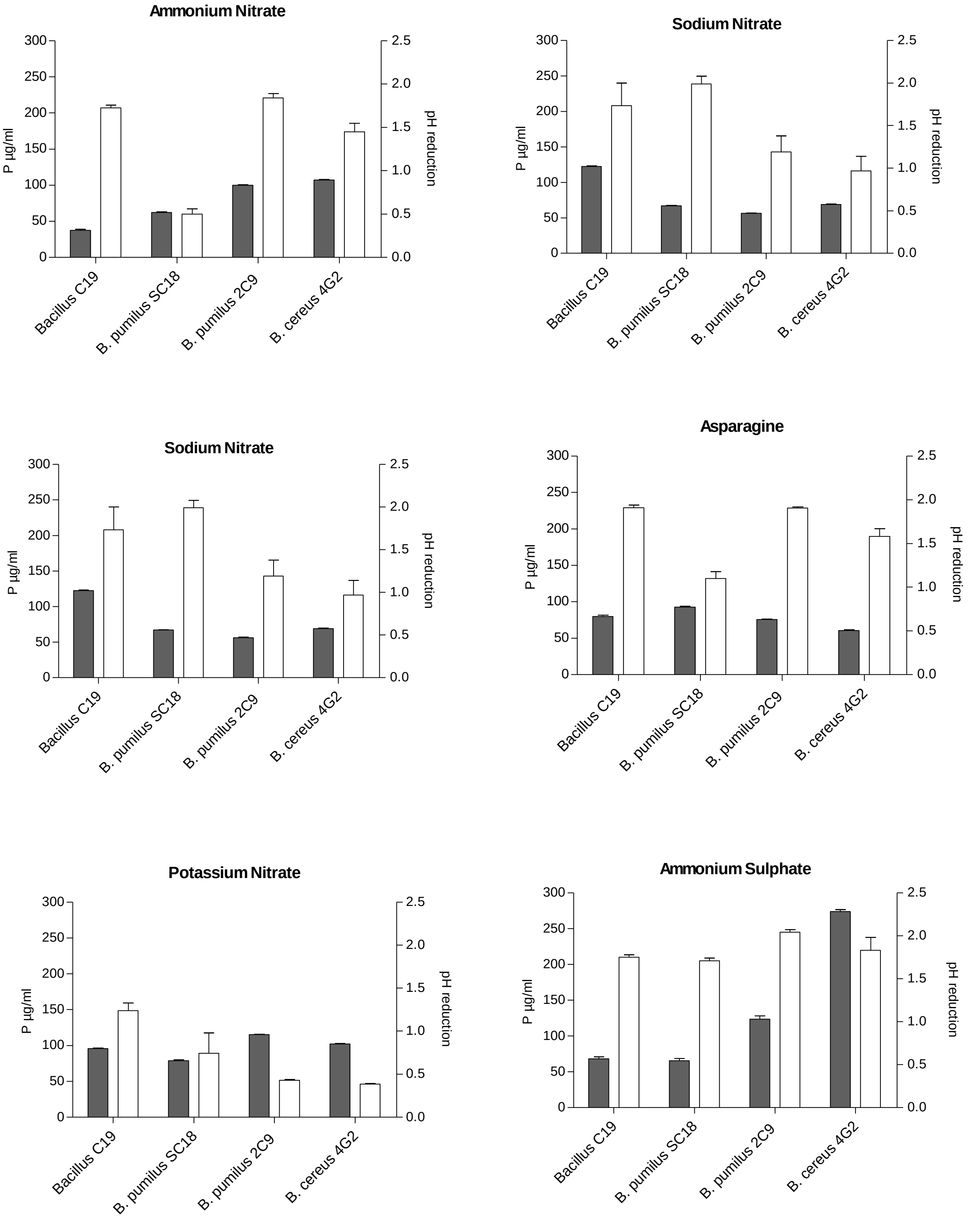


Figure 5.2: Influence of nitrogen sources on P solubilization (filled bars) and pH reduction (empty bars) by different bacterial isolates in presence of different C sources in PKV media having TCP equivalent to 2.3 g P_2O_5/l grown for 15 days

Table 5.2: Influence of carbon sources on acid phosphatase and phytase activity by different bacterial isolates in presence of different nitrogen sources in PKV media having TCP equivalent to 2.3 g P_2O_5/l grown for 15 days

Bacterial isolates	**Acid Phosphatase (µM/ml/h)**	**Phytase activity (µM/ml/h)**
Ammonium Nitrate		
Bacillus C19	81.6±0.4c	9879±76ab
B. pumilus SC18	220.2±1.2a	9572±128b
B. pumilus 2C 9	28.1±0.4d	10281±85a
B. cereus 4G2	90.8±1.2b	9803±68ab
Sodium nitrate		
Bacillus C19	43.9±1.1d	10384±68a
B. pumilus SC18	**278.4±1.1a**	10204±111a
B. pumilus 2C 9	223.9±1.4b	10102±42a
B. cereus 4G2	123.7±1.9c	10016±76a
Urea		
Bacillus C19	19.7±1.7a	**11058±76a**
B. pumilus SC18	2.6±0.4b	10204±76bc
B. pumilus 2C 9	6.0±0.8b	9760±59c
B. cereus 4G2	6.0±0.9ba	**10332±119b**
Asparagine		
Bacillus C19	41.8±1.9b	10315±153a
B. pumilus SC18	72.8±1.2a	**10495±162a**
B. pumilus 2C 9	13.4±0.9c	10170±111a
B. cereus 4G2	3.4±1.9d	9888±119a
Potassium nitrate		
Bacillus C19	**101.8±2.5a**	10598±60a
B. pumilus SC18	53.1±1.6c	9726±98b
B. pumilus 2C 9	59.7±1.1c	**105378±102a**
B. cereus 4G2	87.9±1.9b	9760±93b
Ammonium Sulphate		
Bacillus C19	50.5±1.2c	10777±68a
B. pumilus SC18	23.1±2.2d	10409±93b
B. pumilus 2C 9	**247.3±2.5a**	10435±51b
B. cereus 4G2	**176.3±1.6b**	9461±22c

Values sharing a common letter within the column and within the N source are not significant at $P < 0.05$.

Values are Mean ± SD, n=3.

5.1.3 Solubilization of RRP using best C and N sources

The solubilization of RRP (1% P_2O_5) was tested using best combination of C and N source for a particular isolate. The solubilization increased drastically as compared to PKV media (used during screening) when sucrose + urea and glucose + asparagine were used in the media for *Bacillus* C19 and *B. pumilus SC18* respectively as C and N sources. *B. pumilus 2C9* and *B. cereus 4G2* showed maximum solubilization in C and N source used in PKV media. The solubilization was same as observed during screening. There was no significant difference in the pH reduction by all the four isolates. Enzyme activity was higher in *B. pumilus 2C9* as compared to other isolates (Table 6.3).

Table 5.3: Acid phosphatase and phytase activity by different bacterial isolates in different C sources

C and N sources	**Bacterial isolates**	**pH reduction**	**Soluble P (µg/ml)**	**Acid Phosphatase (µM/ml/h)**	**Phytase activity (µM/ml/h)**
Sucrose + Urea	*Bacillus C19*	1.9±0.02a	**71.4±6.06ab**	54.1±3.5c	7789±510b
Glucose + Asparagine	*B. pumilus SC18*	2.0±0.08a	**87.7±4.90ab**	83.9±3.7b	10408±460ab
Glucose + Ammonium sulphate	*B. pumilus 2C 9*	1.5±0.17a	**65.0±4.27b**	136.5±6.9a	11467±582a
Glucose + Ammonium sulphate	*B. cereus 4G2*	2.2±0.20a	**90.8±2.54a**	79.9±2.9bc	9908±360ab

Values sharing a common letter within the column are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

5.2 Effect of carbon and nitrogen sources on solubilization of TCP by fungal isolates

To study the effect of different carbon and nitrogen sources on the growth and phosphate solubilizing activity of four efficient fungal isolates (*Aspergillus tubingensis (AT6), A.niger, A.tubingensis (AT1)* and *P.oxalicum)*; mannitol or xylose or fructose or sucrose or arabinose as C sources and ammonium nitrate or sodium nitrate or potassium nitrate or urea or asparagine as nitrogen sources were used in the growth media.

5.2.1 Carbon sources

As showed in Fig. 5.3, *A. tubingensis (AT6), A. niger, A. tubingensis (AT1)* and *P. oxalicum* were able to solubilize TCP with all carbon sources. However, the solubilization efficiency as well as acidification of the culture media varied with different carbon sources. Among the carbon sources tested, mannitol was the best for phosphate solubilization by all the isolates. The type of organic acid varied with different carbon sources (Table 5.5). It was observed that in general there was no clear relationship between P solubilization and growth (Table 5.4).

The phosphate solubilization activity in different carbon sources in AT6 were in the following order: mannitol > sucrose > glucose > fructose > arabinose > xylose. The dry biomass was highest in the media having mannitol. The maximum pH reduction was observed in sucrose followed by fructose. Acid phosphatase and phytase activity were higher in case of xylose and fructose respectively (Table 5.4). The main organic acid produced by AT6 was oxalic acid however in media having glucose malic and succinic acid and in sucrose gluconic acid was exudated in significant amount by the fungus (Table 5.5).

In case of *A. niger* the solubilization pattern was: mannitol > sucrose > glucose > xylose > arabinose > fructose. The maximum pH reduction was in the medium having sucrose as compared to other C sources. Acid phosphatase activity was maximum in mannitol, whereas phytase activity was maximum in sucrose by *A. niger* (Table 5.4). In case of mannitol the main organic acids produced were oxalic acid, malic acid and succinic acids. In sucrose amended media *A. niger* exudated oxalic acid, gluconic acid, malic acid and succinic acid in significant amount. In the media having glucose; oxalic acid, malic acid, succinic acid and formic acid were the main organic acids produced by *A. niger*. In media having arabinose, xylose and fructose the main organic acid produced was oxalic acid (Table 5.5).

In AT1 the phosphate solubilization activity was in the order: mannitol > glucose > sucrose > arabinose > xylose > fructose. The highest pH reduction was seen in case of fructose and sucrose. Acid phosphatase activity was maximum in xylose and the phytase activity was maximum in frucose by AT1 (Table 5.4). The main organic acid secreted in presence of manniol and glucose were oxalic acid, malic acid and succinic acid. In other C sources oxalic acid was produced in significantly high amount (Table 5.5).

Different carbon sources in relation to phosphate solubilization activity by *P. oxalicum* were in the following order: mannitol > glucose > xylose > sucrose > arabinose > fructose. The reduction of pH was much lesser in case of arabinose followed by xylose compared to other carbon sources. The maximum biomass was observed in the presence of mannitol (Table 5.4). Maximum acid phosphatase activity was observed in case of mannitol whereas phytase activity was higher in case of glucose followed by fructose as compared to other carbon sources. The main organic acids excreted by *P. oxalicum* in presence of different carbon sources were oxalic acid, citric acid, gluconic acid, malic acid, succinic acid and formic acid. Oxalic acid was exudated by *P. oxalicum* in all the carbon sources. The predominant organic acids in different carbon sources were: malic acid in mannitol and arabinose, oxalic acid in xylose and fructose and formic acid in sucrose and glucose (Table 5.5). The total organic acids produced were higher in case of glucose as compared to other carbon sources.

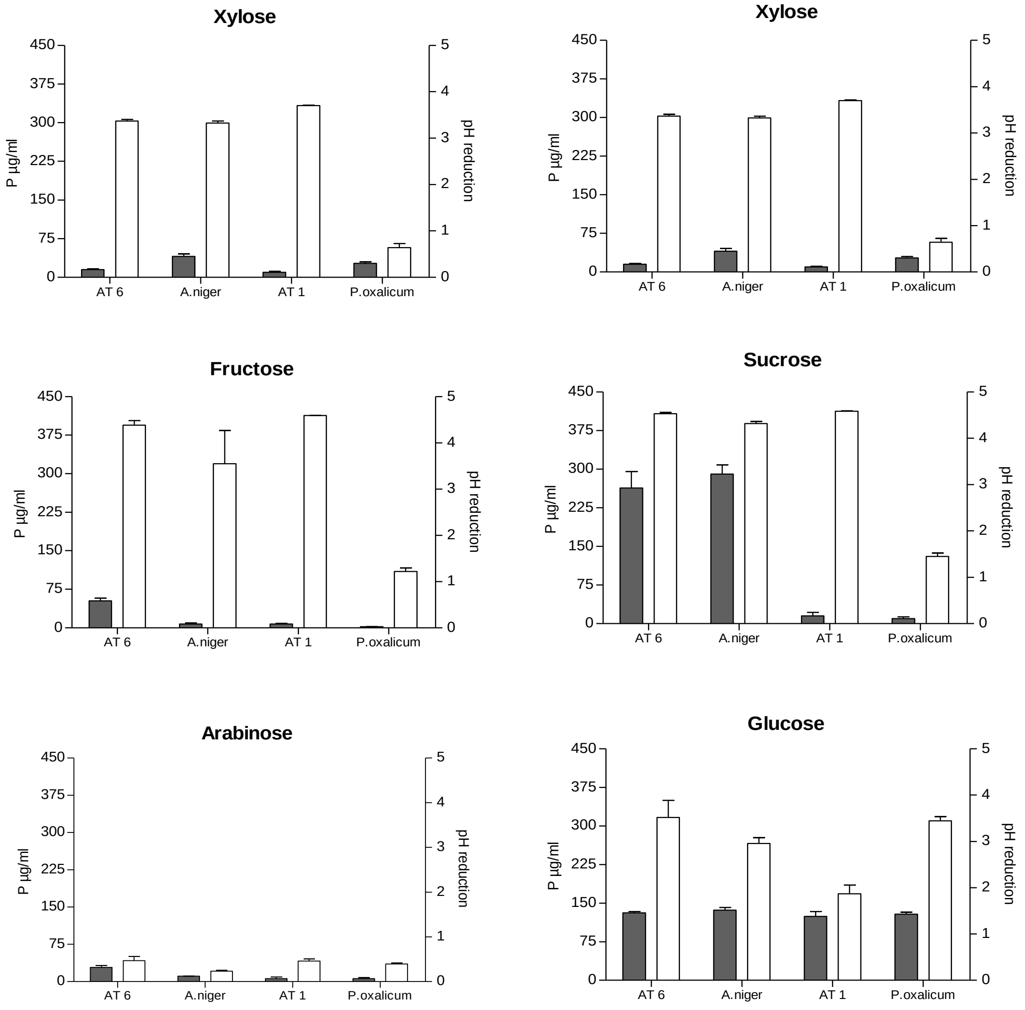


Figure 5.3: Influence of carbon sources on P solubilization (filled bars) and pH reduction (empty bars) by fungal isolates in presence of different C sources in PKV media having TCP equivalent to 2.3 g P_2O_5/l grown for 7 days.

Table 5.4: Influence of carbon sources on acid phosphatase, phytase and fungal dry biomass by different fungal isolates in presence of different C sources in PKV media having TCP equivalent to 2.3g P_2O_5/l grown for 7 days

Fungal isolates	**Acid phosphatase (µM/ml/h)**	**Phytase (µM/ml/h)**	**Dry Biomass (mg)**
Mannitol			
A.tubingensis (AT6)	127.86±15.98bc	615±108b	**853±9a**
A. niger	**789.04±46.56a**	940±30a	**572±19c**
A.tubingensis (AT1)	83.13±17.21c	678±103ab	**496±23d**
P. oxalicum	**404.85±29.95b**	598±145b	**610±25b**
Xylose			
A.tubingensis (AT6)	**196.68±24.45b**	24532±9992a	442±12b
A. niger	379.04±25.34a	13135±1004ab	422±10b
A.tubingensis (AT1)	**334.42±6.23ab**	12087±1599ab	484±16a
P. oxalicum	50.87±2.71c	2159±168b	331±18c
Fructose			
A.tubingensis (AT6)	184.96±13.3c	**139730±18663a**	478±10a
A. niger	651.95±13.2a	134376±14637a	443±49a
A.tubingensis (AT1)	263.45±4.5b	129136±10676ab	458±5a
P. oxalicum	12.81±3.4d	70981±5911b	334±14b
Sucrose			
A.tubingensis (AT6)	94.42±17.2b	136121.00±11049b	474±12a
A. niger	106.03±3.2b	**157620.30±17255ab**	548±70a
A.tubingensis (AT1)	131.63±6.4a	**181956.70±572a**	490±53a
P. oxalicum	6.78±0.9c	6485.00±326c	275±17b
Arabinose			
A.tubingensis (AT6)	1.30±0.4b	6443±374b	249±31a
A. niger	3.02±0.3a	3171±964c	257±19a
A.tubingensis (AT1)	0.29±0.2b	10772±1537a	238±11a
P. oxalicum	1.10±0.5b	6444±474b	244±8a
Glucose			
A.tubingensis (AT6)	0.57±0.1c	119904±8129a	298±11a
A. niger	2.40±0.9b	113545.00±15918a	214±9d
A.tubingensis (AT1)	2.89±0.4b	119075.30±10376a	251±8b
P. oxalicum	62.1±1.3a	**109506.70±23740a**	234±7c

Values sharing a common letter within the column and within the C sources are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Table 5.5: Influence of carbon sources on organic acid exudation (nMoles/ml) by fungal isolates in presence of different C sources in PKV media having TCP equivalent to 2.3 g P_2O_5/l grown for 7 days

Fungal isolates	**Oxalate**	**Citrate**	**Gluconate**	**Malate**	**Succinate**	**Formate**
Mannitol						
A.tubingensis (AT6)	**811±32a**	0	0	13±1b	0	0
A.niger	184±15c	0	0	**4313±221a**	**2157±122b**	0
A.tubingensis (AT1)	141±7c	0	0	**790±67a**	**460±12b**	0
P. oxalicum	199±11c	0	0	**8111±435a**	3560±238b	0
Xylose						
A.tubingensis (AT6)	308±21a	0	0	15±0b	0	0
A.niger	267±12a	0	0	0	14±1b	0
A.tubingensis (AT1)	1309±97a	0	0	53±2b	0	0
P. oxalicum	305±23a	2	0	24±1b	0	0
Fructose						
A.tubingensis (AT6)	155±3a	26±0b	0	0	0	0
A.niger	179±5a	12±1b	0	0	0	0
A.tubingensis (AT1)	2162±98a	0	0	112±7b	0	0
P. oxalicum	204±18a	0	0	20±0b	0	0
Sucrose						
A.tubingensis (AT6)	653±23b	0	**1233±117a**	0	0	0
A.niger	351±21c	15±0d	918±67a	1042±97a	577±22b	0
A.tubingensis (AT1)	3660±111a	0	0	0	18±0b	0
P. oxalicum	193±12c	21±1d	0	189±13c	730±51b	1855±107a
Arabinose						
A.tubingensis (AT6)	397±16a	0	181±12b	22±0c	0	0
A.niger	280±11a	0	0	0	182±14b	0
A.tubingensis (AT1)	184±8a	144±5b	106±9c	18±0d	0	0
P. oxalicum	355±25b	19±0e	292±18c	752±65a	102±9d	0
Glucose						
A.tubingensis (AT6)	356±25c	0	0	1550±39a	833±35b	35±0d
A.niger	246±18c	0	0	392±23b	256±13c	2277±121a
A.tubingensis (AT1)	280±11c	0	0	778±35a	394±24b	58±1d
P. oxalicum	253±19d	154±12e	0	6274±231b	3611±112c	16473±569a

Values sharing a common letter within the column and within the C sources are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

5.2.2 Nitrogen sources

A. tubingensis (AT6), A. niger, A. tubingensis (AT1) and *P. oxalicum* were able to solubilize TCP with all nitrogen sources (Fig.5.4). The solubilization efficiency as well as acidification and enzyme activity in the culture media varied with different nitrogen sources. The type and amount of organic acid varied with different nitrogen sources. In general there was no clear relationship between P solubilization and growth. Potassium nitrate significantly increased P solubilization by all the fungal isolates as compared to other nitrogen sources (Table 5.6).

The phosphate solubilization activity in different nitrogen sources in AT6 was in the following order: potassium nitrate > sodium nitrate > asparagine > ammonium nitrate > urea > ammonium sulphate. The pH reduction was maximum in sodium nitrate followed by ammonium sulphate. Acid phosphatase and phytase activity was higher in case of ammonium nitrate and ammonium sulphate respectively (Table 5.6). The main organic acids produced by AT6 in media having potassium nitrate were formic acid, oxalic acid, citric acid and malic acid, whereas in ammonium nitrate and sodium nitrate in addition to oxalic acid, citric acid and formic acids, significant amounts of succinic acid was also observed (Table 5.7).

In case of *A. niger*, the solubilization pattern was: potassium nitrate > sodium nitrate > ammonium nitrate > asparagine > urea > ammonium sulphate. The maximum pH reduction was in the medium having ammonium sulphate compared to other C sources. Acid phosphatase activity was maximum in ammonium nitrate and phytase activity was maximum in ammonium sulphate by *A. niger* (Table 5.6). In case of potassium nitrate main organic acids produced were oxalic acid and citric acid. In sodium nitrate amended media, *A. niger* exudated oxalic acid, citric acid, succinic acid, formic acid and acetic acid in significant amount. In media having ammonium nitrate; oxalic acid, malic acid, citric acid and formic acid were the main organic acids produced by *A. niger*. In media having urea and ammonium sulphate as N source, the main organic acids produced were malic acid and formic acid (Table 5.7).

In AT1, the phosphate solubilization activity was in order: potassium nitrate > ammonium nitrate > sodium nitrate > asparagine > ammonium sulphate > urea. The highest pH reduction was in case of sodium nitrate followed by ammonium nitrate. Acid phosphatase and phytase activity was maximum in sodium nitrate by AT1 (Table 5.6). The main organic acid secreted in presence of potassium nitrate was oxalic acid and citric acid. In ammonium nitrate and sodium nitrate amended medium the main organic acids were oxalic acid, citric acid and formic acid. In other N sources also oxalic acid and citric acid were main organic acids but the amount was less (Table 5.7).

In *P. oxalicum*, P solubilization in relation to nitrogen sources was in the following order: potassium nitrate > asparagine > ammonium nitrate > sodium nitrate > urea > ammonium sulphate. Acidification of the culture media was observed in all the cases, maximum pH reduction was observed in case of ammonium nitrate. Though high soluble P was observed in the presence of potassium nitrate, more growth was observed with many of the other nitrogen sources. The maximum enzyme activity was determined in medium having sodium nitrate (Table 5.6). The organic acids excreted by *P. oxalicum* in case of all the nitrogen sources were oxalic acid and citric acid. The predominant organic acid in different nitrogen sources was: malic acid in ammonium nitrate and asparagine, succinic acid in sodium nitrate and potassium nitrate and formic acid in urea and ammonium sulphate. The total organic acids produced were higher in case of ammonium sulphate as compared to other carbon sources (Table 5.7).

The results showed that mannitol as carbon source and potassium nitrate as nitrogen source was best for maximum P solubilization of TCP by all the fungal isolates.

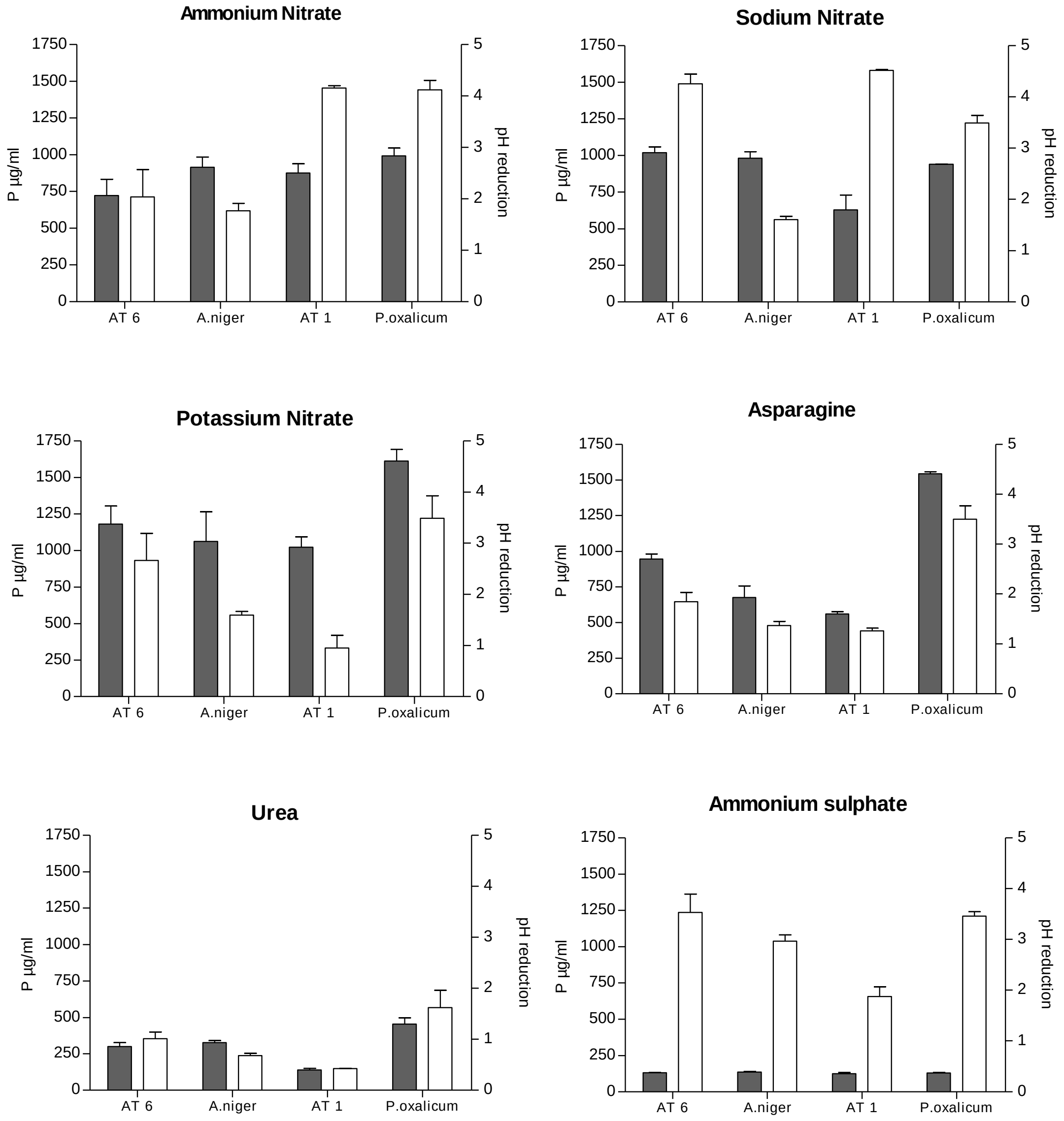


Figure 5.4: Influence of nitrogen sources on P solubilization (filled bars) and pH reduction (empty bars) by bacterial isolates in presence of different N sources in PKV media having TCP equivalent to 2.3 g P_2O_5/l grown for 15 days

Table 5.6: Influence of nitrogen sources on acid phosphatase, phytase and fungal dry biomass by fungal isolates in presence of different N sources in PKV media having TCP equivalent to 2.3 g P_2O_5/l grown for 7 days

Fungal isolates	**Acid phosphatase (µM/ml/h)**	**Phytase activity (µM/ml/h)**	**Dry Biomass (mg)**
Ammonium Nitrate			
A.tubingensis (AT6)	**749.69±265.9c**	80986±13181b	403±6a
A.niger	**2023.99±84.4a**	90762±12975b	393±13a
A.tubingensis (AT1)	1179.47±93.0b	115595±574a	415±7a
P. oxalicum	624.42±167.7c	113193±1078a	423±12a
Sodium Nitrate			
A.tubingensis (AT6)	199.5±42.1d	113586±6977a	438±17a
A.niger	1188.5±40.0b	109175±20698a	392±9a
A.tubingensis (AT1)	**662.3±178.8c**	**132124±7735a**	450±21a
P. oxalicum	**1429.9±130.8a**	**132393±9160a**	**431±35a**
Urea			
A.tubingensis 6	58.1±0.5b	38671±273b	391±18a
A.niger	693.1±49.5a	31422±5371b	385±8a
A.tubingensis (AT1)	58.5±2.6b	12884±42c	401±13a
P. oxalicum	4.1±0.2c	54453±3607a	362±15a
Asparagine			
A.tubingensis (AT6)	612.7±17.7a	89829±18719ab	**467±12a**
A.niger	307.0±4.4c	96706±7530a	**409±4b**
A.tubingensis (AT1)	377.6±16.5b	58989±6169c	445±6b
P. oxalicum	365.9±2.9b	78355±10414b	**431±13b**
Potassium Nitrate			
A.tubingensis (AT6)	464.42±54.5c	86350±15255a	461±27a
A.niger	306.57±34.8d	101345±3973a	398±40a
A.tubingensis (AT1)	1313.99±0.9a	45920±4237b	**468±21a**
P. oxalicum	739.26±89.5b	82601±14627a	423±29a
Ammonium Sulphate			
A.tubingensis (AT6)	0.57±0.1c	**119903±8128a**	298±11a
A.niger	2.40±0.9b	**113545±15918a**	214±9d
A.tubingensis (AT1)	2.89±0.4b	119075±10376a	251±8b
P. oxalicum	62.1±1.3a	109507±23740a	234±7c

Values sharing a common letter within the column and within the N sources are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Table 5.7: Influence of nitrogen sources on organic acid exudation (nMoles/ml) by fungal isolates in presence of different N sources in PKV media having TCP equivalent to 2.3 g P_2O_5/l grown for 7 days

Fungal isolates	**Oxalate**	**Citrate**	**Gluconate**	**Malate**	**Succinate**	**Formate**	**Acetate**
Ammonium nitrate							
A.tubingensis (AT6)	2112±110a	1657±122b	0	0	501±36d	1363±121c	0
A.niger	738±23c	5340±134a	0	332±11d	0	1680±111b	0
A.tubingensis (AT1)	855±44a	554±26b	0	27±0d	0	419±35c	0
P. oxalicum	699±52b	406±32c	57±2e	**19910±967a**	12±1f	65±2d	0
Sodium nitrate							
A.tubingensis (AT6)	557±32c	964±45b	0	0	5524±228a	89±3e	261±11d
A.niger	1496±89b	2870±203a	0	0	742±23c	0	752±21c
A.tubingensis (AT1)	3968±124a	1674±122b	0	0	0	0	0
P. oxalicum	574±47b	116±9d	476±23c	0	1489±104a	0	13±0e
Urea							
A.tubingensis (AT6)	304±14a	0	35±0c	56±1b	0	0	0
A.niger	382±17c	401±32c	0	1812±123a	0	1622±55b	0
A.tubingensis (AT1)	290±13b	547±36a	0	213±5c	0	0	39±0d
P. oxalicum	285±18c	293±11c	0	6440±456b	0	8058±486a	0
Asparagine							
A.tubingensis (AT6)	376±16a	281±23b	0	0	0	0	0
A.niger	327±11a	199±16b	0	58±0c	0	0	0
A.tubingensis (AT1)	298±15a	165±11b	0	21±0c	0	0	0
P. oxalicum	253±15b	191±8c	0	985±62a	44±1d	20±0e	0
Potassium nitrate							
A.tubingensis (AT6)	657±34b	291±8c	0	133±3d	0	**2096±113a**	0
A.niger	368±9b	244±12c	0	25±0d	17±0e	**2927±142a**	0
A.tubingensis (AT1)	443±32b	121±8c	0	0	31±0d	**517±27a**	0
P. oxalicum	418±28c	252±22d	0	684±46b	3589±281a	36±1e	0
Ammonium Sulphate							
A.tubingensis (AT6)	356±25c	0	0	1550±39a	833±35b	35±0d	0
A.niger	246±18c	0	0	392±23b	256±13c	2277±121a	0
A.tubingensis (AT1)	280±11c	0	0	778±35a	394±24b	58±1d	0
P. oxalicum	253±19d	154±12e	0	6274±123b	3611±112c	16473±1367a	0

Values sharing a common letter within the column and within the N sources are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

5.2.3 Effect of carbon and nitrogen sources on solubilization of RRP by fungal isolates

All the four isolates were studied for the effect of mannitol as carbon source and potassium nitrate as nitrogen source on the solubilization of RRP (equivalent to 0.16% P_2O_5) as these sources were found to be best for solubilization of TCP. There was an increase in solubilization by all the isolates except *P. oxalicum* when mannitol as carbon source and potassium nitrate as nitrogen source was used instead of normal PKV media (having glucose as C and ammonium sulphate as N source). There was no significant difference in the reduction of pH (Table 5.8 and 5.10). Acid phosphatase activity decreased, whereas phytase activity and dry biomass of the fungi increased significantly in media having mannitol and potassium nitrate in all the isolates (Table 5.10). In all the four isolates, oxalic acid increased whereas gluconic acid, malic acid, formic acid and acetic acid decreased significantly in presence of mannitol and potassium nitrate. In case of *P. oxalicum* there was seven folds decrease in malic acid in media having mannitol and potassium nitrate as compared to PKV media (Table 5.11).

Table 5.8: pH reduction, soluble P, acid phosphatase, phytase and fungal biomass by fungal isolates in presence of glucose and ammonium sulphate as C and N source respectively in PKV media having RRP equivalent to 1.5 g P_2O_5/l grown for 7 days

Fungal isolates	**pH reduction**	**Soluble P (µg/ml)**	**Acid Phosphatase activity (µM/ml/h)**	**Phytase activity (µM/ml/h)**	**Dry biomass (mg)**
*A.tubingensis (*AT6)	3.4±0.1a	**212±11c**	2505±78b	7935±603b	127±3c
A.niger	3.1±0.0a	**298±9b**	271±9c	13277±998a	179±4b
*A.tubingensis (*AT1)	3.3±0.2a	**286±5b**	2756±93a	8897±421b	171±5b
P.oxalicum	3.5±0.2a	**343±18a**	111±4d	11329±1251a	206±7a

Values sharing a common letter within the column are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Table 5.9: Organic acid exudation (nMoles/ml) by fungal isolates in presence of glucose and ammonium sulphate as C and N source respectively in PKV media having RRP equivalent to 1.5 g P_2O_5/l grown for 7 days

Fungal cultures	Oxalate	Gluconate	Malate	Succinate	Formate	Acetate
*A.tubingensis (*AT6)	447±82c	**10070±3071a**	2552±791b	0	357±7d	539±13c
A.niger	331±19c	**8958±295a**	5397±587b	249±6d	0	57±2e
*A.tubingensis (*AT1)	343±34c	**8421±1004a**	1849±251b	212±7d	0	127±14e
P.oxalicum	411±4c	4306±1045b	**9435±804a**	0	0	92±3d

Values sharing a common letter within the column are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Table 5.10: Influence on pH reduction, soluble P, acid phosphatase, phytase and dry biomass by fungal isolates when mannitol as carbon source and potassium nitrate as nitrogen source was used in PKV media having RRP equivalent to 1.5 g P_2O_5/l grown for 7 days

Fungal cultures	pH reduction	Soluble P (µg/ml)	Acid Phosphatase (µM/ml/h)	Phytase (µM/ml/h)	Dry biomass (mg)
*A.tubingensis (*AT6)	3.6±0.2a	**313±4b**	16±1.3a	28999±1256b	349±13b
A.niger	3.3±0.2a	**422±9a**	8±0.7b	23469±1200b	336±19b
*A.tubingensis (*AT1)	3.2±0.03a	**311±10b**	15±0.7a	43829±3029a	450±21a
P.oxalicum	3.0±0.1b	**248±5c**	10±0.5b	15122±177c	337±18b

Values sharing a common letter within the column are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Table 5.11: Influence on organic acid exudation (nMoles/ml) by fungal isolates when mannitol as carbon source and potassium nitrate as nitrogen source was used in PKV media having RRP equivalent to 1.5 g P_2O_5/l grown for 7 days

Fungal cultures	Oxalate	Gluconate	Malate	Succinate	Acetate
*A.tubingensis (*AT6)	**6006±331a**	0.0	2891±136b	22±2d	364±29c
A.niger	**5024±232a**	0.0	3150±245b	84±7c	0.0
*A.tubingensis (*AT1)	**2392±112a**	0.0	1717±124b	288±25c	0.0
P.oxalicum	**4628±113a**	2424±138b	1330±212c	12±1d	0.0

Values sharing a common letter within the column are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Chapter 6

Solubilization of metal phosphates by fungal isolates

6.1 Solubilization of different metal phosphates

All the fungal isolates were able to solubilize the inorganic metal phosphates in liquid media to a significant level. Higher solubilization was accompanied by higher fungal biomass in culture media.

6.1.1 Aluminium Phosphate

All the four isolates, *A. tubingensis* (AT6), *A. niger*, *A. tubingensis* (AT1) and *P. oxalicum* solubilized aluminium phosphate in liquid media to a significant level. The solubilization of $AlPO_4$ released 163 µg/ml of P by *P. oxalicum* followed by 158.73 µg/ml of P by AT1 (Fig. 6.1). The reduction in pH of the media was less than 4.2 in all the four isolates after seven days of incubation. The biomass was maximum in *P. oxalicum* and minimum in AT6. The phytase activity was more than acid phosphatase by all the isolates. AT1 showed highest acid phosphatase activity and AT6 showed maximum phytase activity followed by *P. oxalicum* (Table 6.1). Both the isolates of *A. tubingensis* exudated oxalic acid in significant amount, whereas *A. niger* beside oxalic acid, exudated gluconic acid and formic acid in presence of $AlPO_4$. In *P. oxalicum,* malic acid exudation was highest followed by oxalic acid (Table 6.2).

6.1.2 Zinc Phosphate

$Zn_3(PO_4)_2$ was solubilized by all isolates to the maximum extent except AT6, which showed lesser soluble P than other three isolates. The solubilization of $Zn_3(PO_4)_2$ by the three isolates *A. niger,* AT1 and *P. oxalicum* was highest even more than TCP. The pH reduction in these three isolates was more than 3.0 (Fig. 6.1). The highest solubilization was observed in *P. oxalicum* followed by *A. niger*. *P. oxalicum* exhibited greatest dry biomass and acid phosphatase as well as phytase activities in presence of $Zn_3(PO_4)_2$. AT6 showed minimum reduction in pH, lowest dry biomass, enzyme activity and organic acid exudation, which directly relates to least solubilization. In case of *A. niger,* the highest amount of organic acid exudated was formic acid followed by citric acid and oxalic acid. AT1 showed significant amount of formic acid and phytase production in presence of $Zn_3(PO_4)_2$. Major organic acids produced by *P. oxalicum* were citric acid, malic acid, formic acid and oxalic acid in the culture filtrate (Table 6.3).

6.1.3 Copper Phosphate

In case of Cu_2OHPO_4 the solubilization by the three strains of *Aspergillus* was 70 to 82 µg/ml where as by *P. oxalicum,* the solubility level was very poor i.e. 23 µg/ml. The poor solubilization did not coincide with poor pH reduction of media except in AT1 (Fig. 6.1). However, low solubilization was associated with low fungal dry biomass. *A. niger* showed highest enzyme activity, whereas *P. oxalicum* showed highest pH reduction (Table 8.1). The main organic acid exudated by *A. niger* was oxalic acid and citric acid, whereas beside oxalic acid other three isolates showed gluconic acid and formic acid exudation in significant amount (Table 6.2).

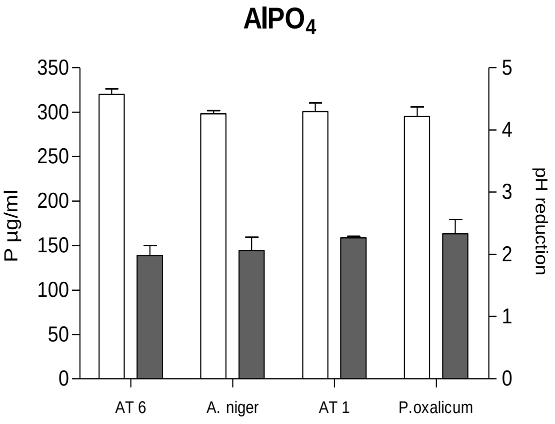
6.1.4 Ferric Phosphate

The solubilization of ferric phosphate as well as dry fungal biomass was significantly higher in *A. niger* and *P. oxalicum* as compared to AT6 and AT1. The pH reduction in general, was more than 4.0 by all the isolates (Fig. 6.1). In AT6, acid phosphatase was more than phytase activity. AT1 showed highest acid phosphatase and phytase activity. In *A. niger* and *P.oxalicum*, phytase activity was more than acid phosphatase activity (Table 6.1). AT6 and *P. oxalicum* exudated highest amount of formic acid followed by oxalic acid whereas in case of *A. niger* and AT1, oxalic acid was the major organic acid (Table 6.2).

6.1.5 Cobalt Phosphate

$Co_3(PO_4)_2$ was solubilized by all the four isolates to a significant level. Maximum solubilization was observed in AT1 (183 µg/ml) followed by *A. niger* (176 µg/ml) (Fig. 6.1). In case of AT1, the pH reduction was around 3.8 and the dry biomass was directly associated with soluble P. Maximum reduction of pH (4.2) was observed in case of *P.oxalicum* where soluble P was 158 µg/ml. The acid phosphatase activity was least, whereas phytase activity was highest in all the isolates as compared to other metal phosphates (Table 6.1). The *Aspergillus* strains showed maximum production of gluconic acid. The other main organic acids secreted were oxalic acid, citric acid, malic acid, succinic acid and acetic acid. *P. oxalicum* exudated succinic acid in the highest quantity followed by gluconic acid and acetic acid in the culture media in presence of $Co_3(PO_4)_2$ (Table 6.2).

The effect of different concentrations of mixture of commercial organic acids and oxalic acid (separately) on solubilization of different metal phosphates was determined (Table 6.3). Results showed high content of soluble P with reduction in the pH in all the metal-P except Al-P.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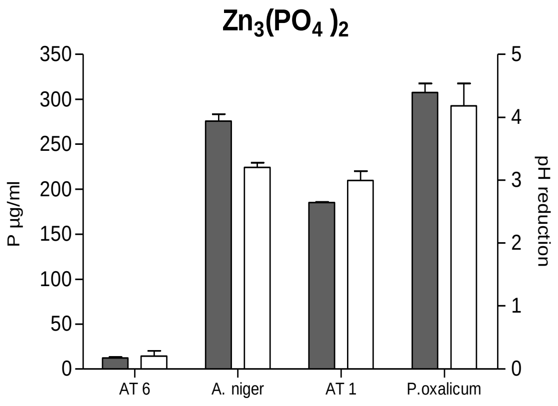
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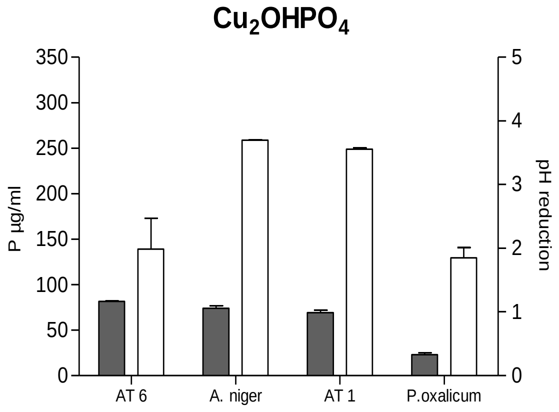
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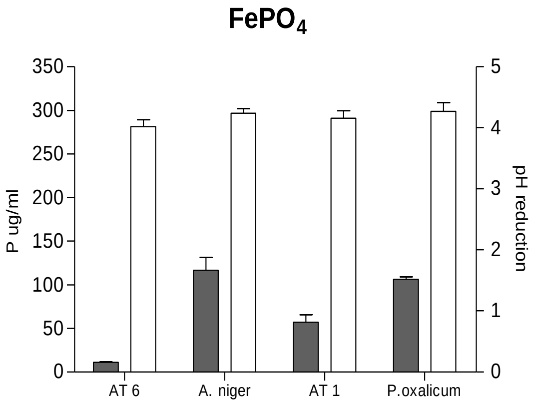
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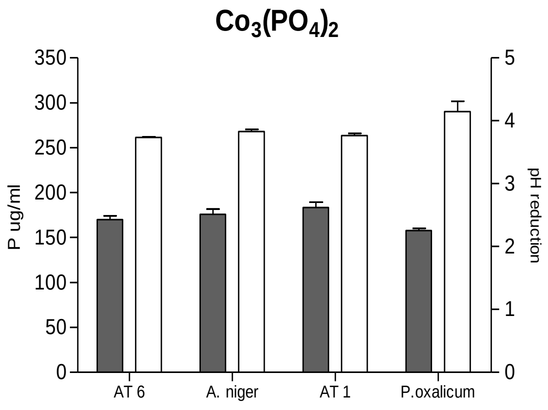
$Co_3(PO_4)_2$

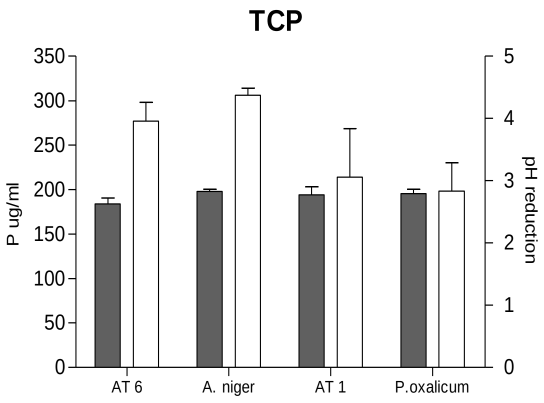
TCP

Figure 6.1: P solubilization (filled bars) and pH reduction (empty bars) by different fungal isolates in presence of different metal phosphates (5 mM) in media grown for 7 days

Table 6.1: Dry fungal biomass, acid phosphatase and phytase activity in different metal phosphates by the fungal isolates in presence of different metal phosphates (5 mM) in media grown for 7 days

Name of isolates	**Dry Biomass (mg)**	**Acid phosphatase Activity (µM/ml/h)**	**Phytase Activity (µM/ml/h)**
		$AlPO_4$	
A.tubingensis (AT6)	198±20b	36.79±1.3d	**489±39a**
A.niger	215±37b	87.00±3.5c	426±26a
A.tubingensis (AT1)	235±11b	180.01±8.8a	284±22b
P.oxalicum	**282±31a**	**126.25±8.5b**	443±19a
		$Zn_3(PO_4)_2$	
A.tubingensis (AT6)	96±13c	1.41±0.06b	2153±105ab
A.niger	**293±26a**	2.27±.12b	3042±60ab
A.tubingensis (AT1)	130±37b	2.81±0.5b	2506±125b
P.oxalicum	172±34b	**45.82±4.7a**	**3218±26a**
		Cu_2OHPO_4	
A.tubingensis (AT6)	**180±13a**	6.36±0.8c	336±052b
A.niger	174±17a	**126.14±8.6a**	604±30a
A.tubingensis (AT1)	107±2b	48.51±5.2b	341±43b
P.oxalicum	78±1c	1.52±0.1c	262±45b
		$Fe\ PO_4$	
A.tubingensis (AT6)	119±12d	667.32±73.3b	450±15c
A.niger	**854±9a**	827.54±65.0b	6858±747b
A.tubingensis (AT1)	650±60b	**1238.18±133.6a**	**24538±1059a**
P.oxalicum	287±96c	83.88±6.4c	7849±410b
		$Co_3(PO_4)_2$	
A.tubingensis (AT6)	216±4b	1.52±0.02b	**76698±826a**
A.niger	259±12b	2.16±0.34b	66777±807b
A.tubingensis (AT1)	**335±85a**	1.12±0.06b	49669±1031ab
P.oxalicum	110±9c	**7.22±0.06a**	63649.97±1144b
		TCP	
A.tubingensis a	153±43a	49.15±3.75c	170.84±9.87c
A.niger	**226±13a**	132.27±7.91b	239.18±17.09b
A.tubingensis b	198±17a	**287.97±12.01a**	**353.10±15.35a**
P.oxalicum	204±35a	104.53±0.883b	250.58±11.39b

Values sharing a common letter within the column and within the P sources are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Table 6.2: Organic acids exudation (nM/ml) by fungal isolates in response to metal phosphates (5 mM) in media

Fungal Isolates	**Oxalate**	**Citrate**	**Gluconate**	**Malate**	**Succinate**	**Formate**	**Acetate**
TCP							
A.tubingensis (AT6)	**199±11a**	-	-	77±3b	-	-	-
A.niger	**1903±4a**	-	-	-	-	-	30±1b
A.tubingensis (AT1)	**1523±87a**	212±9b	-	-	-	-	-
P.oxalicum	**5192±121a**	-	-	-	**1095±12b**	-	-
$AlPO_4$							
A.tubingensis (AT6)	**4129±223**	-	-	-	-	-	-
A.niger	1108±112c	-	**9033±145a**	-	-	1478±103b	-
A.tubingensis (AT1)	**4857±324a**	-	-	-	-	214±18b	-
P.oxalicum	154±12b	-	-	**276±2a**	-	55±1c	-
$Zn_3(PO_4)_2$							
A.tubingensis (AT6)	4±0b	-	8±0a	-	-	-	-
A.niger	265±28c	615±22b	-	-	-	**7877±216a**	-
A.tubingensis (AT1)	21±1b	1±0c	-	-	-	**290 ±15a**	-
P.oxalicum	141±5b	120±9c	-	17±1d	-	**267±11a**	-
Cu_2OHPO_4							
A.tubingensis (AT6)	206±18c	-	**2480±167a**	2±0d	-	957±44b	-
A.niger	**7019±382a**	883±77b	4±0c	-	-	-	-
A.tubingensis (AT1)	190±19c	96 ±2d	2152±156b	-	25±1e	**3276±285a**	-
P.oxalicum	210±15d	130±11e	1453±48b	-	333±16c	**1797±21a**	-
Fe PO_4							
A.tubingensis (AT6)	674±14b	-	-	-	282±11c	**2052±27a**	-
A.niger	**1580±105a**	-	-	5±0d	-	26 ±2b	17±c
A.tubingensis (AT1)	**926±77a**	-	-	12±1c	10±1c	62±5b	-
P.oxalicum	1681±25b	-	-	89±5c	23±1d	**4185±61a**	-
$Co_3(PO4)_2$							
A.tubingensis (AT6)	418±9f	712±14e	**20751±1102a**	1554±24c	4132±69b	880±21d	434±11f
A.niger	2304±178b	398±21d	**27838±985a**	822±41c	88±3e	-	113±19e
A.tubingensis (AT1)	233±15d	531±33c	**28003±127a**	759±33b	664±39c	14±2e	3±0f

P.oxalicum	241±9f	896±15d	1954±38b	500±21e	**4208±58a**	-	1376±23c

Values sharing a common letter within the column and within the P sources are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Table 6.3: Solubilization of different Metal Phosphates by commercially available organic acids

Mixture of Organic Acids (succinic, citric and oxalic acid)

Metal-P	**One Hour**				**Two Hours**				**Three Hours**				**Four Hours**			
5mM	**6mM**		**10mM**		**6mM**		**10mM**		**6mM**		**10mM**		**6mM**		**10mM**	
	µg/ml	**pH**	**µg/ml**	**pH**	**µg/ml**	**pH**	**µg/ml**	**pH**	**µg/ml**	**pH**	**µg/ml**	**pH**	**µg/ml**	**pH**	**µg/ml**	**pH**
$AlPO_4$	14.46	2.17	17.75	1.99	25.54	2.17	31.53	1.99	35.42	2.16	**42.01**	1.98	35.72	2.15	41.41	1.99
Cu_2OHPO_4	188.11	2.22	197.69	2.10	219.85	2.22	**253.98**	2.10	217.75	2.22	253.08	2.11	218.35	2.22	251.29	2.10
$Co_3(PO_4)_2$	283.02	2.40	295.30	2.20	287.22	2.40	**296.20**	2.20	289.01	2.39	297.99	2.20	288.71	2.40	297.40	2.19
$FePO_4$	110.27	3.53	148.29	3.45	134.22	3.53	**158.17**	3.45	133.92	3.50	151.89	3.42	134.22	3.49	151.89	3.43
$ZnPO_4$	316.56	2.45	**345.00**	2.12	322.84	2.45	344.40	2.12	322.25	2.42	341.71	2.10	322.54	2.41	343.20	2.11
Oxalic Acid																
$AlPO_4$	17.16	2.24	16.56	2.01	25.84	2.23	30.63	2.00	35.12	2.24	35.12	2.00	35.12	2.24	**38.71**	2.00
Cu_2OHPO_4	193.20	2.25	205.18	2.08	191.71	2.24	**231.83**	2.06	192.90	2.24	222.84	2.05	195.00	2.24	221.35	2.05
$Co_3(PO_4)_2$	233.92	2.06	256.08	2.02	280.03	2.06	**280.33**	2.00	279.43	2.06	280.03	2.00	279.73	2.06	277.34	2.00
$FePO_4$	129.43	2.27	165.66	2.05	152.19	2.26	**180.03**	2.06	161.47	2.27	168.35	2.07	162.07	2.27	168.65	2.07
$ZnPO_4$	246.80	2.41	315.66	2.25	245.00	2.42	**315.06**	2.24	238.41	2.42	314.46	2.26	239.61	2.42	312.37	2.26

6.2 Carbon sources

The solubilization of different metal phosphates was tested by using different carbon sources in the Pikovskaya media.

6.2.1 Aluminium Phosphate ($AlPO_4$)

The solubilization of aluminium phosphate by AT6 was maximum when fructose was used as carbon source in the medium. In this case, the dry biomass as well as phytase activity was also higher amongst the other carbon sources used in the media.

A. niger, AT1 and *P. oxalicum* solubilized aluminium phosphate to a maximum extent in presence of glucose, in which the pH reduction was higher than other C sources (Fig. 6.2). However, in case of AT1, there was no significant difference in solubilization in media having glucose and mannitol. The dry biomass of *P. oxalicum* was maximum in case of glucose. *A. niger* and AT1 showed maximum dry biomass in presence of xylose in which the solubilization was quite low. In all the cases, phytase activity was more than acid phosphatase activity. Phytase activity increased when C sources other than glucose were used (Table 6.4).

6.2.2 Zinc Phosphate [$Zn_3(PO_4)_2$]

AT6 showed maximum solubilization of zinc phosphate in presence of fructose in the medium (Fig. 6.3). In this case, acid phosphatase activity was highest when compared to other carbon sources. *A. niger* solubilized maximum of zinc phosphate in case of mannitol which coincides with higher pH reduction. Fructose proved to be the best carbon source for solubilization of zinc phosphate by AT1. Phytase activity was maximum in presence of fructose when compared with other carbon sources (Table 6.5). In case of *P.oxalicum,* the solubilization and dry biomass was highest in case of glucose and sucrose respectively. However, the enzyme activity was maximum in case of fructose and maximum pH reduction was observed in fructose followed by glucose.

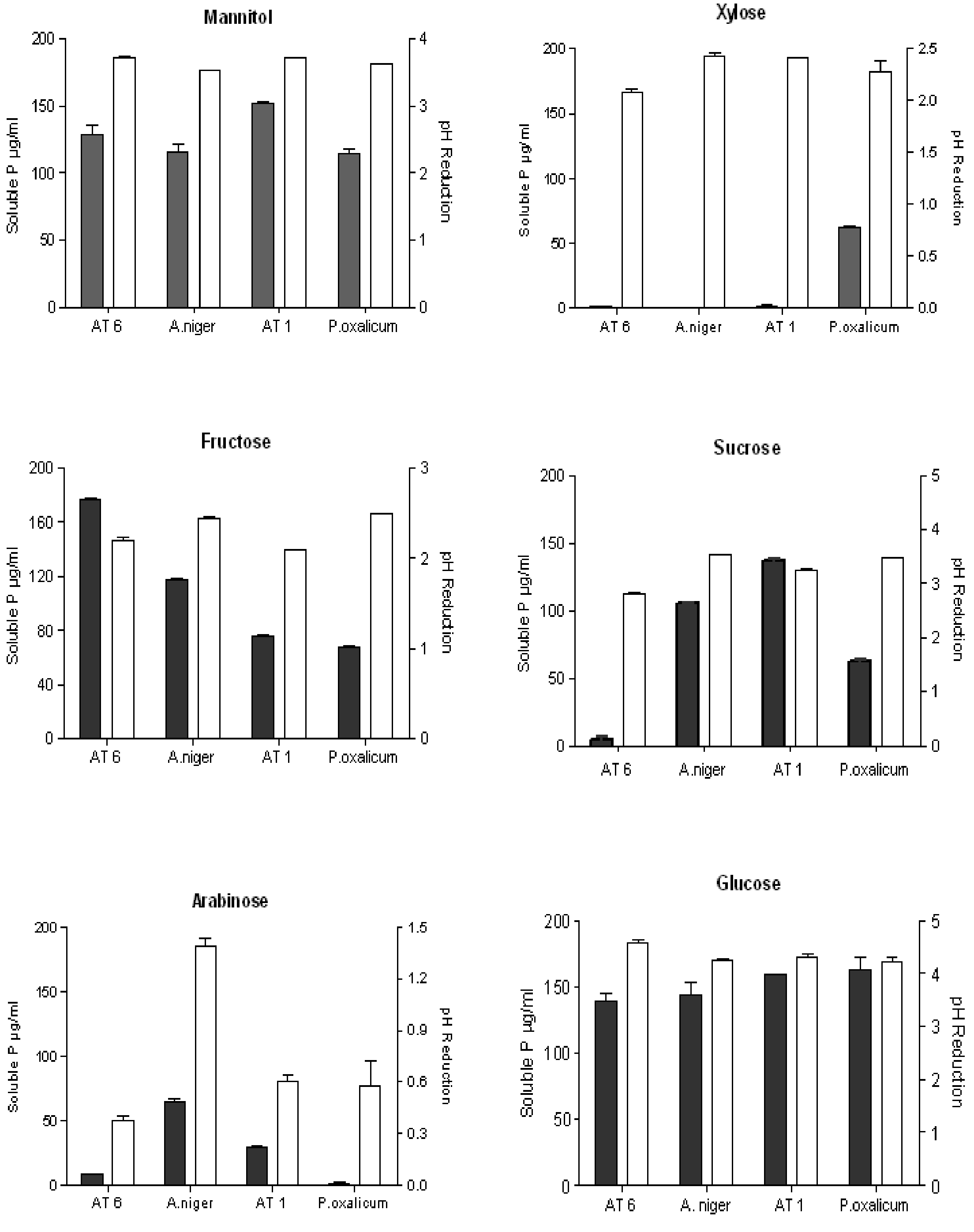

Figure 6.2: Influence of carbon sources on P solubilization (filled bars) and pH reduction (empty bars) by fungal isolates in presence of different C sources in PKV media having aluminium phosphate equivalent to 5 mM grown for 7 days

Table 6.4: Acid phosphatase and phytase activity by fungal isolates in presence of different C sources in PKV media having aluminium phosphate equivalent to 5mM grown for 7 days

Fungal isolates	**Acid phosphatase (µM/ml/h)**	**Phytase (µM/ml/h)**	**Dry Biomass (mg)**
Mannitol			
A.tubingensis (AT6)	204±8a	41488±224b	256±7a
A.niger	130±6b	38299±278d	240±6ab
A.tubingensis (AT1)	127±5b	40370±35c	249±9a
P.oxalicum	110±6b	94034±309a	209±7b
Xylose			
A.tubingensis (AT6)	417±8b	27570±353d	248±9b
A.niger	**619±7a**	**90451±378a**	**252±12b**
A.tubingensis (AT1)	228±1d	41965±216b	**300±9a**
P.oxalicum	294±7c	36289±181c	123±7c
Fructose			
A.tubingensis (AT6)	164±4d	**55552±198a**	**257±4a**
A.niger	236±2c	45320±537b	242±6a
A.tubingensis (AT1)	**583±15a**	**46832±450b**	221±13a
P.oxalicum	**399±2b**	41985±319c	222±13a
Sucrose			
A.tubingensis (AT6)	**529±11b**	15329±224d	162±11b
A.niger	407±2c	16965±394c	177±1b
A.tubingensis (AT1)	561±18a	19741±215b	225±9a
P.oxalicum	238±2d	22205±451a	221±9a
Arabinose			
A.tubingensis (AT6)	204±13a	218±6c	92±2b
A.niger	190±3a	103±12c	119±7a
A.tubingensis (AT1)	100±4b	28585±876b	104±6ab
P.oxalicum	45±3c	**120525±2176a**	117±1a
Glucose			
A.tubingensis (AT6)	36.79±1.3d	489±39a	198±20c
A.niger	87.00±3.5c	426±26a	215±17b
A.tubingensis (AT1)	180.01±8.8a	284±22b	235±31b
P.oxalicum	126.25±8.5b	443±19a	**282±13a**

Values sharing a common letter and within the column within the C sources are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

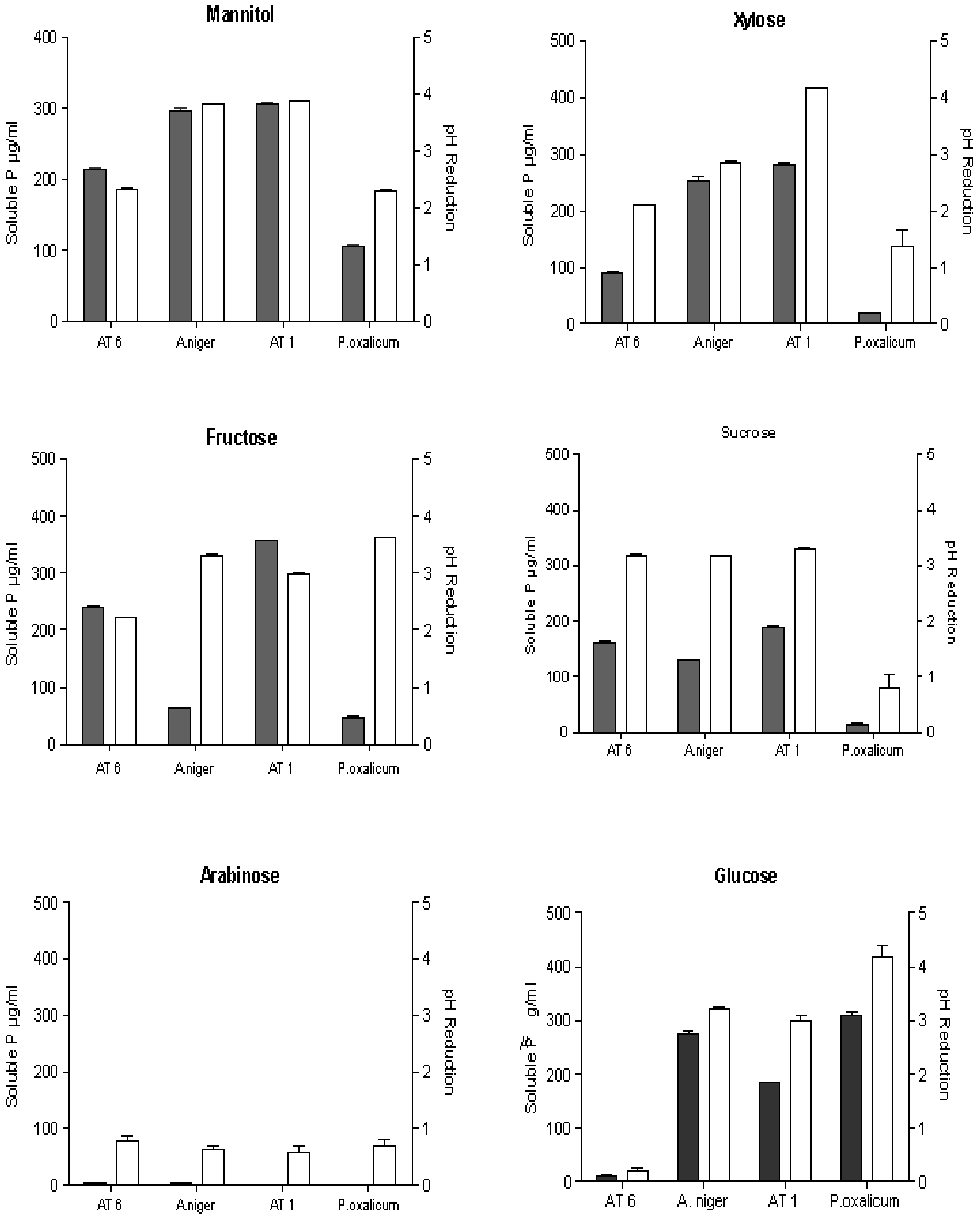

Figure 6.3: Influence of carbon sources on P solubilization (filled bars) and pH reduction (empty bars) by fungal isolates in presence of different C sources in PKV media having zinc phosphate equivalent to 5 mM grown for 7 days

Table 6.5: Acid phosphatase and phytase activity by fungal isolates in presence of different C sources in PKV media having zinc phosphate equivalent to 5 mM grown for 7 days

Fungal isolates	**Acid phosphatase (µM/ml/h)**	**Phytase (µM/ml/h)**	**Dry Biomass (mg)**
Mannitol			
A.tubingensis (AT6)	162±2a	29620±199c	197±3c
A.niger	93±3c	44988±313b	**262±3a**
A.tubingensis (AT1)	83±1d	54640±90a	236±1b
P. oxalicum	114±4b	26555±449d	139±1d
Xylose			
A.tubingensis (AT6)	214±1b	**60108±271a**	**205±4b**
A.niger	219±2b	**55842±450b**	161±2c
A.tubingensis (AT1)	402±2a	55365±326b	**342±2a**
P. oxalicum	3±0c	34653±781c	197±2b
Fructose			
A.tubingensis (AT6)	**337±2b**	42130±170b	124±1d
A.niger	**483±1a**	34073±368c	210±1b
A.tubingensis (AT1)	219±0c	**61641±269a**	285±2a
P. oxalicum	200±6d	32375±701d	163±3c
Sucrose			
A.tubingensis (AT6)	112±3b	28398±231c	144±3c
A.niger	95±4c	39603±210b	217±1a
A.tubingensis (AT1)	198±1a	45693±326a	128±1d
P. oxalicum	2±0d	23966±542d	**200±2b**
Arabinose			
A.tubingensis (AT6)	170±3a	5843±109d	177±6a
A.niger	72±6b	11953±169c	128±9c
A.tubingensis (AT1)	53±3c	12802±161a	145±5bc
P.oxalicum	39±1c	13651±107b	158±5ab
Glucose			
A.tubingensis (AT6)	1±0b	2153±105ab	96±13b
A.niger	2±0b	3042±60ab	133±26a
A.tubingensis (AT1)	3±1b	2506±125b	117±37ab
P.oxalicum	46±4a	3218±26a	172±34a

Values sharing a common letter within the column and within the C sources are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

6.2.3 Copper Phosphate (Cu_2OHPO_4)

The solubilization of copper phosphate was highest in the media having sucrose as C source. In case of AT6, the total enzyme activity was maximum (Fig. 6.4). *A. niger* showed maximum solubilization as well as dry biomass in case of mannitol. However, the maximum enzyme activity was observed in sucrose. AT1 showed higher solubilization efficiency in case of sucrose, which coincides with higher pH reduction and enzyme activity than any other carbon source. In case of *P. oxalicum*, solubilization observed was 100 times higher in arabinose than other carbon sources. In this case, the pH reduction was maximum as compared to other C sources (Table 6.6).

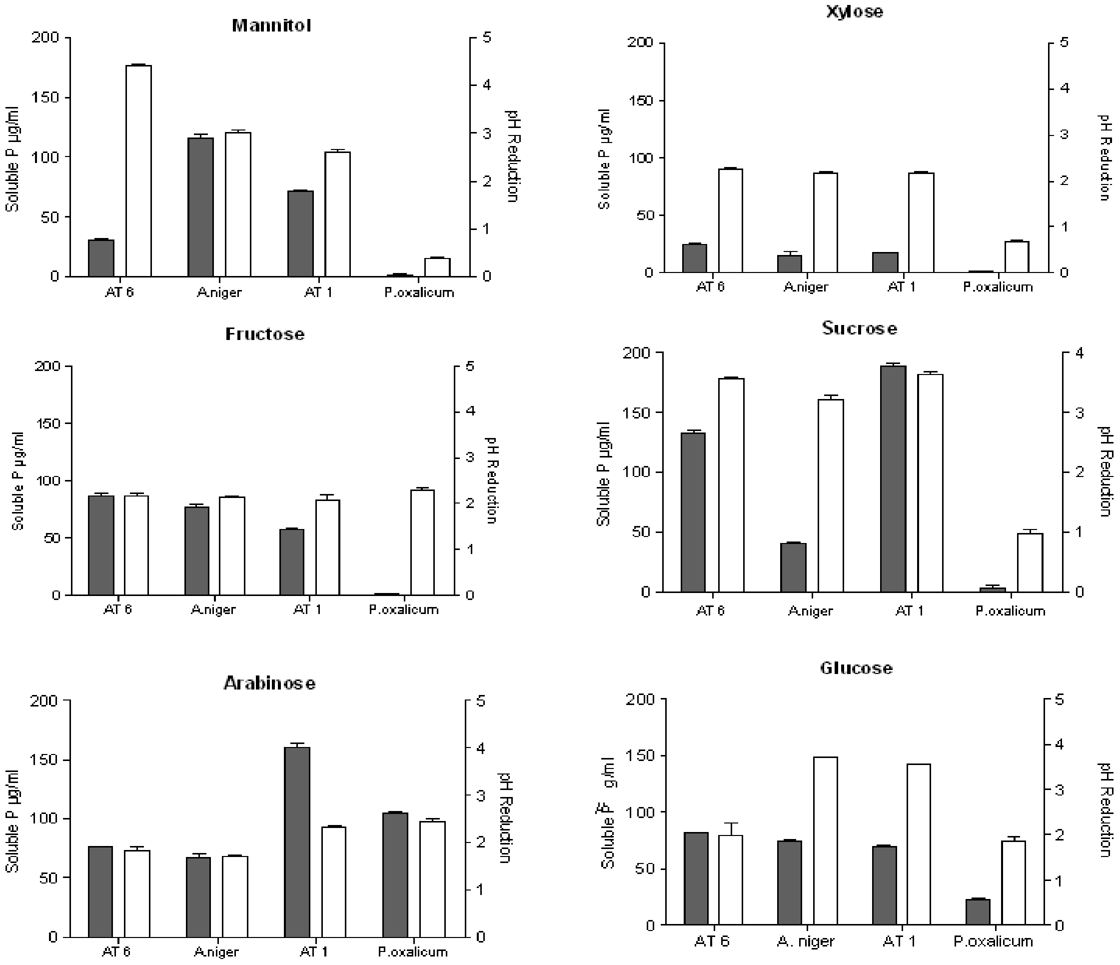

Figure 6.4: Influence of carbon sources on P solubilization (filled bars) and pH reduction (empty bars) by fungal isolates in presence of different C sources in PKV media having copper phosphate equivalent to 5 mM grown for 7 days

Table 6.6: Acid phosphatase and phytase activity by fungal isolates in presence of different C sources in PKV media having copper phosphate equivalent to 5 mM grown for 7 days

Fungal isolates	**Acid phosphatase (μM/ml/h)**	**Phytase (μM/ml/h)**	**Dry Biomass (mg)**
Mannitol			
A.tubingensis (AT6)	**174±3b**	34488±408d	203±6b
A.niger	248±1a	53688±456a	**269±10a**
A.tubingensis (AT1)	61±2c	45755±309b	238±16ab
P.oxalicum	5± 0d	36207±235c	141±2c
Xylose			
A.tubingensis (AT6)	43±2a	52010±368b	**213±5b**
A.niger	274±3b	46998±443d	164±7b
A.tubingensis (AT1)	**688±2c**	48862±244c	**299±21a**
P.oxalicum	5±0d	**58617±219a**	**208±6b**
Fructose			
A.tubingensis (AT6)	25±2b	**65535±285a**	156±9b
A.niger	58±2a	50539± 326c	216±9ab
A.tubingensis (AT1)	69±5a	54992±272b	285±10a
P.oxalicum	30±1b	38423±573d	168±10b
Sucrose			
A.tubingensis (AT6)	**139±2c**	63733±199b	148±10c
A.niger	549±7b	**58762±378c**	225±16a
A.tubingensis (AT1)	625±1a	**83347±556a**	128±4d
P.oxalicum	**103±6d**	49421±400d	199±7b
Arabinose			
A.tubingensis (AT6)	139±5a	26016±1105c	100±10b
A.niger	68±2c	29807±599ab	98±6b
A.tubingensis (AT1)	110±7b	28771±494bc	90± 11b
P.oxalicum	61± 3c	32375±109a	275±3a
Glucose			
A.tubingensis (AT6)	6±1c	336±052b	180±13a
A.niger	126±8a	604±30a	174±17a
A.tubingensis (AT1)	49±5b	341±43b	107±2b
P.oxalicum	2±0c	262±45b	78±1c

Values sharing a common letter within the column and within the C sources are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

6.2.4 Ferric phosphate ($FePO_4$)

The solubilization of ferric phosphate by AT6 was maximum when xylose was used as carbon source in the medium (Fig. 6.5). In this case, the acid phosphatase activity was also highest amongst the other carbon sources used. *A. niger* solubilized iron phosphate to a maximum extent in presence of glucose in which reduction of pH as well as acid phosphatase activity was higher than other C sources. In case of AT1, the solubilization was maximum in media having xylose. The solubilization, dry biomass and pH reduction was maximum by *P. oxalicum* in case of glucose (Table 6.7).

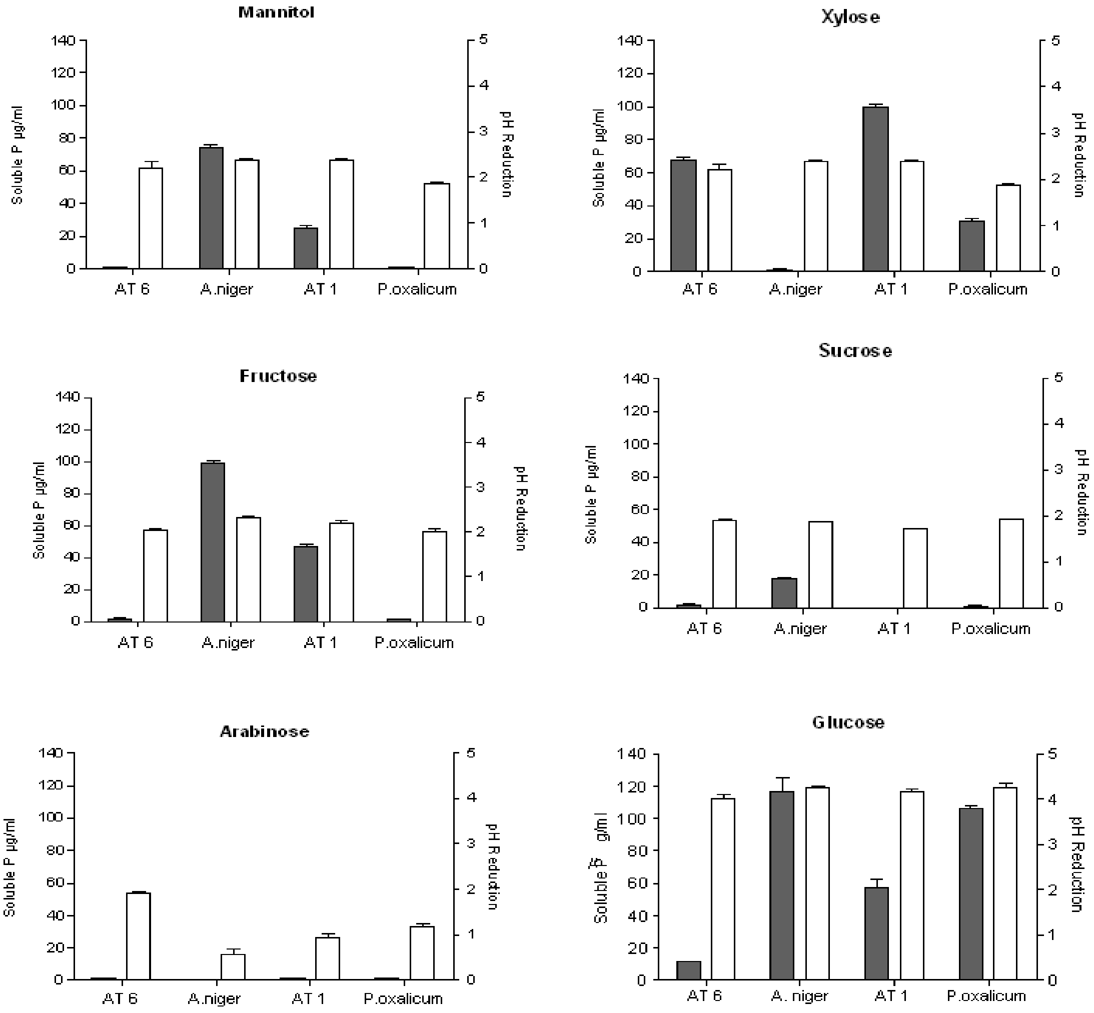

Figure 6.5: Influence of carbon sources on P solubilization (filled bars) and pH reduction (empty bars) by fungal isolates in presence of different C sources in PKV media having ferric phosphate equivalent to 5 mM grown for 7 days

Table 6.7: Acid phosphatase and phytase activity by fungal isolates in presence of different C sources in PKV media having ferric phosphate equivalent to 5 mM grown for 7 days

Fungal isolates	**Acid phosphatase (µM/ml/h)**	**Phytase (µM/ml/h)**	**Dry Biomass (mg)**
Mannitol			
A.tubingensis (AT6)	116±1c	204740±313d	196±3b
A.niger	149±2b	215365±574c	165±2c
A.tubingensis (AT1)	248±3a	241877±364b	175±2c
P.oxalicum	92±1d	**248484±775a**	238±5a
Xylose			
A.tubingensis (AT6)	480±1c	235269±305a	165±4b
A.niger	619±2b	237154±517a	184±7a
A.tubingensis (AT1)	773±2a	241110±75a	169±4b
P.oxalicum	20±2d	228372±145a	173±3b
Fructose			
A.tubingensis (AT6)	183±1b	**255194±198a**	**212±5b**
A.niger	152±1c	**248194±442b**	210±5b
A.tubingensis (AT1)	365±3a	**254925±557a**	154±7c
P.oxalicum	2±0d	186327±485c	243±12a
Sucrose			
A.tubingensis (AT6)	322±1b	215552±590c	131±7d
A.niger	142±3c	221123±599b	165±3c
A.tubingensis (AT1)	660±3a	204471±485d	183±5b
P.oxalicum	**99±3d**	242581±239a	211±4a
Arabinose			
A.tubingensis (AT6)	228±6b	42731±556b	84±4b
A.niger	664±9a	45900±124a	70±5bc
A.tubingensis (AT1)	130±6c	38630±312d	58±2c
P.oxalicum	43±7d	40225±244c	107±5a
Glucose			
A.tubingensis (AT6)	**667±73b**	450±15c	119±12d
A.niger	828±65b	6858±747b	854±9a
A.tubingensis (AT1)	1238±134a	24538±1059a	650±60
P.oxalicum	84±6c	7849±410b	287±16c

Values sharing a common letter within the column and within the C sources are not significant at $P < 0.05$. Values are Mean ± SD.

6.2.5 Cobalt Phosphate [$Co_3(PO_4)_2$]

In case of AT6, the solubilization of cobalt phosphate was highest along with highest pH reduction in the media having mannitol as C source. *A. niger* showed maximum solubilization in case of fructose in which acid phosphatase activity was highest amongst the C sources. AT1 also showed higher solubilization efficiency in presence of fructose. In case of *P. oxalicum*, the highest solubilization among all the carbon sources was in sucrose. The maximum pH reduction was in glucose followed by sucrose (Fig. 6.7).

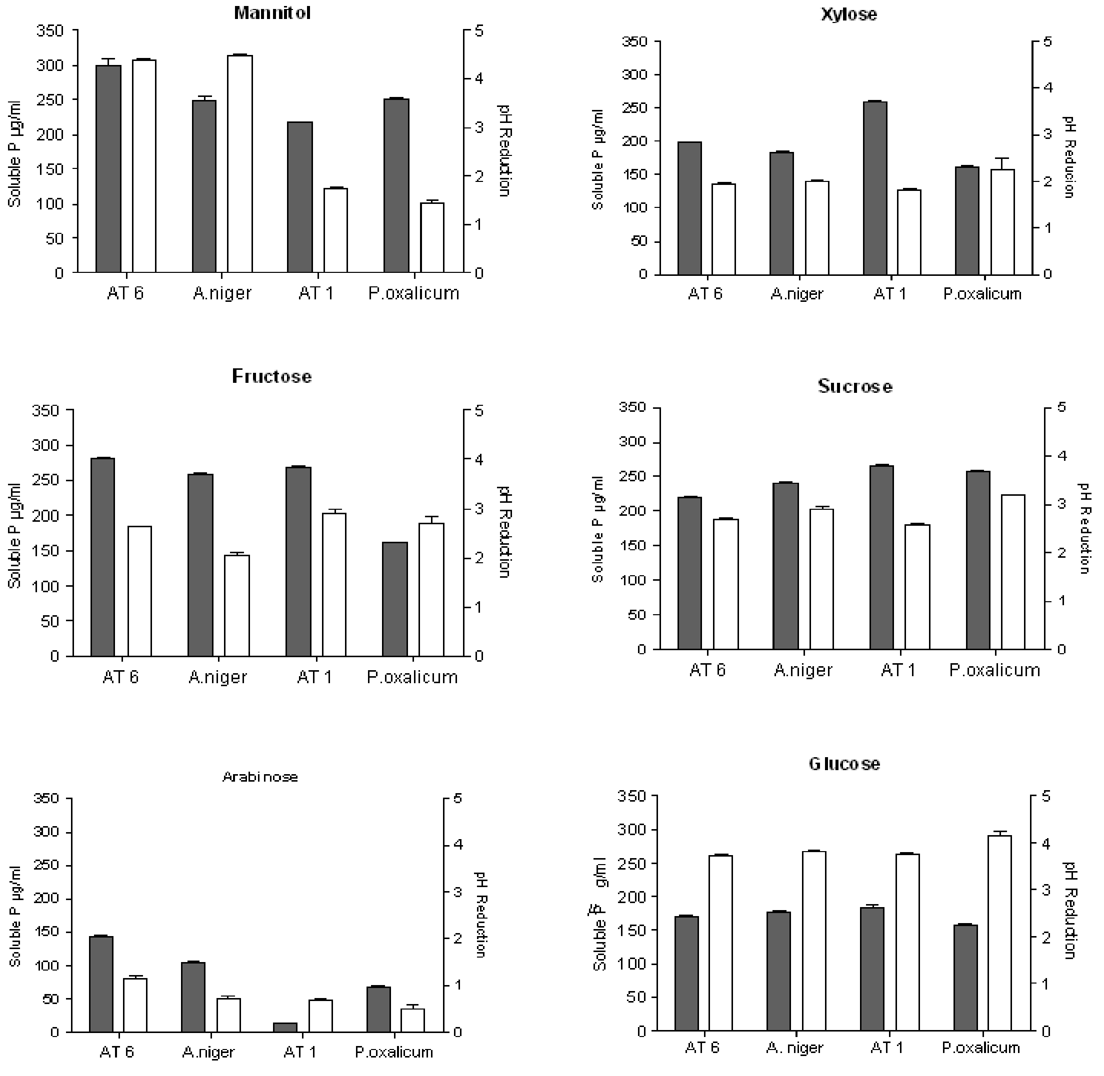

Figure 6.6: Influence of carbon sources on P solubilization (filled bars) and pH reduction (empty bars) by fungal isolates in presence of different C sources in PKV media having cobalt phosphate equivalent to 5 mM grown for 7 days.

Table 6.8: Acid phosphatase and phytase activity by fungal isolates in presence of different C sources in PKV media having cobalt phosphate equivalent to 5 mM grown for 7 days

Fungal isolates	**Acid phosphatase (µM/ml/h)**	**Phytase (µM/ml/h)**	**Dry Biomass (mg)**
Mannitol			
A.tubingensis (AT6)	125±1c	79847±490b	197±3c
A.niger	149±2b	58348± 33d	**263±5a**
A.tubingensis (AT1)	145±3b	**112220±252a**	237±4b
P.oxalicum	387±2a	64955± 826c	144±6d
Xylose			
A.tubingensis (AT6)	166±6ab	61910±498c	**211±4b**
A.niger	178±3a	72680±603b	166±5c
A.tubingensis (AT1)	**166±9ab**	79930±324a	**345±4a**
P.oxalicum	161±4b	71168± 454b	207±8b
Fructose			
A.tubingensis (AT6)	81±9c	76926±519b	127±3d
A.niger	**212±2b**	73550±521c	215±3b
A.tubingensis (AT1)	55± 1d	84693± 503a	284±8a
P.oxalicum	**610±4a**	63836±380d	170±5c
Sucrose			
A.tubingensis (AT6)	120±1b	67772±893c	149±3c
A.niger	80±1d	**79288±701b**	224±07a
A.tubingensis (AT1)	102±1c	86806±2606a	128±3d
P.oxalicum	474±12a	75021±253b	202±4b
Arabinose			
A.tubingensis (AT6)	**236±3a**	**92937±556a**	157±5b
A.niger	53± 3d	33183±287d	124±3c
A.tubingensis (AT1)	93±4c	46542±305c	153±4b
P.oxalicum	117±3b	**81794±522b**	**213±2a**
Glucose			
A.tubingensis (AT6)	2±0b	76698±826a	66±4c
A.niger	2±0b	66777±807b	59±2c
A.tubingensis (AT1)	1±0b	49669±1031ab	335±15a
P.oxalicum	7 ±0a	63650±1144b	110±9b

Values sharing a common letter within the column and within the C sources are not significant at $P < 0.05$. Values are Mean ± SD.

6.3 N sources

The solubilization of different metal phosphates was tested by using different nitrogen sources in the Pikovskaya media.

6.3.1 Aluminium Phosphate ($AlPO_4$)

The solubilization of aluminium phosphate by AT6 was maximum when ammonium sulphate was used as nitrogen source in the medium (Fig. 6.7). In this case, the pH reduction was highest amongst the other nitrogen sources used in the media. *A. niger* solubilized aluminium phosphate to a maximum extent in presence of asparagine, in which the pH reduction was higher than other N sources. AT1 and *P. oxalicum* showed highest solubilization of aluminium phosphate in presence of ammonium sulphate in Pikovskaya media. The dry biomass of all the four fungi was maximum in case of ammonium sulphate (Table 6.9).

6.3.2 Zinc Phosphate [$Zn_3(PO_4)_2$]

AT6 showed maximum solubilization of zinc phosphate in presence of asparagine in the medium (Fig. 6.8). In this case, pH reduction as well as acid phosphatase activity was higher compared to other nitrogen sources. *A. niger* solubilized maximum of zinc phosphate in case of ammonium sulphate which coincides with pH reduction. Potassium nitrate proved to be best nitrogen source for solubilization of zinc phosphate by AT1. Acid phosphatase activity was maximum when compared with other nitrogen sources in AT1 (Table 6.10). *P. oxalicum* showed highest the solubilization in presence of ammonium sulphate

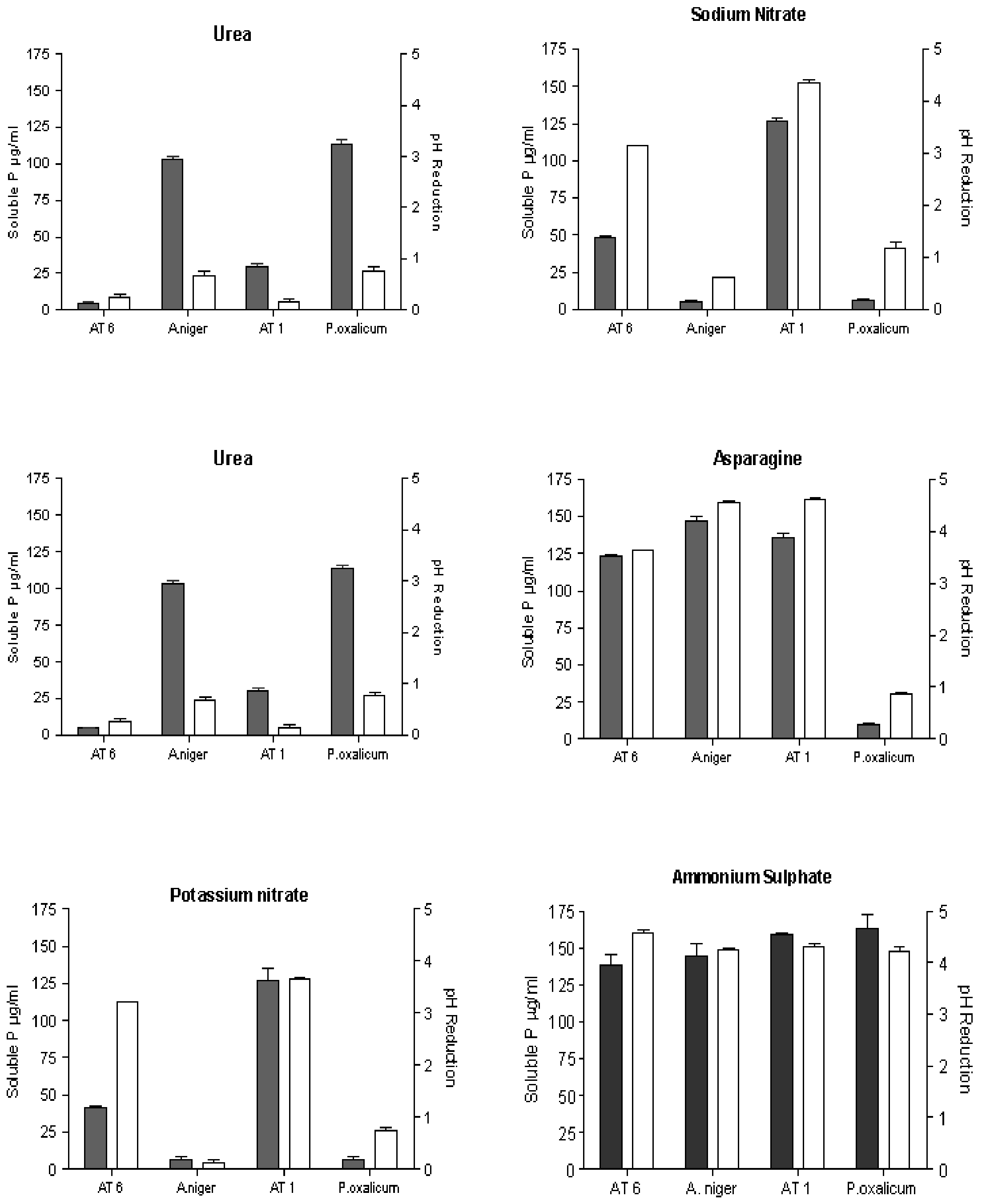

Figure 6.7: Influence of N sources on P solubilization (filled bars) and pH reduction (empty bars) by fungal isolates in PKV media supplemented aluminium phosphate equivalent to 5 mM grown for 7 days

Table 6.9: Acid phosphatase and phytase activity by fungal isolates after 7 days in presence of different N sources in PKV media having aluminium phosphate equivalent to 5 mM.

Fungal isolates	**Acid phosphatase (μM/ml/h)**	**Phytase (μM/ml/h)**	**Dry Biomass (mg)**
Ammonium Nitrate			
A.tubingensis (AT6)	**795±2a**	33845±378d	220±5a
A.niger	796±2a	18332± 354b	170±3b
A.tubingensis (AT1)	**798±7a**	56132±274a	130±03d
P.oxalicum	76±4b	25312±244c	140±3c
Sodium Nitrate			
A.tubingensis (AT6)	275±11b	87883±239a	240±3a
A.niger	778±9a	8432±693d	190±6b
A.tubingensis (AT1)	298±16b	**58596± 377b**	200±4b
P.oxalicum	8±0c	10337±272c	100±6c
Urea			
A.tubingensis (AT6)	725±3b	10006±325c	**280±8a**
A.niger	468±2c	16344±219b	220±7b
A.tubingensis (AT1)	774±7a	19720±161a	**290±9a**
P.oxalicum	**365±9d**	17089±259b	**290±4a**
Asparagine			
A.tubingensis (AT6)	406±2b	51720±268a	220±8a
A.niger	629±3a	**36600±377b**	230±7a
A.tubingensis (AT1)	277±6c	33328±451c	220±9a
P.oxalicum	8±0d	11663±171d	120±5b
Potassium Nitrate			
A.tubingensis (AT6)	393±3c	**123611±359a**	260±3a
A.niger	**952±4a**	32831±592c	**290±5b**
A.tubingensis (AT1)	410±2b	34860±515b	160±4b
P.oxalicum	164±3d	21439±697d	070±3c
Ammonium Sulphate			
A.tubingensis (AT6)	36.79±1.3d	489±39a	198±20c
A.niger	87.00±3.5c	426±26a	215±17b
A.tubingensis (AT1)	180.01±8.8a	284±22b	235±31b
P.oxalicum	126.25±8.5b	443±19a	282±13a

Values sharing a common letter within the column and within the nitrogen sources are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

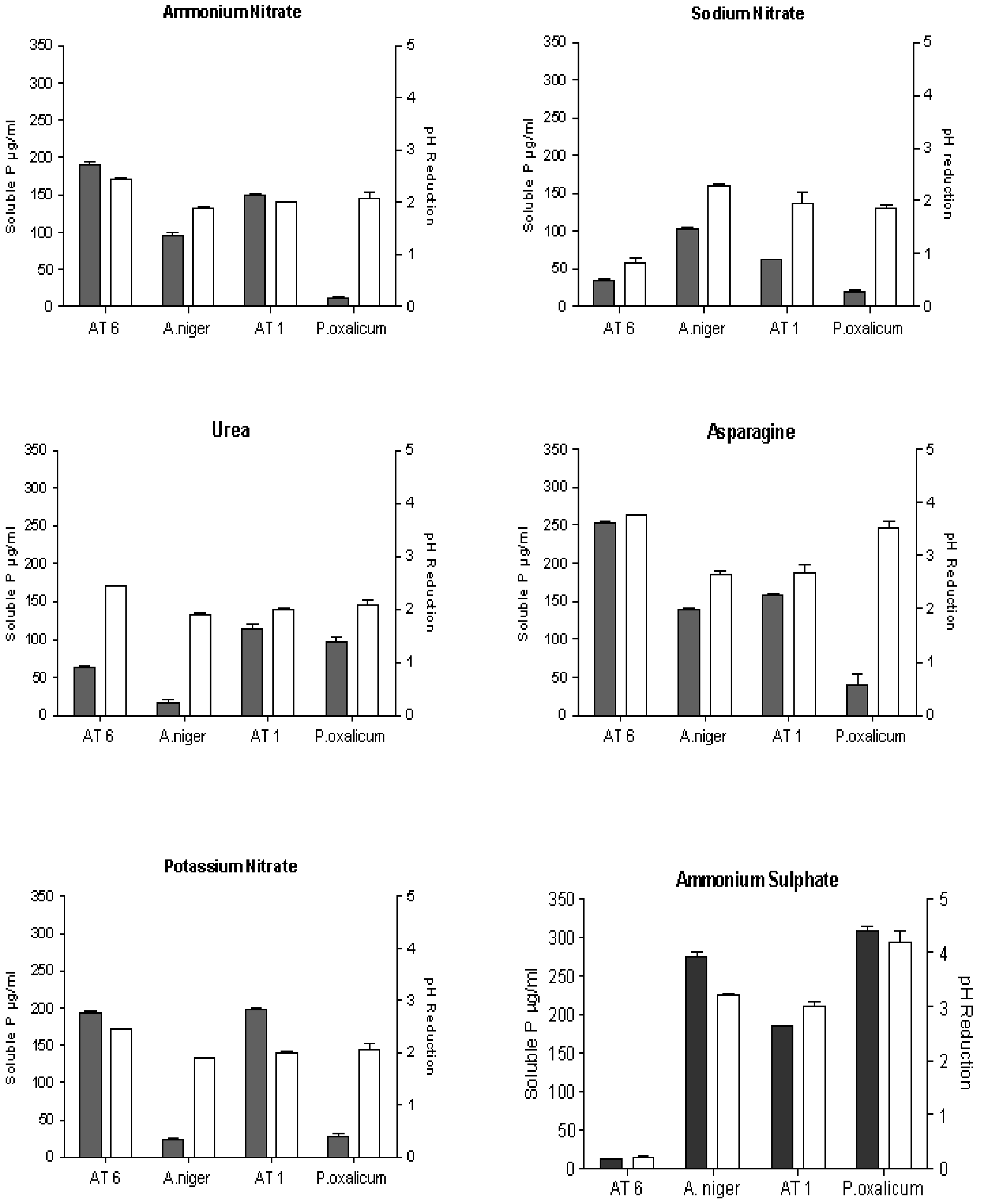

Figure 6.8: Influence of N sources on P solubilization (filled bars) and pH reduction (empty bars) by different fungal isolates in PKV media supplemented with zinc phosphate equivalent to 5 mM grown for 7 days

Table 6.10: Acid phosphatase and phytase activity by fungal isolates in presence of different N sources in PKV media having zinc phosphate equivalent to 5 mM grown for 7 days

Fungal isolates	**Acid phosphatase (µM/ml/h)**	**Phytase (µM/ml/h)**	**Dry Biomass (mg)**
Ammonium Nitrate			
A.tubingensis (AT6)	240±2a	28398±203a	226±7a
A.niger	8±0c	20755±415b	170±5b
A.tubingensis (AT1)	90±2b	27590±420a	128±8c
P.oxalicum	2±0c	15764±493c	145±5bc
Sodium Nitrate			
A.tubingensis (AT6)	**334±6a**	30801±251b	237±6a
A.niger	50±3c	**31236±218b**	185±4b
A.tubingensis (AT1)	147±2b	**38858±298a**	201±5b
P.oxalicum	23±3d	24649±422c	98±6c
Urea			
A.tubingensis (AT6)	269±2b	30904±238c	**272±9a**
A.niger	**406±2a**	25913±415d	**231±6b**
A.tubingensis (AT1)	83±2c	38692±364a	**290±6a**
P.oxalicum	**90±3c**	**34529±322b**	**299±7a**
Asparagine			
A.tubingensis (AT6)	320±9a	17359±344d	215±5a
A.niger	41±3c	5905±560a	230±7a
A.tubingensis (AT1)	98±2b	12098±251b	218±9a
P.oxalicum	13±0d	9219±325c	117±6b
Potassium Nitrate			
A.tubingensis (AT6)	272±3b	**39541±462a**	254±6a
A.niger	59±1c	15267±789c	161±4b
A.tubingensis (AT1)	**902±11a**	36144±388b	160±9b
P.oxalicum	53±2c	16302±273c	63±3c
Ammonium Sulphate			
A.tubingensis (AT6)	1±0 b	2153±105ab	96±13b
A.niger	2 ±0b	3042±60ab	133±26a
A.tubingensis (AT1)	3±1b	2506±125b	117±37ab
P.oxalicum	46 ±5a	3218±26a	172±34a

Values sharing a common letter within the column and within the nitrogen sources are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

6.3.3 Copper Phosphate (Cu_2OHPO_4)

In AT6, *A.niger* and AT1, the solubilization was maximum in presence of potassium nitrate, whereas this high solubilization was not associated with high pH reduction and dry fungal biomass. *P. oxalicum* showed higher solubilization in sodium nitrate than other nitrogen sources (Fig. 6.9). In this case, the acid phosphatase activity was maximum as compared to other N sources (Table 6.11).

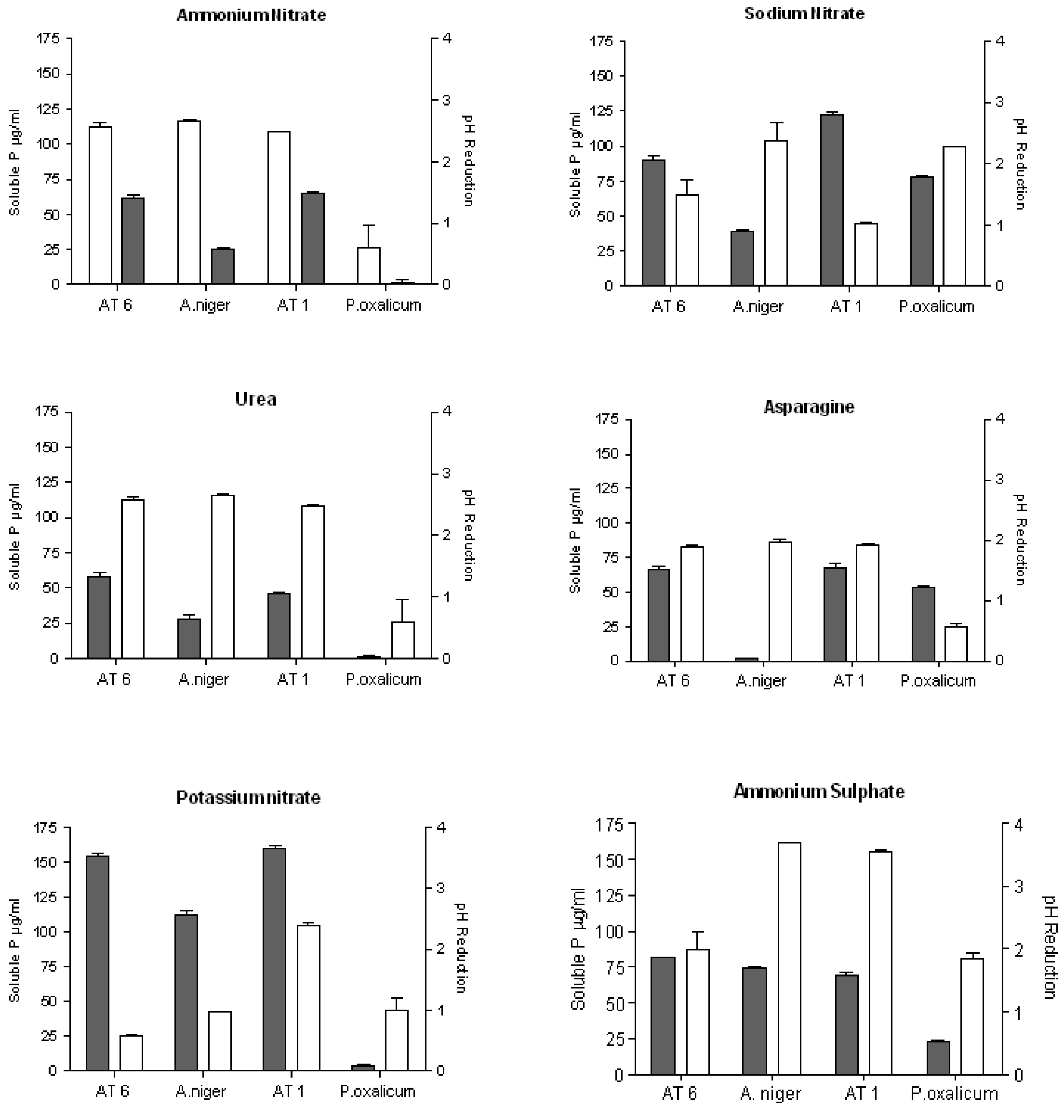

Figure 6.9: Influence of N sources on P solubilization (filled bars) and pH reduction (empty bars) by different fungal isolates in PKV media supplemented with copper phosphate equivalent to 5 mM grown for 7 days

Table 6.11: Acid phosphatase and phytase activity by fungal isolates in presence of different N sources in PKV media having copper phosphate equivalent to 5 mM grown for 7 days

Fungal isolates	**Acid phosphatase (μM/ml/h)**	**Phytase (μM/ml/h)**	**Dry Biomass (mg)**
Ammonium Nitrate			
A.tubingensis (AT6)	26±2a	109361±572a	220±7a
A.niger	31±3a	104660±529b	169±9b
A.tubingensis (AT1)	14±1b	110583±259a	120±6d
P.oxalicum	2±0d	102941±475b	135±8c
Sodium Nitrate			
A.tubingensis (AT6)	43±1c	**129328±651ab**	245±3a
A.niger	91±3b	**121996±1942c**	183±3b
A.tubingensis (AT1)	14±1d	135231±198a	195±5b
P.oxalicum	178±3a	**127857±1684bc**	87±7c
Urea			
A.tubingensis (AT6)	107±3a	103334±547a	**265±5b**
A.niger	95±3a	107477±1124b	**219±4c**
A.tubingensis (AT1)	39±2ab	105778±618bc	**282±5ab**
P.oxalicum	9±0b	102630±999c	**289±6a**
Asparagine			
A.tubingensis (AT6)	**419±12a**	41633±583c	206±7a
A.niger	**315±2b**	42897±305c	**219±6a**
A.tubingensis (AT1)	**470±14a**	46728±831b	202±8a
P.oxalicum	**104±5c**	64147±756a	108±6b
Potassium Nitrate			
A.tubingensis (AT6)	223±2a	112655±326b	250±4a
A.niger	146±2b	113835±373ab	146±6b
A.tubingensis (AT1)	56±2c	**115347±443a**	155±6b
P.oxalicum	49±2c	108491±224c	54±3c
Ammonium Sulphate			
A.tubingensis (AT6)	6±0c	336±052b	180±13a
A.niger	126±8a	604±30a	174±17a
A.tubingensis (AT1)	49±5b	341±43b	107±2b
P.oxalicum	2±0c	262±45b	78±1c

Values sharing a common letter within the column and within the different nitrogen sources are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

6.3.4 Ferric phosphate ($FePO_4$)

The maximum solubilization by AT6 was observed when sodium nitrate was used as nitrogen source in the medium (Fig. 6.10). The acid phosphatase activity in AT6 was also highest amongst the other nitrogen sources (Table 6.12). *A.niger*, AT1 and *P. oxalicum* solubilized ferric phosphate to a maximum extent in presence of ammonium sulphate as N source, which coincided with higher pH reduction.

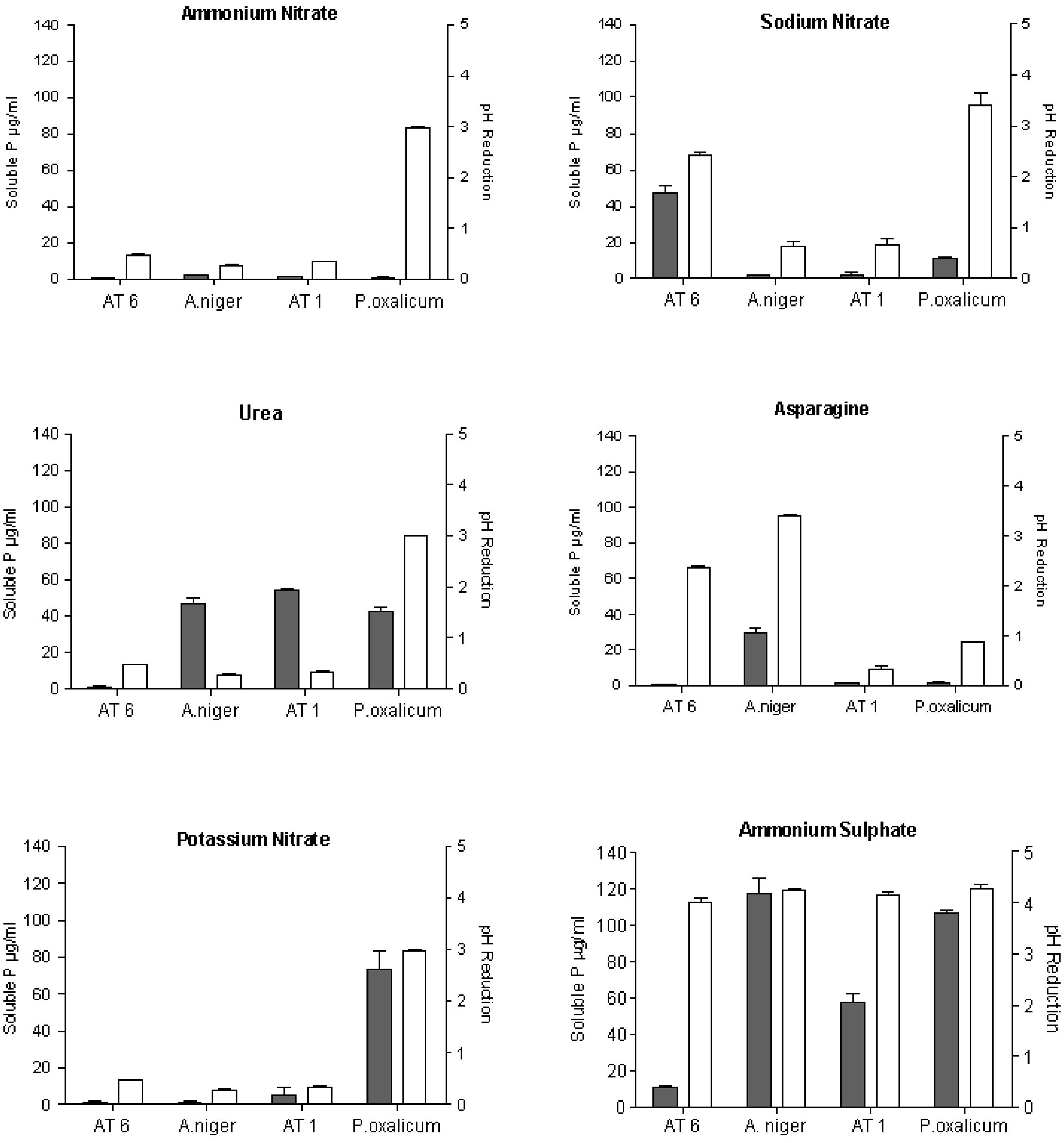

Figure 6.10: Influence of N sources on P solubilization (filled bars) and pH reduction (empty bars) by fungal isolates in PKV media supplemented with ferric phosphate equivalent to 5 mM grown for 7 days

Table 6.12: Acid phosphatase and phytase activity by fungal isolates in presence of different N sources in PKV media having ferric phosphate equivalent to 5 mM grown for 7 days

Fungal isolates	**Acid phosphatase (μM/ml/h)**	**Phytase (μM/ml/h)**	**Dry Biomass (mg)**
	Ammonium Nitrate		
A.tubingensis (AT6)	843±13a	104515±596b	179±5b
A.niger	867±6a	111702±469a	172±7b
A.tubingensis (AT1)	862±4a	104970±288b	203±5a
P.oxalicum	78±1b	104018±719b	170±5b
	Sodium Nitrate		
A.tubingensis (AT6)	855±3a	83823±796a	201±6a
A.niger	861±8a	7997±129b	185±7ab
A.tubingensis (AT1)	133±3b	9757±368c	170±3b
P.oxalicum	65±4c	25561±395d	97±4c
	Urea		
A.tubingensis (AT6)	766±7a	244176±1698b	164±4c
A.niger	759±3a	306850±592a	146±3c
A.tubingensis (AT1)	749±23a	**254987±542b**	204±6b
P.oxalicum	**557±74b**	**249706±1668b**	**294±7a**
	Asparagine		
A.tubingensis (AT6)	859±44a	**289328±694a**	**532±2a**
A.niger	238±3b	**239930±5375b**	159±5c
A.tubingensis (AT1)	839±4a	246537±707ab	200±7b
P.oxalicum	76±5c	241442±3986ab	107±6d
	Potassium Nitrate		
A.tubingensis (AT6)	**872±10a**	106358±5184a	178±11ab
A.niger	**882±4a**	106358±4842a	191±6a
A.tubingensis (AT1)	507±12b	108160±1920a	179±6ab
P.oxalicum	50±2c	106027±420a	156±3b
	Ammonium Sulphate		
A.tubingensis (AT6)	667±73 b	450±15c	119±12d
A.niger	828±65b	6858±747b	**854±9a**
A.tubingensis (AT1)	**1238±134a**	24538±1059a	**650±60**
P.oxalicum	84±6c	7849±410b	287±16c

Values sharing a common letter within the column and within the nitrogen sources are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

6.3.5 Cobalt Phosphate [$Co_3(PO_4)_2$]

The maximum cobalt phosphate solubilization was observed in AT6 along with high acid phosphatase activity in the media having ammonium nitrate (Fig. 6.11). *A. niger* showed maximum solubilization in case of potassium nitrate, AT1 in presence of sodium nitrate and P. *oxalicum* in media having asparagine as a N sources. All these isolates showed highest acid phosphatase as well as phytase activity in the respective N sources as compared to other N sources used in the study (Table 6.13).

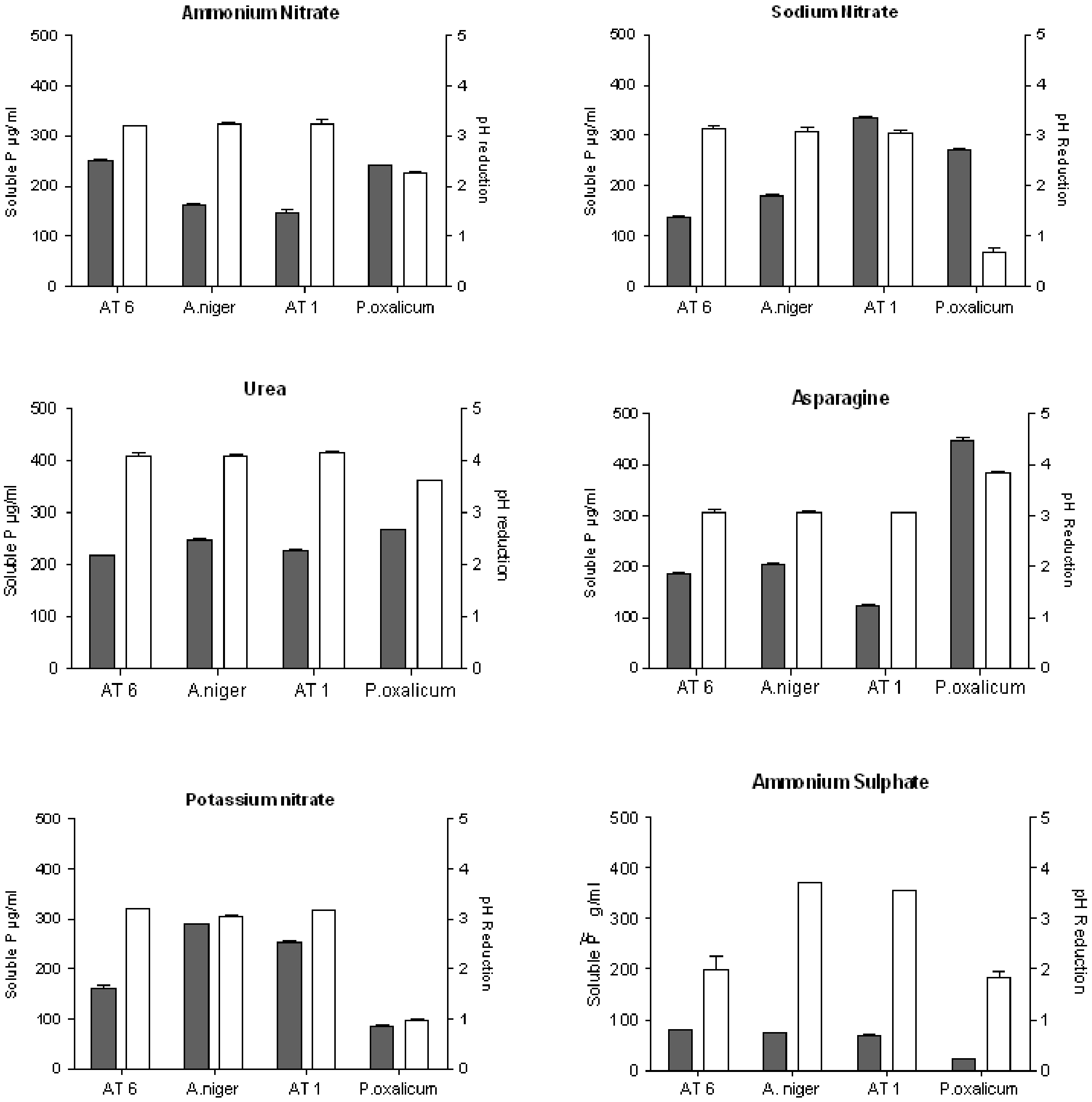

Figure 6.11: Influence of N sources on P solubilization (filled bars) and pH reduction (empty bars) by fungal isolates after 7days in presence of different N sources in PKV media having cobalt phosphate equivalent to 5 mM

Table 6.13: Acid phosphatase and phytase activity by fungal isolates in presence of different N sources in PKV media having cobalt phosphate equivalent to 5 mM grown for 7 days

Fungal isolates	**Acid phosphatase (µM/ml/h)**	**Phytase (µM/ml/h)**	**Dry Biomass (mg)**
		Ammonium Nitrate	
A.tubingensis (AT6)	**169±3b**	77921±451b	219± 3a
A. niger	66±1d	61310±564d	170±6b
A.tubingensis (AT1)	95±3c	65245±233c	132±2c
P.oxalicum	419±2a	92274±326a	149±9bc
		Sodium Nitrate	
A.tubingensis (AT6)	71±2c	**94821±292a**	243±7a
A.niger	12±1d	71955±794b	188±6b
A.tubingensis (AT1)	**153±4b**	95029±991a	201± 5b
P.oxalicum	630±1a	86992±611ab	97± 5c
		Urea	
A.tubingensis (AT6)	60±2b	47267±342c	**273± 6a**
A.niger	50±1b	51823±428b	221± 6b
A.tubingensis (AT1)	27±2c	59653±581a	284±5a
P.oxalicum	105±4a	60377±971a	**287±6a**
		Asparagine	
A.tubingensis (AT6)	167±4b	51181±759c	212±4a
A.niger	110±2c	64437±1565b	224±4a
A.tubingensis (AT1)	104±2c	49234±719c	211±6a
P.oxalicum	**801±14a**	86806±580a	113±4b
		Potassium Nitrate	
A.tubingensis (AT6)	30±3c	58824±557c	247±4a
A.niger	**144±3a**	**76802±428b**	155±8b
A.tubingensis (AT1)	91±2b	**91636±1910a**	154±1b
P.oxalicum	14±1d	33183±467d	55± 8c
		Ammonium Sulphate	
A.tubingensis (AT6)	2±0 b	76698±826a	66±4c
A.niger	2 ±0b	66777±807b	59±2c
A.tubingensis (AT1)	1±0b	49669±1031ab	**335±15a**
P.oxalicum	7±0a	63649.97±1144b	110±9b

Values sharing a common letter within the column and within the nitrogen sources are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Chapter 7

Enhancement of fertilizer value of rock phosphate

Field evaluation of PSMs

Phosphate solubilizing microorganisms (PSMs) were inoculated to Rajasthan rock phosphate (RRP) amended alkaline soils and studied the growth and yield of wheat and maize sown in two consecutive seasons. An agricultural field was used for the experiment having soil with sand: 66%; silt: 15.45%; clay 18.75% (Sandy loam); pH of 8.1; available P: 1.6 mg kg^{-1}; EC: 0.3 mS/cm; CEC: 12.7meq/100g; Total N: 0.10% and organic carbon: 0.09%.

7.1 Wheat experiment

Inoculation of P solubilizing microorganisms significantly increased the growth of wheat plants compared to non-inoculated ones (Plate 1). The shoot height increased in RRP amended soil than normal soil inoculated with these fungi and bacteria for e.g. the shoot height of the RRP fertilized soil inoculated with *P. oxalicum* was 1.5 times higher than that of non-inoculated and non-amended soil (Table 7.1). The shoot as well as root dry biomass increased significantly in RRP amended soil in all the inoculated treatments. Shoot and root dry biomass was maximum in the plants having bacterial consortium as inoculum in RRP fertilized soil. An increase in the yield of wheat was also recorded with the application of P solubilizing fungi as compared to non-inoculated RRP amended or non-amended control soil. In the treatment having RRP and *P. oxalicum,* the yield was maximum i.e. 42% higher than control. However, the yield in the treatment having RRP and bacteria was same as that of control. The Inoculation with P solubilizing fungi and bacteria considerably increased P uptake and enhanced the plant growth, even in the absence of rock phosphate. Inoculation by these P solubilizing isolates in the plots having

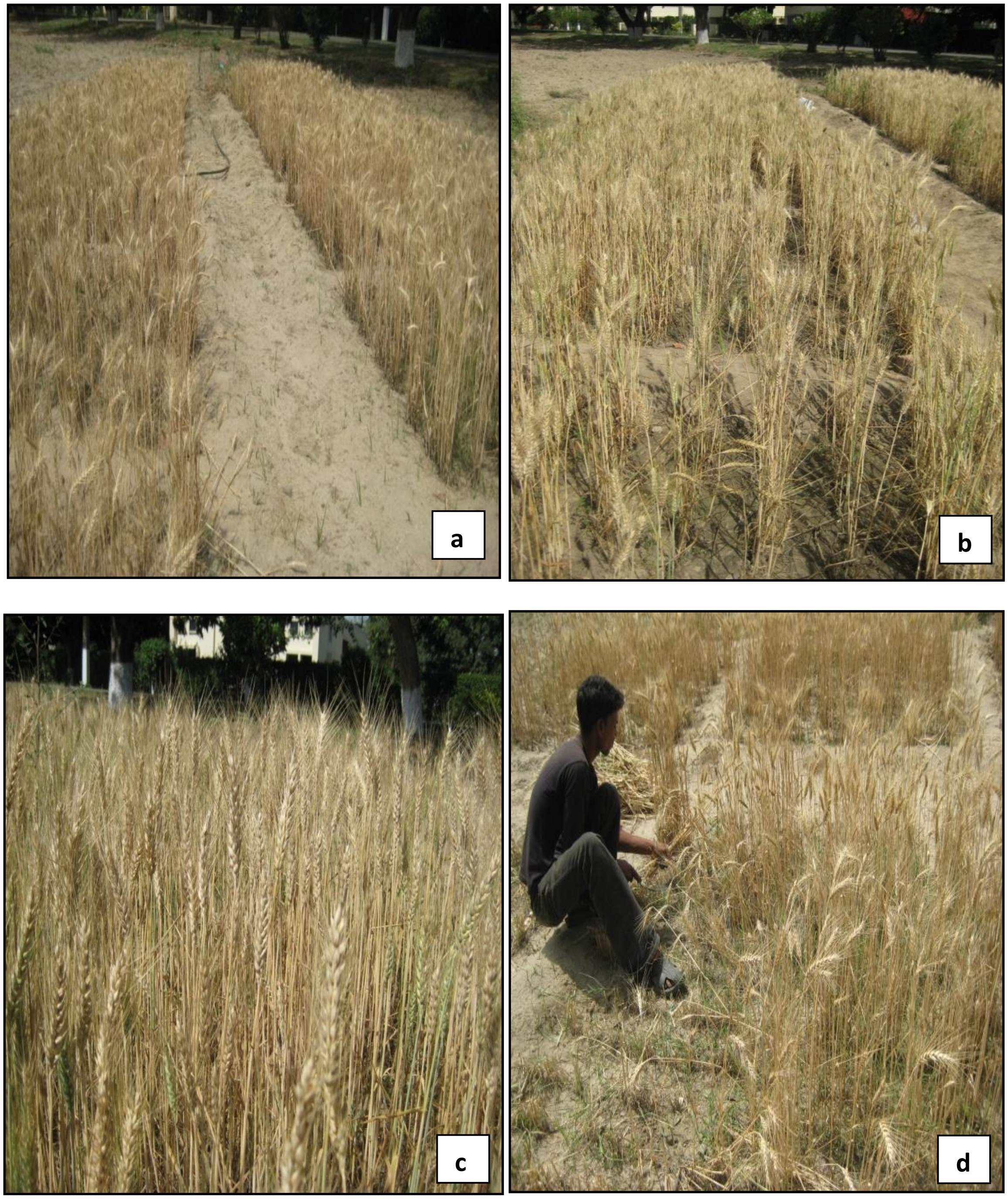

Plate 1: Wheat experiment under field conditions. a and b: showing experimental design of wheat crop, c: ripened wheat crop, d: harvesting of the crop

RRP fertilization increased total P contents of wheat plants parts (in shoot, grain and roots) in amounts higher than those reported with plants receiving inoculum without fertilization. The P content in roots was higher than the other plant parts in all the treatments. The total P levels were higher in RRP amended and inoculated soil than RRP amended non-inoculated control (Table 7.2). The available P increased 3 to 4 times in RRP amended soils inoculated with PSMs compared to non-amended and non-inoculated control soil. In general, inoculation of these PSMs increased the available P compared to the respective control treatments. Inoculation of PSMs increased the organic carbon as well as nitrogen content in both RRP amended and non-amended soils compared to control treatment (Table 7.3). There was not much change in the pH of the soil compared to initial soil values. The electric conductivity (EC) and value of total dissolved salts (TDS) decreased in all the treatments when compared to the initial values of soil (Table 7.4). When both these parameters were compared among the different treatments after wheat harvest, inoculated and RRP amended treatments showed more decrease in the values as compared to their respective controls. Different enzyme activities were tested for organic phosphate dissolution in the soil (Table 7.5). Acid phosphatase activity was very less in all the treatments. Alkaline phosphatase activity was higher in all the inoculated treatments as compared to the non-inoculated controls. Phytase activity was higher than phosphatase activity in all the treatments.

Viability of inoculated fungi and bacteria was tested, one week after inoculation in the field soil and again after 20 weeks (i.e. one week before harvesting). The viable cell count of fungi after one week of inoculation was 2.8-3.0 x 10^6 and that of bacteria was 7.5 x 10^6 on Pikovskaya agar (PKV) plate. However, after 20 weeks the viability of inoculated fungi and bacteria was found to be 7.4-8.2 x 10^7 and 8.7 x 10^7 respectively. Microbial growth was observed in the non-inoculated soil samples, but no halo zones around the colonies were observed, which showed that phosphate solubilizing microorganism were absent in the non-inoculated control soils.

Aflatoxins in the wheat grain samples were tested by HPLC analysis and these results revealed that the aflatoxins such as B1, B2, G1 and G2 were absent in the grains.

Table 7.1: Growth parameters and P uptake in wheat plants as affected by Rajasthan rock phosphate (RRP) and different inoculums

Treatments	**Yield in ($kg/4m^2$)**	**Shoot length (cm)**	**Shoot dry wt (g)**	**Root dry wt (g)**
Soil	0.76±0.02f	43±2c	1.07±0.04e	0.60±0.02d
Soil+AT6	1.00±0.02d	51±1c	1.28±0.04d	0.94±0.02ab
Soil+*A.niger*	1.10±0.04c	50±3c	1.24±0.09d	0.87±0.02bc
Soil+AT1	0.97±0.02d	51±3c	1.30±0.03d	0.76±0.02c
Soil+*P.oxalicum*	1.19±0.01b	58±2ab	1.67±0.02c	0.64±0.02d
Soil+FC	0.80±0.01f	61±2a	1.89±0.05ab	0.85±0.01bc
Soil+BC	0.78±0.02f	55±2b	1.45±0.03d	0.92±0.02ab
Soil +RRP	0.77±0.02f	50±3c	1.48±0.11d	0.73±0.05c
Soil+RRP+AT6	1.10±0.02c	51±3c	1.71±0.09c	0.96±0.06ab
Soil+RRP+*A.niger*	1.10±0.02c	59±2ab	1.82±0.03b	0.90±0.06ab
Soil+RRP+AT1	1.05±0.01c	60±1a	1.89±0.02ab	1.14±0.07a
Soil+RRP+*P.oxalicum*	**1.29±0.03a**	62±2a	1.92±0.04ab	0.73±0.05c
Soil+RRP+FC	0.87±0.01e	**63±3a**	1.93±0.05ab	0.99±0.08ab
Soil+RRP+BC	0.79±0.01f	**63±2a**	**2.00±0.06a**	**1.19±0.06a**

Values sharing a common letter within the column are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Table 7.2: Total P in different tissues of wheat plants as affected by Rajasthan rock phosphate (RRP) and different inoculums

Treatments	**Total P (mg/kg)**			
	Grain	**Stem**	**Root**	**Seed Cover**
Soil	52±1a	21±1d	69±2i	37±2g
Soil+AT6	204±6c	48±6b	219±1g	132±11d
Soil+*A. niger*	57±3fg	45±5bc	163±3h	62±3f
Soil+AT1	123±6e	43±6bc	254±6ef	78±6f
Soil+*P.oxalicum*	74±5f	31±6bc	244±2f	139±3c
Soil+FC	35±3cd	19±1d	223±7g	135±4cd
Soil+BC	144±5d	24±6d	298±4d	95±4e
Soil+RRP	153±5d	24±1d	272±7e	43±1g
Soil+RRP+*AT6*	**297±7a**	52±5b	**520±12a**	141±5cd
Soil+RRP+*A. niger*	209±9c	57±5b	218±3g	207±6b
Soil+RRP+AT1	240±10b	45±5bc	269±8e	153±7c
Soil+RRP+*P.oxalicum*	203±10c	54±5b	266±6e	**449±6a**
Soil+RRP+FC	260±50b	**116±3a**	457±9b	140±6cd
Soil+RRP+BC	246±9b	32±3cd	412±10c	100±6e

Values sharing a common letter within the column are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Table 7.3: Soil characteristics of wheat plants as affected by Rajasthan rock phosphate (RRP) and different inoculums

Treatments	**Total P (mg/kg)**	**Available P (mg/kg)**	**Carbon (%)**	**Organic matter (mg)**	**Nitrogen (%)**
Soil	329±10e	1.57±0.13d	0.10±0.00abc	0.17±0.01abc	0.13±0.01c
Soil+AT6	472±9cd	2.84±0.06cd	0.11±0.00abc	0.18±0.01abc	0.18±0.01b
Soil+*A.niger*	340±6e	2.82±0.22cd	0.11±0.00abc	0.18±0.01abc	0.17±0.01b
Soil+AT1	552±15c	2.75±0.11cd	0.08±0.01c	0.15±0.01c	0.18±0.01b
Soil+*P.oxalicum*	353±6de	2.91±0.03cd	0.09±0.00abc	0.16±0.01abc	0.21±0.02b
Soil+FC	537±12c	2.57±0.02cd	0.09±0.00c	0.15±0.00c	0.20±0.02b
Soil+BC	343±6de	2.55±0.19cd	0.11±0.01abc	0.16±0.01abc	0.15±0.01b
Soil+RRP	1245±27a	3.85±0.27bc	0.09±0.00c	0.15±0.01c	0.18±0.01b
Soil+RRP+*AT6*	1208±22a	**5.24±0.29ab**	0.11±0.00a	**0.20±0.01a**	0.21±0.02b
Soil+RRP+*A. niger*	1219±36a	4.88±0.47b	0.11±0.01ab	0.19±0.01ab	0.22±0.01b
Soil+RRP+AT1	939±11b	4.90±0.30b	0.09±0.00bc	0.16±0.01bc	**0.25±0.02a**
Soil+RRP+*P.oxalicum*	1225±20a	**5.20±0.27ab**	0.11±0.00ab	0.19±0.01ab	**0.27±0.02a**
Soil+RRP+FC	1030±15b	**6.81±0.80a**	0.11±0.00abc	0.18±0.00abc	0.20±0.02b
Soil+RRP+BC	1056±19b	**5.44±0.04ab**	0.10±0.01abc	0.17±0.01abc	0.17±0.00c

Values sharing a common letter within the column are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Table 7.4: Soil characteristics of wheat plants as affected by RRP and different inoculums

Treatments	**pH**	**EC (mScm^{-1})**	**TDS (ppm)**
Soil	8.03±0.01a	0.18±0.01bcd	0.130±0.00b
Soil+AT6	7.86±0.01bc	0.15±0.00e	0.135±0.01b
Soil+*A.niger*	7.88±0.01b	0.16±0.00e	0.140±0.02b
Soil+AT1	7.80±0.01cd	0.18±0.00bcd	0.160±0.02a
Soil+*P.oxalicum*	7.86±0.01bc	0.17±0.01de	0.160±0.03a
Soil+FC	7.85±0.02bc	0.17±0.00cde	0.160±0.03a
Soil+BC	7.59±0.01g	0.17±0.01de	0.160±0.03a
Soil+RRP	7.86±0.01bc	0.20±0.00ab	0.150±0.00a
Soil+RRP+*AT6*	7.75±0.01de	0.19±0.00abc	0.155±0.00a
Soil+RRP+*A. niger*	**7.49±0.01h**	0.18±0.00bcd	0.150±0.01a
Soil+RRP+AT1	7.68±0.03f	**0.21±0.00a**	**0.160±0.00a**
Soil+RRP+*P.oxalicum*	**7.65±0.01fg**	**0.20±0.01ab**	0.150±0.00a
Soil+RRP+FC	7.83±0.02bc	**0.20±0.01ab**	0.155±0.00a
Soil+RRP+BC	7.70±0.02ef	**0.20±0.00ab**	0.155±0.00a

Values sharing a common letter within the column are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Table 7.5: Enzyme activities in the soil of wheat plants as affected by RRP and different inoculums

Treatments	**Acid Phosphatase (µM/g/h)**	**Alkaline Phosphatase (µM/g/h)**	**Phytase activity (µM/g/h)**
Soil	4.9±0.3e	65.0±0.3fg	39811±556a
Soil+AT6	5.1±0.1e	68.8±0.5g	44118±3230a
Soil+*A.niger*	5.9±0.1de	101.5±0.5c	44639±294a
Soil+AT1	5.3±0.0e	77.0±3.9ef	40333±228a
Soil+*P.oxalicum*	11.9±0.6cd	123.6±0.7b	40529±229a
Soil+FC	15.9±1.0bc	123.9±2.3b	43139±1141a
Soil+BC	11.0±0.5cd	144.5±0.4a	40366±1435a
Soil+RRP	10.8±0.2cd	69.1±1.6fg	44280±1892a
Soil+RRP+*AT6*	11.8±0.3c	131.5±1.7b	**52566±1566a**
Soil+RRP+*A. niger*	31.3±0.6a	87.3±3.5de	**50805±1043a**
Soil+RRP+AT1	12.7±0.4c	129.5±1.9b	42878±750a
Soil+RRP+*P.oxalicum*	**20.4±3.2b**	124.7±0.7b	47673±65a
Soil+RRP+FC	**28.0±1.0a**	100.0±5.0cd	**48032±2121a**
Soil+RRP+BC	14.4±0.2c	109.2±1.7c	**49337±816a**

Values sharing a common letter within the column are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

7.2 Maize Experiment

The yield of maize significantly increased in RRP amended soils inoculated with the PSMs compared to other treatments (Plate 2). AT6 and *P. oxalicum* when inoculated in RRP amended soil showed more than 82% increase in yield whereas other inoculated RRP amended treatments showed 60-70 % increase in yield compared to non-inoculated and non-amended control (Table 7.6). The yield of maize was 1.5 times more in the RRP amended treatment inoculated with bacterial consortium than the control soil. The shoot height increased in the PSM inoculated and RRP amended treatments. Shoot height as well as shoot dry biomass was found to be highest in plot having *A. niger* and RRP in the soil. The dry biomass of shoot and root were significantly increased in soils inoculated with these P solubilizing fungi and bacterial consortium compared to controls. The growth parameters were higher in RRP amended soil inoculated with the PSMs compared to non-amended soils (Table 7.6). Phosphate uptake in the plant parts was higher in the inoculated treatments than non-inoculated control. The P uptake in grain was significantly higher in most of the RRP amended PSM inoculated treatments compared to their control soils (Table 7.7). In general the P levels were increased both in RRP amended and non-amended soils inoculated with PSMs (Table 7.8). After harvesting maize, the total P content decreased in the soil to less than one third of the P content present initially. The addition of PSMs increased the available P of the soil to more than five folds as compare to control soil. The highest increase was observed where RRP fertilization was involved for example in RRP amended soil inoculated with *P. oxalicum*

Plate 2: Maize experiment under field conditions. a: showing experimental design of maize crop, b: ripened maize cobs, c: harvested maize cobs and d: corn cobs for growth and yield parameters

and fungal consortium. The increase in available P in this case was more than ten folds than the control soil. In bacterial consortium inoculated and RRP amended treatment, the available P in the soil was more than five times higher as compared to control. A general increase in organic carbon occurred in soil after harvesting the maize as compared to control soil. The total N content increased in the treatments having RRP along with inoculum, the maximum N content was observed in fungal consortium inoculated RRP amended soil.

The pH of the soil decreased compared to the initial pH of the soil in all the treatments (Table 7.9). This reduction was higher in the treatments where RRP amended soil was inoculated with PSMs. Electric conductivity as well as TDS reduced significantly in all the treatments after maize harvest as compared to the initial values. Acid phosphatase activity increased more than ten times in all treatments as compared to initial values. Phytase activity was higher than phosphatase activity in all the treatments (Table 7.10).

The viability of inoculated P solubilizing microorganisms was tested one week after inoculation in maize field, which was found to be 7.1 - 8.0 x 10^6 and 8.8 x 10^6 for fungi and bacteria respectively. After eleven weeks, the viability of inoculated fungus and bacteria was 4.9 - 5.8 x 10^9 and 6.2 x 10^9 respectively. In control soils where inoculum was not added, it was found that no halo zones were present around the grown colonies which suggested that phosphate solubilizing microorganism were absent in the control soils.

Table 7.6: Growth parameters and P uptake in maize plants as affected by Rajasthan rock phosphate (RRP) and different inoculums

Treatments	Yield (kg/4m^2)	Shoot length (cm)	Shoot dry wt (g)	Root dry wt (g)
Soil	1.73±0.01d	140±9b	31±3c	3.1±0.7c
Soil+AT6	2.35±0.01c	145±3b	37±8c	4.0±1.9c
Soil+*A. niger*	2.77±0.01b	147±7b	51±3ab	4.0±0.4c
Soil+AT1	2.88±0.01b	162±4a	48±7b	7.5±0.4b
Soil+*P.oxalicum*	1.80±0.01d	146±8b	31±3c	3.3±0.3c
Soil+FC	1.82±0.01d	150±3b	39±4c	3.3±0.4c
Soil+BC	1.75±0.02d	140±5b	60±7ab	6.8±2.1b
Soil+RRP	2.44±0.01c	150±8b	49±4b	6.3±1.9b
Soil+RRP+AT6	2.78±0.02b	152±3b	50±10b	7.0±1.5b
Soil+RRP+*A. niger*	2.95±0.02b	**167±10a**	**81±7a**	7.8±0.8b
Soil+RRP+AT1	**3.15±0.03a**	**163±5a**	61±13ab	7.9±0.7b
Soil+RRP+*P.oxalicum*	**3.10±0.02a**	150±5b	55±12ab	6.4±1.6b
Soil+RRP+FC	2.69±0.02c	152±5b	60±6ab	**9.4±1.6a**
Soil+RRP+BC	2.58±0.01c	149±8b	**72±4a**	**9.8±1.8a**

Values sharing a common letter within the column are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Table 7.7: Total P in different tissues of maize plants as affected by Rajasthan rock phosphate (RRP) and different inoculums

Treatments	**Total P (mg/kg)**		
	Grain	**Stem**	**Root**
Soil	91±5d	72±3d	69±4e
Soil+AT6	143±13c	121±5b	141±4c
Soil+*A.niger*	130±7c	118±6c	140±5c
Soil+AT1	143±9c	116±7c	166±10c
Soil+*P. oxalicum*	149±18c	130±7b	98±3d
Soil+FC	150±2c	111±4c	105±8d
Soil+BC	141±4c	85±5d	121±9d
Soil+RRP	152±4c	89±5d	116±7d
Soil+RRP+AT6	167±5b	**160±3a**	185±3b
Soil+RRP+*A. niger*	163±0b	131±5b	183±5b
Soil+RRP+AT1	185±7b	134±4b	**237±9a**
Soil+RRP+*P.oxalicum*	178±11b	140±6b	166±7c
Soil+RRP+FC	**243±10a**	125±5b	188±8b
Soil+RRP+BC	156±4c	134±7b	153±4c

Values sharing a common letter within the column are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Table 7.8: Soil characteristics of maize plants as affected by Rajasthan rock phosphate (RP) and different inoculums

Treatments	**Total P in (mg/kg)**	**Available P (mg/kg)**	**Carbon (%)**	**Organic matter (mg)**	**Nitrogen (%)**
Soil	197.46±5.43e	1.64±0.09g	0.65±0.03a	1.11±0.05a	0.18±0.01c
Soil+AT6	284.42±9.06bcde	3.11±0.10f	0.70±0.03a	1.21±0.05a	0.29±0.02b
Soil+*A. niger*	318.84±18.12abc	3.65±0.16ef	0.72±0.03a	1.23±0.05a	0.21±0.02
Soil+AT1	248.19±12.68cde	3.01±0.15f	0.72±0.04a	1.25±0.06a	0.20±0.01
Soil+*P. oxalicum*	320.65±19.93acb	3.29±0.17f	0.75±0.04a	1.29±0.06a	0.24±0.01
Soil+FC	295.29±12.68bcd	3.54±0.05ef	0.71±0.02a	1.22±0.04a	0.21±0.02
Soil+BC	217.39±10.87de	3.42±0.12f	0.76±0.04a	1.31±0.07a	0.18±0.01c
Soil+RRP	304.35±10.87bcd	4.69±0.13de	0.71±0.02a	1.22±0.04a	0.21±0.01c
Soil+RRP+*AT6*	356.88±16.30ab	5.68±0.12cd	0.72±0.01a	1.23±0.02a	0.32±0.02b
Soil+RRP+*A. niger*	344.20±14.49ab	6.40±0.47c	0.73±0.01a	1.26±0.02a	0.25±0.02c
Soil+RRP+AT1	364.13±16.30ab	5.99±0.15c	0.75±0.04a	1.29±0.06a	0.31±0.02b
Soil+RRP+*P.oxalicum*	396.74±19.93a	**10.38±0.43b**	**0.81±0.01a**	**1.40±0.02a**	0.32±0.02b
Soil+RRP+FC	409.42±32.61a	**11.97±0.13a**	**0.79±0.05a**	**1.37±0.09a**	0.43±0.02a
Soil+RRP+BC	403.99±9.06a	**9.36±0.16b**	0.77±0.03a	1.33±0.05a	0.39±0.02a

Values sharing a common letter within the column are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Table 7.9: Soil characteristics of maize plants as affected by Rajasthan rock phosphate (RP) and different inoculums

Treatments	pH	EC (mScm^{-1})	TDS (ppm)
Soil	8.04±0.07a	0.08±0.01bcda	0.05±0.00bcd
Soil+AT6	7.08±0.02bc	0.04±0.00fg	0.03±0.00d
Soil+*A. niger*	6.98±0.02cd	0.06±0.00def	0.04±0.00cd
Soil+AT1	6.95±0.04cde	0.08±0.00bcda	0.05±0.00bcd
Soil+*P. oxalicum*	6.83±0.10defg	0.05±0.00ef	0.03±0.01d
Soil+FC	7.10±0.06bc	0.07±0.00cdef	0.04±0.01cd
Soil+BC	7.25±0.07b	0.09±0.00abcd	0.06±0.01bc
Soil+RRP	7.2±0.03b	0.02±0.00g	0.14±0.01a
Soil+RRP+*AT6*	**6.66±0.06g**	**0.11±0.00ab**	0.07±0.00b
Soil+RRP+*A. niger*	6.76±0.04efg	0.09±0.00abc	0.06±0.00bc
Soil+RRP+AT1	6.85±0.06def	**0.11±0.00a**	0.07±0.00b
Soil+RRP+*P.oxalicum*	**6.71±0.05fg**	0.09±0.00abc	0.05±0.01bcd
Soil+RRP+FC	6.80±0.03defg	0.09±0.00abc	0.06±0.00bc
Soil+RRP+BC	6.75±0.03fg	0.09±0.00bcda	0.06±0.00bc

Values sharing a common letter within the column are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Table 7.10: Enzyme activities in the soil of wheat plants as affected by Rajasthan rock phosphate (RP) and different inoculums

Treatments	**Acid phosphatase (µM/g/h)**	**Phytase (µM/g/h)**
Soil	276.8±1.2c	9416±356d
Soil+AT6	256.4±0.8e	10340±616bcd
Soil+*A. niger*	284.4±0.7b	10589±128abcd
Soil+AT1	268.7±0.5d	9866±188cd
Soil+*P. oxalicum*	246.8±0.8f	10487±120abcd
Soil+FC	247.4±0.8f	9651±343d
Soil+BC	281.6±0.8bc	10413±184abcd
Soil +RRP	284.1±0.8b	11836±176abcd
Soil+RRP+AT6	284.9±0.8b	12173±461abc
Soil+RRP+*A. niger*	286.6±0.8ab	12628±815ab
Soil+RRP+AT1	285.6±0.8ab	12701±598ab
Soil+RRP+*P.oxalicum*	284.6±0.8b	**12819±739a**
Soil+RRP+FC	**293.1±0.8a**	11411±677abcd
Soil+RRP+BC	282.4±0.8bc	11513±402abcd

Values sharing a common letter within the column are not significant at $P < 0.05$. Values are Mean ± SD, n=3.

Chapter 8

Discussion

Discussion

Isolation and diversity of phosphate solubilizing microorganisms (PSMs)

Soil microorganisms play an important role in soil processes that determine plant productivity. For successful functioning of introduced microbial bioinoculants and their influence on soil health, exhaustive efforts have been made to explore soil microbial diversity, their distribution and behavior in soil habitats (Hill 2000). The era of molecular microbial ecology has uncovered only a part of novel microbiota, most of which is based on rRNA and rDNA analysis (Torsvik and Overseas 2002). In present study, P solubilizing bacteria and fungi were isolated on Pikovskaya (PKV) agar plates and further quantitative solubilization and screening was done in PKV broth having tricalcium phosphate (TCP) or Rajasthan rock phosphate (RRP) as P source. Several authors attribute the solubilization of inorganic insoluble phosphate by microorganisms to the production of organic acids and chelating oxo acids from sugars (Leyval and Barthelin 1989, Yadav and Dadarwal 1997). Therefore, most of the quantitative tests to assay the relative efficiency of the phosphate solubilizing microorganisms are based on the lowering of pH, owing to production of organic acids into the surrounding medium (Bajpai and Sundara Rao 1971; Gaind and Gaur 1989; Rose 1957). The initial isolation of phosphate solubilizers is usually made by using a medium suspended with insoluble phosphates such as tri calcium phosphates (Tilak 1993). The production of clearing zones around the colonies of the organism is an indication of the presence of phosphate solubilizing organisms. Such cultures are isolated and the extent of phosphate solubilization is determined quantitatively, by biochemical methods (Subbarao 1993;

Tilak 1993). Fungi produced larger halo zones than bacteria. These results are in accordance to Chabot et al. (1993) and Nahas (1996). Similar results were also found by Kucey (1983, 1987); Kumar and Narula (1999).

Bacterial diversity

All the strains maintained their phosphate solubilizing activity after being re-sown many consecutive times in Pikovskaya medium (Igual et al. 2001). Preferentially endospore-forming Gram positive rods were found regarding other bacteria examined in the rhizospheric soil of mind landfills of rock phosphate mines. This can be compared with the results of Silva and Nahas (2002) that larger proportion (100%) of spore forming Gram positive rods were found in uncultivated soil fertilized with rock phosphate compared to other treatments.

A measurement of physiological activity of microorganisms is an approach, allowing the study of different characteristics and metabolic diversity of P solubilizing microbial communities. The Biolog technique is one of the methods which rely on measurements of utilizing different carbon substrates by microorganisms. Bacterial respiration reduces the tetrazolium dye to formazan in active cells, so that the pattern of colored wells (different carbon sources) represents a metabolic fingerprint. Measurements of substrate use enable qualifying microbial metabolic capabilities and hence functional diversity of a microbial community (Stefanowicz 2006). The Biolog plates enable characterization of a community on the basis of so-called individual physiological pro-files which are obtained as a result of inoculation of bacterial isolates onto a plate (Garland 1997). Several investigators have successfully used Biolog plates to differentiate between microbial communities (Garland 1996; Grayson and Campbell

1996; Guckert et al. 1996; Victorio et al. 1996). Miller et al. (1993) reported that Biolog plates can be efficient between 70 to 100 % for identification of the bacterial isolates. McCarthy and Murray (1996) used Biolog plates on 103 Gram negative isolates cultured from a contaminated aquifer. They were able to tentatively identify 55 of the isolates and kept records of the carbon source utilization pattern of the remaining 48 isolates for future characterization. Numerous lines of evidence suggest that aerobically incubated Biolog plates may be a poor method to detect the taxa (Konopka et al. 1998).

Identification of bacterial isolates

Polyphasic taxonomical studies, which include phenotypic, genetic and phylogenetic information, have been widely used in microbial diversity studies (Vandamme et al. 1996). Regarding genetic and phylogenetic characterizations, molecular techniques such as DNA sequencing have been used (Eisen 1995). The sequence of the 16S rRNA gene has been widely used as phylogenetic marker in microbial ecology (Ludwig et al. 1998), since the extent of divergence in the sequence of this gene provides an estimate of the phylogenetic distance existing between different species (Igual et al. 2001). The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel 2001).

The sequences of the 16S rRNA gene of the 2C9 and SC18 strains showed 99 % identity with the sequence of the 16S rRNA gene reported in the Genbank for *Bacillus pumilus,* C19 had 99% similarity with uncultured *Bacillu*s *spp.* and 4G2 strain showed 99 % identity to *Bacillus cereus*. These results were similar to the classification obtained with biochemical testing as they all were identified as *Bacillus* species.

There are studies reporting bacteria from the *Bacillus* genus and Enterobacteriaceae family with potential to promote P solubilization (Souchie et al. 2006). Rodriguez and Fraga (1999) related that strains from *Pseudomonas*, *Bacillus* and *Rhizobium* are among the genera with the greatest potential for P solubilization. Kucey (1983) reported that mainly the biofertilizer bacteria are genus of *Pseudomonas* and *Bacillus*. Those bacteria are able to solubilize available forms of Fe, Ca, Mg, Al bound P. This solubilization effect is generally due to the production of organic acids.

Fungal diversity

The identification of fungal species by classic taxonomy is based mainly on the use of morphological markers. However, the number of these markers available is generally low, which makes difficult the classification and/or identification of related species. The development of molecular biology techniques for the genetic differentiation of species has resulted in substantial advances in taxonomy due to their sensitivity and specificity. The amplification of internal transcribed spacer (ITS1-5.8S-ITS2) of ribosomal DNA (rDNA) by the polymerase chain reaction (PCR) (Mullis and Faloona 1987), combined with sequencing of the amplicon and analysis of similarity between the sequences obtained and those already deposited in the gene bank, has been frequently employed for identification of fungal species.

In this study out of 14 isolates, 13 were identified as genus *Aspergillus* whereas one belongs to *Penicillium.* Classical taxonomy has been utilized and it does not allow the discrimination of *A. tubingensis* from *A. niger.* These two species have a high morphological similarity and *A. tubingensis* was considered as a subspecies of *A. niger*. The utilization of molecular methods allowed a better distinction of the *A. niger* group until 1991 (Kusters-Van Someren et al. 1991; Varga et al. 1993, 1994; Accensi et al. 1999, 2001) and the classification of *A. niger* and *A. tubingensis* as distinct species. The amplification of the ITS1-5.8S-ITS2 region of rDNA for the 13 *Aspergillus* isolates, using the universal primers ITS1 and ITS4 (White et al. 1990) originated a fragment of approximately 600 bp. This result is in accordance to Henry et al. (2000) who found ITS1-5.8S-ITS2 amplicons of sizes varying between 565 and 613 bp.

In the present study, the distinction between *A. niger* and *A. tubingensis* was determined by RFLP analysis. This analysis was based on the presence of the restriction site for the endonuclease *RsaI* (GT/AC) in ITS1 sequence from *A. niger* and its absence in *A. tubingensis* (Accensi et al. 1999).

Thus all the identified P solubilizing fungi belong to either *Aspergillus* or to *Penicillium* genus. Several authors have described *Aspergillus* or *Penicillium* as major genus of P solubilizing fungi (Whitelaw et al. 1999; Seshadri et al. 2004; Wakelin et al. 2004; Wang et al. 2005).

Solubilization of TCP and RRP

It is well known that many microorganisms isolated from the soil are able to dissolve different kinds of rock phosphates in a liquid culture (Kucey 1983; Goenadi et al. 2000; Vazques et al. 2000). In the present study, the bacteria as well as fungi solubilized the

TCP and RRP to a significant level. The solubilization coincided with drop in the pH of the media. Achal et al. (2007); Pradhan and Sukla (2005) reported that P solubilizing activity is associated with a drop in pH, however, some reports do not show such a trend (Kucey 1983). This pH reduction was attributed to the varying diffusion rates of different organic acids secreted by the tested organisms. Pradhan and Sukla (2005); Reddy et al. (2002) reported that *Aspergillus* sp. showed significant drops in pH and high P solubilization. After certain incubation period (seven days for fungi and fifteen days for bacteria), the solubilization started decreasing. However, in case of fungi the dry biomass kept on increasing with the time. It is generally accepted that the major mechanism of solubilization of insoluble phosphate is the action of organic acid synthesis and exudation by bacteria (Kucey 1983, Goenadi et al. 2000, Vazques et al. 2000). Banik and Dey (1983) reported that *Bacillus* spp. strains produce oxalic acid, 2-ketogluconic acid and succinic acids capable of solubilizing phosphate. According to Goldstein (2000), the bioprocess of rock phosphate ore involves much more processes than simple acid dissolution. He assumed that the bacteria form biofilms on the surface of the ore particles, which produce unique physicochemical conditions. That result in true biocatalytic events that greatly enhance the rate and efficiency of phosphorus bioleaching from the phosphates. Nautiyal et al. (2000), Kang et al. (2002), Nahas (1996) and Kim et al. (1997) reported that rock phosphate solubilization was associated with sharp decline in pH. Nahas (1996) showed that the solubilization of insoluble phosphates depends upon a multitude of factors including decrease in pH, microorganisms and the insoluble phosphate used.

Increase in P concentration in the medium containing phosphate solubilizing fungi, was related to the secretion of organic-acid-types metabolites, which should correlate with the pH of the medium (Illmer and Schinner 1992; Illmer et al. 1995; Narsian et al. 1995). However, they failed to establish a clear cut relationship between phosphate solubilization and pH. On the other hand, Kim et al. (1997, 1998) showed a strong relationship between a drop in the pH and a drastic increase in soluble phosphate concentration.

The reduction in phosphate solubilizing activity of fungi was observed after 7 days of incubation period. This may be attributed to the availability of a soluble form of phosphate that has an inhibiting effect on further P solubilization (Narsian et al. 1995). The decrease in P concentration at the beginning stages of the experiment confirms the findings of Seshadri et al. (2000) who stated that the existing P is utilized for growth and development of the organism during this period. Probably soluble P was removed from the cultures at the early periods as rapidly as it was solubilized. The increase of P concentration in the later stages might be due to release of more P from the insoluble phosphate source to fulfill the increased demand of nutrients by the fungi. The cell lysis and P precipitation brought about by organic metabolites can also increase the released P during the later stages. The formation of an organo-P compound induced by organic metabolites released, reduces the amount of available P (Illmer and Schinner 1992) might also be responsible for final soluble P concentration. Another explanation for this might be the utilization of soluble form of phosphate by fungus for its growth and development due to depletion of nutrients in the medium in the later stages.

Considering the case of bacteria, the length of the incubation period (15 days) was quite long and the corresponding effects on increasing the soluble P contents was insignificant. Thus inoculation of P solubilizing bacteria (PSB) on TCP or RRP appeared to be an impractical method for bioactivation of these insoluble phosphates at industrial scale. In addition, the solubilization of TCP was higher than rock phosphate. This was in consistent with results reported by Nahas (1996), that in general the rock phosphate has low P solubility as compared to calcium phosphate. The solubilization of TCP as well as RRP was higher in case of fungi than bacteria. Fungi have been reported to possess greater ability to solubilize insoluble phosphates than bacteria (Cunningham and Kuiack 1992). However Alam et al. (2002) reported that bacteria are more effective in phosphorus solubilization than fungi.

The increase in soil weathering and the enhancement of nutrient availability in soil are frequently associated with the production of organic acids. The production and release of organic acids attribute to ion chelation and solubilization of inorganic P sources (Illmer and Schinner 1995). Rashid et al. (2004) and Illmer and Schinner (1995) reported exudation of organic acids by all the phosphate solubilizing microorganisms.

The major portion of the P that is applied to soil rapidly becomes ‘fixed’ into inorganic and organic fractions which are poorly available to plants. These fixed fractions of soil P accounts for a major component of the large pool of P occurring in most soils (Sanyal and De Datta 1991). Of particular significance is the occurrence of soil organic P as phytate. Anderson (1980) reported that phytates (derivatives of inositol hexaphosphates) account for large component of the organic P (some 20–50% of the total soil organic P), yet appear to be only poorly utilized by plants (Hayes et al. 2000; Richardson et al. 2000). Phytates may readily undergo physical and chemical reactions in soil environments, rendering them unavailable for plant uptake (McKercher and

Anderson 1989). Phosphatases (phytase and acid phosphatase) produced by soil microorganisms play a major role in mineralization of organic forms of soil P to release phosphate (Raghothama 1999). Acid phosphatase and phytase enzyme activity were significantly high in P solubilizing fungi in presence of TCP as well as RRP. Relwani et al. (2008) also reported that apart from other mechanisms, both acid phosphatase and phytase play a major role in P solubilization.

Effect of carbon and nitrogen sources on phosphate solubilization by bacterial isolates:

The bacterial isolates were evaluated in the presence of five carbon and five nitrogen sources, by replacing glucose and $(NH_4)_2SO_4$, respectively in the Pikovskaya media. All four strains demonstrated diverse levels of phosphate solubilization activity in the presence of various carbon and nitrogen sources. The role of carbon source is important in phosphate solubilization as the production of acid which was common mechanism of solubilization (Di Simine et al. 1998) was affected by the carbon source and the nature of acid produced is more important than the quantity of the acid (Agnihotri 1970). Glucose was the best carbon sources for the phosphate solubilization by strains *Bacillus pumilis SC18*, *B. pumilis 2C9* and *B. cereus 4G2*. Nautiyal et al. (2000) also showed that in some bacterial isolates glucose proved to be one of the best C sources for insoluble phosphate dissolution. Sucrose was identified as best carbon source for phosphate solubilization for *Bacillus C19*. Reyes et al. (1999a) and Relwani et al. (2008) showed that for *P. rugulosum* and *A. tubingensis* sucrose was the best carbon source for solubilization of insoluble P forms.

All nitrogen sources were utilized for phosphate solubilization by the four bacterial strains. *Bacillus C19* and *B. pumilis SC18* showed maximum solubilization in presence of organic source of nitrogen. Gaur (1990) reported that asparagine was second preferred nitrogen source after ammonium nitrate by *A. awamori.* Cerezine et al. (1988) reported that inorganic N sources were preferred over organic sources by *A. niger* in P solubilization process.

Bacillus pumilis 2C9 and *B. cereus 4G2* showed maximum solubilization in presence of ammonium sulphate in the medium. Ammonium sulphate yeast extract glucose (AYG) medium (Halder et al. 1991), PVK medium (Pikovskaya 1948) and National Botanical Research Institute's phosphate growth medium devoid of yeast extract (NBRIY) medium (Nautiyal 1999) are used for maximum P solubilization and all the three happen to contain ammonium sulphate as nitrogen source. The presence of ammonium in the growth medium of *Penicillium cyclopium* was reported to result in the development of inorganic acid by a proton exchange mechanism (Roos and Luckner 1989). However, another mechanism did not require the presence of ammonium and probably involved the excretion of organic acid metabolites by phosphate solubilizing fungi (Thomas 1985; Asea et al. 1988). Our results revealed that phosphate solubilization by bacterial isolates varied when ammonium, nitrates and organic sources of nitrgen were used. This study suggests that, contrary to the previous reports on PSB (Halder et al. 1991; Abd-Alla 1994) more versatile mechanism(s) for phosphate solubilization may be adopted by PSB. Thus the two mechanisms for fungi for the generation of acid may not be the only mechanisms for phosphate solubilizers (Roos and Luckner 1989; Thomas 1985; Asea et al. 1988).

Effect of carbon and nitrogen sources on phosphate solubilization by fungal isolates: Four mechanisms of solubilization were reported in fungi: (i) acidolysis, (ii) complexolysis, (iii) redoxolysis, and (iv) the mycelium functioning as a sink (Burgstaller and Schinner 1993). The first two mechanisms occur as a result of the efflux of protons from hyphae, the production of siderophores (for iron) and the production of organic acids (Gadd 2000). Generally, fungi acidify their nutrient medium during growth, although this can depend on the nitrogen source (Illmer and Schinner 1995; Whitelaw et al. 1999). Acidification can result from four main processes: (i) excretion of protons via proton-translocating plasma membrane ATPase; (ii) uptake of nutrients in exchange for protons; (iii) excretion of organic acids; and (iv) acidification through carbon dioxide produced by fungal respiratory activity (Burgstaller and Schinner 1993). The production of organic acids in turn provides a source of protons for solubilization (Gadd 1999).

Amongst above mentioned mechanisms for phosphate solubilization, the most recognized one is through the production of organic acids (Reyes et al. 2006). The production and release of organic acids (mainly citric acid, oxalic acid, malic acid and gluconic acid) attribute to ion chelation and solubilization of inorganic P sources (Cunningham and Kuiack 1992; Reyes et al. 2006). The nature and amount of organic acids excreted by fungi are mainly influenced by medium pH and buffering capacity, carbon source and the balance of nitrogen and phosphate (Mattey 1992; Reyes et al. 1999a).

In case of *A. tubingensis* (AT6) and *A. niger* the maximum solubilization was in media having mannitol followed by sucrose and glucose. AT6 showed higher amount of gluconic acid and malic acid in case of glucose and sucrose respectively but solubilization was lower than mannitol. This can be attributed to the exudation of highest amount of oxalic acid in case of mannitol followed by sucrose as compared to other C sources. Relwani et al. (2008) also showed that oxalic acid, succinic acid and acetic acid are the main organic acids responsible for solubilization of phosphate by *A. tubingensis*. In *A. niger* malic acid and succinic acid exudation might be responsible for high solubilization in mannitol and sucrose than other C sources. In AT1, the phosphate solubilization activity was highest in mannitol followed by glucose. The main organic acids secreted in presence of manniol and glucose was oxalic acid, malic acid and succinic acids. In other C sources, oxalic acid was produced significantly high amount which suggests that only malic acid and succinic acids are more responsible for solubilization by AT1 than other organic acids. Mannitol and glucose were reported to be the best sources for *A. niger* to solubilize P (Seshadri et al. 2004). Barroso et al. (2006) reported that C sources which resulted in the most extensive solubilization of Ca-P by *A. niger* were maltose and mannitol. These findings support that the solubilization activity of a microorganism is related to its organic acid production, however, the nature of the acid produced is also important which is dependent on the carbon source supplied (Vassileva et al. 1998).

The C source which resulted in the most extensive solubilization of TCP by *P. oxalicum* was mannitol followed by glucose which was in consistence with findings of Barroso et al. (2006). The main organic acids produced in case of mannitol were malic acid and succinic acid. In case of glucose, the total organic acid production was higher but solubilization was lower than mannitol. This can be attributed to the fact that in case of glucose despite having high formic acid exudation, the total concentration of malic acid and succinic acid was lower than mannitol which suggests that the main organic acids involved in solubilization of TCP by *P. oxalicum* are malic acid and succinic acid. No or very low production of malate and succinate in case of xylose, fructose, sucrose and arabinose were reported which directly be related to low solubilization. Mannitol and glucose were also reported to be the best sources for *A. niger* to solubilize P (Seshadri et al. 2004). Reyes et al. (1999a) showed that sucrose was the best carbon source for *P. rugulosum* for solubilization of hydroxyl apatite and $FeSO_4$. Sucrose and glucose significantly increased P solubilization by *A. tubingensis* compared to other carbon sources (Relwani et al. 2008). These studies suggest that different fungi use different carbon sources and depending on the carbon source, the fungi use alternative metabolic pathways to produce organic acids. For the same C source, it is possible that, the nature of the organic acid varies with the phosphate used in the culture medium influencing the solubilization mechanism. In addition, the nature and quantity of organic acids secreted by the fungus were influenced by the C source and the balance between N and P sources (Mattey 1992).

The maximum growth in all the isolates was observed in mannitol which can be attributed to the fact that mannitol is best utilized as a carbon source for solubilization of TCP by these fungi. In other C sources, where P solubilization and growth had no clear relationship, the soluble phosphate produced must have been absorbed by the fungus to sustain its growth, which shows that the C source influenced the solubilization capacity of the fungus. Barroso et al. (2006) reported that fungal growth was influenced as much by the C and N sources as by the phosphate sources added to the culture medium.

Previous reports on PSMs (Lapeyrie et al. 1991; Carlile and Watkinson 2001) have attributed the differences in solubilization (when ammonium and nitrate were used) to the difference in mechanisms for the generation of acidity in the culture. The different nitrogen sources had different effects on solubilization because of differential pH changes that occur in the medium with different nitrogen sources. This may affect the solubilization process, for example, a marked fall in pH generally results during growth on ammonium as sole N source (Carlile and Watkinson 2001). In the present study, the highest P solubilization by all the four fungal isolates was observed in presence of potassium nitrate as compared to other nitrogen sources in the medium. This was in consistent with results of Relwani et al. (2008). In all the strains of *Aspergillus,* the main organic acids exudated in all the C sources were oxalic acid, citric acid, malic acid, succinic acid and formic acid. However, the higher solubilization in potassium nitrate can be attributed to highest amount of formic acid production in this case as compared to other N sources. The total amount of organic acids exudation by the fungi in the medium can be responsible for different level of pH reduction in different N sources.

The main organic acid involved in *P. oxalicum* was succinic acid followed by malic acid in presence of mannitol. Fungal growth was extensive in presence of asparagine and sodium nitrate. Asparagine was next to potassium nitrate followed by ammonium nitrate to show higher solubilization by *P. oxalicum* where the main organic acid involved was malic acid. In case of sodium nitrate, succinic acid was predominantly present in the medium. In media having ammonium sulphate and urea, the soluble P was low may be due to presence of organic acids other than malate or succinate in higher quantity. Thus results reveal that malic acid and succinic acids are the main organic acids which play major role in solubilization of TCP by *P. oxalicum.*

Improved solubilization with nitrate was associated with an increase in the production of organic acids. The oxalic production was higher in case of nitrate. Lapeyrie et al. (1987) suggested that increased production of oxalic acid may be due to the influence of N source on enzymes involved in the production and degradation of oxalic acid. Nitrate, for example, is an inhibitor of oxalic acid oxidase, an enzyme which hydrolyses oxalic acid, and inhibition of this enzyme results in an accumulation of oxalic acid (Meeuse and Campbell 1959). Reyes et al (1999a) also reported that wild type *Penicillium* grows better on nitrate than on ammonium, when relatively insoluble phosphate sources are used. Whitelaw et al. (1999); Pradhan and Sukla (2005) and Asea et al. (1988) found a higher P solubilization from ammonium assimilation by *Penicillium.*

Among ammonium sources of nitrogen, ammonium nitrate showed high soluble P and increased fungal biomass as compared to ammonium sulphate. This was in consistent with Chatterjee and Nautiyal (1981) who reported increased growth and increased fungal biomass of *A. niger* in ammonium nitrate amendment and poor growth, soluble P and poor fungal biomass formation in ammonium sulphate amended medium. The results show higher organic acid production and higher pH reduction in ammonium nitrate as compared to other N sources. The reduction of pH indicates the possibility of the operation of NH_4^+/H^+ exchange mechanism acidifying the medium, as reported by Roos and Luckner (1984). Hence, acidification due to NH_4^+ is more evident rather than NO_3^- because the acidification of the medium is a result of H^+ efflux from hyphae during NH_4^+

uptake (Jacobs et al. 2002a). Ammonium sulfate or ammonium nitrate has been used as a nitrogen source for organic acid production on large scale.

Cerezine et al. (1988) reported that ammonical sources increased the solubilization of fluorapatite by *A. niger* more than organic sources of N. Physiologically, ammonium compounds are preferred since their consumption lowers the pH of the medium which is an additional prerequisite of organic acid production. Urea also showed high production of malic acid and formic acid. It can be used as a good nitrogen source for organic acid production as suggested by Yigitoglu (1992).

Phosphatases (phytase and acid phosphatase) produced by soil microorganisms, play a major role in mineralization of organic forms of soil P to release phosphate (Raghothama 1999). There was a significantly high production of acid phosphatase and phytase enzyme by fungal isolates in culture filtrate which was in consistent with Relwani et al. (2008). Highest acid phosphates activity was recorded in mannitol amongst other C sources, which may be responsible for highest solubilization. In *P. oxalicum* also, high soluble P in mannitol and potassium nitrate coincides with high acid phosphatase activity whereas in case of fructose, arabinose, sucrose, ammonium sulphate and urea, acid phosphatase activity was significantly low this can directly be related to significant low solubilization of TCP. In all the cases, phytase activity was more than phosphatase activity as reported by Aseri et al. (2009) that fungi executes extracellular phytase activity many times more than extracellular phosphatase activity. There was no clear relationship between phytase activity and solubilization. The study suggests that activity of acid phosphatase and phytase varies with varying source of carbon and nitrogen sources in the media and acid phosphatase play a major role in P solubilization, apart from other mechanisms.

All the four fungal isolates were studied for the effect of mannitol as carbon source and potassium nitrate as nitrogen source on the solubilization of RRP as these sources were found to be best for solubilization of TCP. There was an increase in solubilization by all the isolates except *P. oxalicum*. There was no significant difference in reduction of pH. Acid phosphatase activity decreased whereas phytase activity and dry biomass of the fungi increased significantly. In all the four isolates oxalic acid increased whereas gluconic acid, malic acid, formic acid and acetic acid decreased significantly in presence of mannitol and potassium nitrate. In case of *P. oxalicum* there was seven folds decrease in malic acid in media having mannitol and potassium nitrate as compared to PKV media. These studies suggest that different fungi use different carbon and nitrogen sources and depending on the phosphate source, the fungi use alternative metabolic pathways to produce organic acids. Barroso et al. (2006) reported that fungal growth was influenced as much by the C and N sources as by the phosphate sources added to the culture medium. As a consequence of fungus growth, the production of acids and pH decrease in the medium varied influencing the amounts of P solubilized.

Solubilization metal phosphates by fungal isolates

Solubilization of insoluble metal compounds is an important aspect of fungal physiology to release phosphates and essential metal cations into available forms for intracellular uptake by plants and other microorganisms and also for biogeochemical cycling of metal and phosphate (Gadd 2001).

The fungal isolates [*A. tubingensis (AT6), A. tubingensis (AT1), A. niger* and *P. oxalicum*] used in the present study varied in solubilization of the metal phosphates. Phosphate solubilization and phosphate mobilizing mechanisms depends on the nature of the P source and the organisms involved in the process (Oliveira et al. 2008). In present study, all the isolates solubilized aluminium, zinc and cobalt phosphates to greater extent than other metal phosphates. This might be due to metal component of the phosphate as these will influence the availability of phosphate, biomass and growth rate of fungi (Jacobs et al. 2002b). The solubilization of $Ca_3(PO_4)$ was higher than all the metal phosphates by all the isolates except zinc phosphate by *A. niger* and *P. oxalicum.* Barroso and Nahas (2005) also reported that the ability of the fungi to dissolve insoluble phosphates decreased in the following order: P–Ca > P–Al > P–Fe. This might be explained by the difference in the solubilities of these phosphates in solution (Lide and Frederikse 1998). Similarly, Banik and Dey (1983) observed the same tendency of several fungi from soil to solubilize Ca–P, Al–P and Fe–P. Leyval and Berthelin (1985) found that the contents of solubilized P were higher with $Ca_3(PO_4)$ than with $FePO_4$. Phosphate solubilization by *Penicillium radicum* was higher with Ca–P forms than with Fe–P or Al–P forms of phosphates (Whitelaw et al. 1999). The solubilization of $Zn_3(PO_4)_2$ was highest in *P. oxalicum* amongst all the four isolates which may be attributed to the highest concentration of formic acid amongst the other organic acids. The relative order of phosphate solubilization was not consistent when each isolate is analyzed individually. In most of the cases, low solubilization of metal phosphates coincides with low fungal biomass for e.g. in Cu_2OHPO_4 low solubilization as well as low fungal biomass was observed. This was possibly due to inhibition of the fungi by this compound and/or released metal species resulting in a decrease in growth and biomass.

The solubilization of metal phosphates by the fungi was associated with lowering of pH. In case of $Zn_3(PO_4)_2$, the pH reduction was less but solubilization was significantly high except in AT6 in which pH reduction as well as solubilization both were low. However, in case of $FePO_4$, the lowering of pH was not associated with high soluble P. Illmer et al. (1995) reported that sometimes, the pH of culture filtrate was relatively high and yet high P solubilization occurred in the medium, this may be due, to the chelation of organic acids with cations which were present in the insoluble phosphate source. The effects of pH on the solubility and speciation of metals are well documented for example in soil, the mobile concentrations of metals such as Zn, Ni and Cd was increased two folds by unit decrease in pH (Gadd 1999, 2001; Giller et al. 1998; Sayer and Gadd 2001). Cerezine et al. (1988) and Puente et al. (2004) have also observed P solubilization with decrease in pH values. This acidification can result from four main processes: excretion of protons via proton-translocating plasma membrane ATPase; uptake of nutrients in exchange for protons; excretion of organic acids; and acidification through carbon dioxide produced by fungal respiratory activity (Burgstaller and Schinner 1993).

The increased proton content responsible for the pH decrease can arise from the production of organic acids. Carboxylic acids and amino acids such as citric acid, malic acid and histidine are potential ligands for heavy metals and so could play a role in tolerance and detoxification (Clemens 2001; Rauser 1999). The production of metal complexing organic acids assists both essential metal and anionic nutrition of fungi and plants via the solubilization of phosphate and sulphate from insoluble metal-containing substances (Gadd 2001). Solubilization of inorganic P by fungi has been linked to the production of various organic acids, including citric acid (Illmer et al. 1995), gluconic acid and oxalic acid (Gharieb 2000; Sayer and Gadd 1997). The production of organic acids and ensuing complexation and/or precipitation of free metal ions have been implicated in metal tolerance, as the mobility and toxicity of metals is related to speciation (Pettersson et al. 1993).

The process of leaching by fungi can occur by several mechanisms but organic acids occupy a central position in the overall process, supplying both protons and a metal complexing organic acid anion (Gadd 2001). The nature and amount of organic acids excreted by fungi are mainly influenced by the pH and buffering capacity of the environment of the medium, the carbon, phosphorus, and nitrogen sources, and the presence or absence of certain metals and trace elements (Gadd 1999; Gharieb 2000). In the present study there was not much difference in solubilization of TCP by all the four isolates and the main organic acid exudated was oxalic acid. The highest amount of oxalic acid amongst all the four isolates was exudated by *P. oxalicum*. In this case succinic acid was also found to be present in significant amount, whereas it was absent in case of all the three isolates of *Apergillus*. In case of $AlPO_4$, gluconic acid was major organic acid produced by *A. niger* and oxalate by both the isolates of *A. tubingensis*, whereas in *P. oxalicum* malic acid was the main organic acid. Gadagi et al. (2003) also reported that malic acid may have been a main organic acid involved in the solubilization of $AlPO_4$ and $Ca_3(PO_4)_2$. It has been reported that *Aspergillus* can produce significant quantities of citric acid, gluconic acid and oxalic acids, along with other organic acids

(Burgstaller and Schinner 1993). As explained by Gadd (2001), the oxalate ion $C_2O_4^{2-}$, is a bidentate, it can bind to Al^{3+}, Cr^{3+}, Fe^{3+} (three oxalates) or to Cu^{2+}, Zn^{2+} (two oxalates) to form an anionic complex.

The solubilization of $Zn_3(PO_4)_2$ was highest in *P. oxalicum* amongst all the four isolates. In this case, the main organic acid exudated was formic acid. The solubilization of $Zn_3(PO_4)_2$ was higher in *A. niger* than in *A. tubingensis* (AT6 and AT1) which can be attributed to higher formic acid exudation in case of *A. niger*. Large concentrations of citric acid and oxalic acid were also found in presence of $Zn_3(PO_4)_2$ by *A. niger* which also may be responsible for higher metal solubilization. Sayer and Gadd (2001) reported that $Zn_3(PO_4)_2$ induces production of organic acids like oxalic acid and citric acid which are responsible for solubilization and tolerance of Zn^{2+}.

Copper phosphate involved high amount of gluconic acid production by AT6, oxalic acid by *A. niger* and formic acid by AT1 and *P. oxalicum* but even then the solubilization was very low. This can be explained as Cu and Cd show higher affinity for phytochelatins than organic acids as reported by Singh (2006). In the media amended with $FePO_4$, the main organic acid exudated by AT6 was formic acid, whereas *A. niger* and *AT1* produced oxalic acid in high amounts. This supports that beside the mechanism of metal solubilization by production of low molecular weight iron chelating siderophores that specifically solubilize Fe, the organic acid mediated solubilization is ‘redoxolysis’, where organic acids can affect the reduction of Fe(III) to Fe(II) thus increasing iron solubility (Gadd 1999) and oxalic acid can act as a leaching agent for those metals that form soluble oxalate complexes, including Al and Fe (Gadd 2001; Strasser et al. 1994). Gluconic acid was produced in large quantities by three isolates of

Aspergillus when the medium was amended with $Co_3(PO_4)_2$. This was in consistent with Sayer and Gadd (2001) that presence of $Co_3(PO_4)_2$ stimulates gluconic acid production. Thus the important mechanisms, that explains the most extensive solubilization of $Co_3(PO_4)_2$ by the isolates, was the large amount of organic acid especially gluconic acid exudation. *P. oxalicum* exudated succinic acid in significant amount in presence of $Co_3(PO_4)_2$. The important mechanisms, that explains the most extensive solubilization of $Co_3(PO_4)_2$ by all the isolates was, the large amount of organic acid exudation, however, a clear correlation between amounts of acid produced and exposure to a metal, has not been produced to support a widespread role in solubilization (Clemens 2001; Rauser 1999; Sayer and Gadd 2001).

Gadd (1999) reported that oxalic acid, citric acid, malic acid and acetic acid were produced as secondary metabolites in substantial amount as a range of low molecular weight organic acids by fungi and bacteria which can have profound effects on the speciation and mobility of metals, with resultant consequences for microbial and plant nutrition, and nutrient cycling, as well as influences on metal solubility. The results suggest that as a consequence of fungal growth, the production of acids and pH decrease in the medium varied thereby influencing the extent of metal phosphate solubilization.

When the solubilization with commercial organic acids was tested, the high content of available P with reduction in pH was observed in all the metal phosphates except in case of aluminium phosphate. This supports that solubilization of inorganic P sources can be attributed to the production and release of organic acids mainly citric acid, oxalic acid, gluconic acid and succinic acid (Gadd 1999; Gharieb 2000; Sayer and Gadd 2001). The present work also supports the fact that the organic acids present in culture filtrates are as efficient as commercially available organic acids in solubilization of metal phosphates. In case of $AlPO_4$, the pH reduction by organic acid mix or oxalic acid alone could not solubilize it. This was in consistent with the suggestions that organic acids may play an important role, but it is not the only way for solubilizing insoluble phosphates (Salih et al.1989).

Apart from other mechanisms, both acid phosphatase and phytase have important role in phosphate solubilization as reported by Raghothama (1999). The available P gets converted into organo-P compound induced by organic metabolites released by the microorganisms (Illmer and Schinner 1992). Phosphatases (phytase and acid phosphatase) produced by soil microorganisms, play a major role in mineralization of organic forms of soil P to release phosphate. In case of $Zn_3(PO_4)_2$, $FePO_4$ and $Co_3(PO_4)_2$, beside organic acid exudation, phytase activity was very high. This contradicts the finding of Fujita et al. (2003) who reported that Cu^{2+}, Mn^{2+} and Zn^{2+} induced slight inhibition, while Ca^{2+}, Co^{2+}, Fe^{3+}, Mg^{2+} and Fe^{2+} had no effect on the enzyme activities. Most of these enzymes require divalent metal ions to manifest activity but above an optimal concentration, inhibition is observed, which depends on the nature of the metal. Phytate chelates with the calcium, magnesium, iron, cobalt and copper very strongly to form insoluble metal-phytate complexes (Gifford and Clydesdale 1990). These phytates should be dephosphorylated by phytases/acid phosphatases before assimilation (Satyanarayana and Singh 2009).

The study is thus relevant to an understanding of fungal responses towards metal species as well as the possible mechanisms involved in phosphate bioavailability and metal detoxification (particularly the organic acid exudation and enzyme activities), consequently the implications for the biogeochemical cycling of metals and phosphorus.

Effect of carbon and nitrogen sources on metal-P solubilization

The ability to solubilize insoluble phosphates is widespread among microorganisms. Such solubilization is of importance in nutrient cycling as it releases phosphates as well as metal ions into biogeochemical cycles. It plays an important role in phosphate acquisition by microbial and plant species, especially in soils where phosphates can readily become immobile by interactions with soil components (Lapeyrie et al. 1991; Illmer and Schinner 1992). Solubilization can be accomplished by a range of mechanisms, which include the excretion of metabolites such as organic acids, proton extrusion or production of chelating agents (Hughes and Poole 1989; Burgstaller and Schinner 1993; Gadd 1993; Sayer et al. 1995; Sayer and Gadd 1997). Acidic metabolite production appears to be the major mechanism of solubilization exhibited by filamentous fungi, and the production of citric acid and oxalic acids by fungi such as *Aspergillus* and *Penicilium* is well documented (Cunningham and Kuiack 1992; Burgstaller et al. 1994; Sayer and Gadd 1997). A number of factors have been considered due to its effect in acid production and pH lowering by microorganisms, such as the C and N sources (Dixon-hardy et al. 1998; Reyes et al. 1999a, b). The solubilizing ability has also been found to be influenced by the P source in the culture medium (Nahas et al. 1990). The P solubilizing isolates of fungi showed high ability to solubilize both metal phosphates in culture medium so it was important to evaluate this ability by growing the fungus in a medium added with different C or N sources.

Aluminium phosphate was solubilized to the highest by AT6 in presence of fructose and ammonium sulphate. The dry biomass, phytase activity and pH reduction was maximum. *A. niger* solubilized aluminium phosphate to a maximum extent in presence of glucose and asparagine, in which the pH reduction was higher than other C and N sources. AT1 and *P. oxalicum* showed highest solubilization of aluminium phosphate in presence of ammonium sulphate and glucose (Pikovskaya media). Variation of medium composition markedly altered the phosphate mobilization by phosphate solublizing microorganisms (Illmer and Schinner 1992). The increased proton content responsible for the pH decrease can arise from the production of organic acid (Cunningham and Kuiack 1992) or H^+ -excretion that accompanies NH^{4+} assimilation (Beever and Burns 1980).

A. tubingensis (AT6) showed maximum solubilization of zinc phosphate in presence of fructose and asparagine in the medium. In this case, pH reduction as well as acid phosphatase activity was highest when compared to other carbon and nitrogen sources. *A. niger* solubilized maximum of zinc phosphate in case of mannitol and ammonium sulphate which coincides with higher pH reduction. Fructose and potassium nitrate proved to be the best carbon and N source for solubilization of zinc phosphate by AT1. In this case, the phytase and acid phosphates activity was maximum. In case of *P. oxalicum,* the solubilization of zinc phosphate was highest in the medium having glucose and ammonium sulphate which may be due to the highest pH reduction in this case amongst other C and N sources.

The solubilization of copper phosphate was highest in case of AT6 in the media having sucrose. *A. niger* showed maximum solubilization as well as dry biomass in case of mannitol. AT1 showed highest solubilization efficiency in case of sucrose which coincides with highest pH reduction and enzyme activity than any other carbon source. Hefnawy et al. (2009) reported that for solubilization of insoluble phosphates, sucrose as carbon source and sodium nitrate as sole nitrogen source proved to be best for *A.niger*. In case of *P.oxalicum,* 100 times greater solubilization was observed in arabinose than other carbon sources. In this case, the pH reduction was maximum as compared to other C sources. Roos and Luckner (1984) reported that the acidification observed in cultures of *Penicillium cyclopium* results mainly from the uptake of NH_4^+, which is stoichiometrically coupled with H^+ excretion. Cunningham and Kuiack (1992) reported that *Penicillium bilaii* causes a significantly higher reduction of pH in NH_4^+, supplemented medium as compared to a medium supplemented with NO_3^-. In case of AT6, *A. niger* and AT1 the solubilization was maximum in presence of potassium nitrate, whereas this high solubilization was not associated with high pH reduction and dry fungal biomass. *P. oxalicum* showed greater solubilization in sodium nitrate than other nitrogen sources. In this case the pH reduction and acid phosphatase activity was maximum as compared to other N sources. The increased proton content responsible for the pH decrease can arise from the production of organic acids (Cunningham and Kuiack 1992) or H^+ excretion that accompanies NH_4^+ assimilation (Beever and Burns 1980).

The solubilization of ferric phosphate by AT6 was maximum when xylose and sodium nitrate were used in the medium. In this case the acid phosphatase activity was also higher than the other C and N sources used. *A. niger* and *P. oxalicum* solubilized iron phosphate to a maximum extent in the medium having glucose and ammonium sulphate as C and N sources. In this case the reduction of pH was also found to be maximum. In case of AT1, the solubilization was highest in media having xylose and ammonium sulphate. Reyes et al. (1999b) have also shown that $AlPO_4$ solubilization by *P. rugulosum* was significantly better when NH_4^+ was used as the sole N source as compared to NO_3^- , whereas with $FePO_4$ as the P source, higher solubilization was observed with NO_3^- as the sole N source. Dixon-hardy et al. (1998) showed that the glucose level in the medium was shown to be an important factor for the solubilization of zinc and cobalt phosphate. The form of nitrogen source is of importance, e.g. nitrogen in the ammonium form was necessary for increased phosphate solubilization of rock phosphate (Asea et al. 1988). Many fungi are able to utilize both ammonium and nitrate as nitrogen sources (Dixon-hardy et al. 1998). The differential pH changes that occur on the medium with different inorganic nitrogen sources may affect the solubilization process: a marked fall in pH generally results during growth on ammonium as sole N source, for example (Carlile and Watkinson 1994).

In case of cobalt phosphate, the highest solubilization and pH reduction by AT6 was in the media having mannitol and ammonium nitrate. *A. niger* showed maximum solubilization in case of fructose and potassium nitrate in which enzyme activity was highest amongst the other C and N sources. Clearly, the hypothesis that P solubilization is linked to acidification caused by NH_4^+ assimilation, does not hold true for all microorganisms. Fungal solubilization of inorganic P is associated with the production of organic acids, most frequently citrate, gluconate, oxalate and succinate (Cunningham and Kuiack 1992; Gharieb 2000; Reyes et al. 2001; Vassilev et al. 2001; Vazquez et al. 2000). Organic acids may solubilize inorganic P through the release of acidic protons (Reyes et al. 1999b ; Whitelaw et al. 1999) and their abilities to chelate Ca^{+2}, Fe^{+3}, and

Al^{+3} (Kpomblekou and Tabatabai 1994; Whitelaw et al. 1999). These observations are consistent with other reports demonstrating that the mere presence of organic acid does not account for all the soluble P that is solubilized by the microorganisms (Illmer et al. 1995; Illmer and Schinner 1995). Clearly, other factor(s) plays a role in the solubilization of inorganic P. AT1 also showed higher solubilization efficiency in presence of fructose and sodium nitrate. In case of *P. oxalicum* the highest solubilization among all the carbon sources was in sucrose and asparagine.

Hefnawy et al. (2009) reported that nutritional constituent of the culture medium may play an important role in phosphate solublization from its ores. In many fungi, the mechanism of solubilization of insoluble metal compounds is primarily due to excretion of metabolites such as organic acids (Burgstaller and Schinner 1993; Strasser et al. 1994). This process is important in the natural environment because of the solubilization of important nutrients such as essential metals and anionic nutrients e.g. phosphate, and any factor affecting production of metabolites involved in solubilization will therefore effect solubilization. The nature and amount of organic acids excreted by fungi are mainly influenced by medium pH and buffering capacity, carbon source and the balance of nitrogen and phosphate (Mattey 1992). The nutrient effects on the solubilization of insoluble metal phosphates apparently vary greatly according to nutrient source. According to Dixon-hardy et al. (1998) the manipulation of the concentrations as well as source of carbon and nitrogen in the growth medium can clearly alter the rate of solubilization of insoluble metal phosphates by P solubilizing fungi. This response is not only important in the natural environment but also could be useful to optimize the production of organic acids by manipulating the media in laboratory. This may also have potential for biotechnological applications, such as recycling of metals from industrial waste and low-grade byproducts and possibly in the bioremediation of polluted soils.

Field experiment

Microbial populations are key components of the soil-plant entity where they are associated in a framework of interactions affecting plant development (Vassilev et al. 2006). P solubilizing microorganisms can solubilize and mineralize P from inorganic and organic pools of total soil P and may be used as inoculants to increase P availability to plants (Kucey et al. 1989; Richardson 1994, 2001; Illmer et al. 1995; Whitelaw et al. 1999).

Phosphate anions may be immobilized through precipitation with cations such as Ca, Mg, Fe and Al. Organic P in the soil generally accounts for around 50% of total insoluble soil P in soils with high organic matter content, such as the no-tillage management systems (Bayer et al. 2001; Gyaneshwar et al. 2002). A large proportion of the organic P is represented by inositol phosphates and lesser amounts of other phosphate esters as phospholipids (Richardson 2001; Wakelin et al. 2004; Richardson et al. 2005).

Presently, rock phosphate is being chiefly employed to sustain soil P levels in an available form for plants. In this context, PSMs have been reported to solubilize the rock phosphate through the production of organic acids, ion chelation and exchange reaction in the growth environment (Yadav and Dadarwal 1997). As a result of this activity, PSMs play an important role in supplementing P to the plants and allowing a sustainable use of phosphatic fertilizers.

Inoculation with *Aspergillus*

In the present study, significant increase in the yield of wheat as well as maize was recorded with the application of RRP along with the inoculation of P solubilizing *Aspergillus*, AT1, AT6 and *A. niger* in the soil. The inoculation with these fungi in RRP fertilized soils, improved the growth of the wheat and maize plants. Vassilev et al. (2006) and Omar (1998) reported that there is a positive effect of rock phosphate fertilization and phosphate solubilizing fungi on growth of the plants. Kundu and Gaur (1984) reported, increased P uptake and plant growth in various crops inoculated with PSMs. Rock phosphate fertilization as well as fungal inoculum had significant effect on total plant P in this study. This finding was contradictory to Omar (1998) who reported that rock phosphate fertilization had no significant effect on total plant P but it was significantly increased by plant inoculation. The inoculation with P solubilizing *Aspergillus* considerably increased P uptake and enhanced plant growth as compared to uninoculated soil, even in the absence of rock phosphate. These results could be attributed to the ability of these microorganisms to solubilize organic and inorganic phosphorus already present in soil. The RRP fertilized soil inoculated with isolates of *Aspergillus* showed higher organic carbon than non-inoculated ones, may be due to the production of microbial organic colloidal materials and solubilization of P as suggested by Mba (1994). The significant increase in N content of the soil after final harvesting may be due to application of urea in the field. Kucey et al. (1989) also reported that besides providing P, phosphate solubilizing microorganisms produces considerable amounts of N and plant growth promoting substances in the rhizosphere.

Mycotoxins are toxic compounds produced by certain fungi under specific conditions. Among various mycotoxins, aflatoxins have assumed significance due to their deleterious effects on human beings, poultry and livestock. Grain is susceptible to aflatoxins produced either while the crop is growing by fungal species or during storage. Among 18 different types of aflatoxins identified, major members are aflatoxin B1, B2, G1 and G2. The wheat samples were analyzed for these aflatoxins and results came out to be negative which suggests that all the phosphate solubilizing fungi, used in this study can be used as a bio fertilizer for enhancement of plant growth and nutrition.

Inoculation with *Penicillium*

Penicillium strains have been studied for their phosphate solubilizing activity and used as biofertilizers (Whitelaw 2000). In the present study, *P. oxalicum* was tested for its effect on wheat and maize crops in presence of RRP in the field conditions. The field experiments showed highly significant interaction between rock phosphate amendment and inoculation with rock-phosphate-solubilizing *P. oxalicum*. It has been reported that *Penicillium bilaii* effectively colonized roots of canola and increased the P uptake and crop in field conditions (Kucey and Leggett 1989). The growth parameters were higher in RRP fertilized soil inoculated with fungus, compared to control soil. 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 made soluble by P solubilizing fungi from soil reserves and RP (Reyes et al. 2002). The reduction in the soil pH and increase in available P was greater in inoculated treatments as compared to non-inoculated ones which may be attributed to ability of such microorganisms to excrete organic acids, thereby decrease the pH and increase the concentration of phosphorus in soil by mechanisms involving chelation and exchange reactions (Vassilev et al. 1996). The RRP fertilized soil inoculated with *P. oxalicum* showed higher organic carbon than non inoculated ones, may be due to the production of microbial organic colloidal materials and solubilization of P as suggested by Mba (1994). The increase of N content in the soil after final harvesting may be due to application of urea in the crop. The PSMs produces considerable amounts of N and plant growth promoting substances in the rhizosphere (Kucey et al. 1989).

In the present study, the results showed that the addition of rock phosphate along with fungal cultures greatly enhanced the plant growth and nutrient uptake of wheat and maize plants. It is generally thought that PSMs in addition to solubilizing inorganic P also release growth promoting substances (Kucey et al. 1989). The P uptake by roots of wheat was higher than that of shoots in all the treatments. This may be due to the reason given by Vassilev et al. (2006) that P can get diluted in shoots due to high plant growth. A general reduction in salinity was seen over the course of the experiment, which may be attributed to the removal of crop nutrients or to the movement of salts down through the profile as a result of irrigation. The available P increased significantly in all the inoculated and RRP fertilized soils. In case of soil having fungal consortium and RRP the available P in the soil after final harvest was seven times more than the control soil. Improved crop yield in this investigation could also have resulted due to the significant increase of the available P in all the treatments compared to non-inoculated and non-fertilized soil. The application of P solubilizing fungi is recommended as a sustainable way for increasing crop yield under all the field conditions. Many reports support the present study and had shown the improvement in plant growth using P solubilizing fungi (Whitelaw 1997; Richa et al. 2007).

The major portion of the P that is applied to soil rapidly becomes ‘fixed’ into inorganic and organic fractions which are poorly available to plants (Sanyal and De Datta 1991). The available P gets converted into organo-P compound induced by organic metabolites released by the microorganisms (Illmer and Schinner 1992). Phosphatases (phytase, acid phosphatase, etc.) produced by soil microorganisms play a major role in mineralization of organic forms of soil P to release phosphate and *Penicillium* is one of the major genera of phosphatases and phytase producing fungi (Aseri et al. 2009). Therefore in the field study, the phosphatase as well as phytase activity was determined in the soil, which showed that in all the treatments the phytase activity was more than phosphatase activity. Aseri et al. (2009) also reported that fungi execute extracellular phytase activity many times more than extracellular phosphatase activity. That phytase activity decreased after the maize harvest as compared to initial values may be due to slight reduction in pH as supported by Richardson et al. (2005) that decease in pH of soils makes phytase less effective in the soil environment. According to Pandey et al. (2007) the acid phosphatase activity was higher than alkaline phosphatase activity in all the treatments.

Inoculation with bacteria

Phosphate solubilizing bacteria (PSB) are slowly emerging as important organisms used to improve soil health as *in vitro* studies have demonstrated that they dissolve RP and thereby reduce P deficiency in soil (Yosef et al. 1999). Because of the enormous numbers of microbial populations and species in the soil, especially in the rhizosphere, intensive interactions have been established between soil microorganisms and various other soil organisms and plant roots. Plant growth promotion by rhizosphere microorganisms is well established (Bashan 1998). In spite of the deleterious effects of some microorganisms on plants, the beneficial effects are usually greater and the overall results are usually demonstrated by growth promotion and faster germination (Atlas and Bartha 1998). The overall evidence show significant plant growth effects induced by inoculation of some soil bacteria in the rhizosphere (Gerhardson and Wright 2002).

Bacteria belonging to genera *Bacillus, Pseudomonas, Serratia, Enterobacter* etc., are reported to solubilize the insoluble phosphatic compounds and aid in plant growth (Rodriguez and Fraga 1999). In the present study, the four *Bacillus* strains (*B. pumilis SC18, B. pumilis 2C9, Bacillus C19* and *B. cereus 4G2)* were used as bacterial consortium (BC) for field inoculation. After one week of inoculation, the number of viable cells decreased may be because some time is needed for inoculated microorganisms to get adapted in the natural environment. The microbial activity elevated after few weeks when cells became well adapted. The growth parameters as well as yield of wheat as well as maize were higher in treatment having RRP as fertilizer along with bacterial consortium. Chaykovskaya et al. (2001) reported that treatment with phosphate solubilizing bacteria resulted in increased yield of pea and barley. Several results suggest that PSB have the ability to solubilize RP thereby increasing its availability to plants (Goenadi et al. 2000; Lal 2002). PSB are also reported to produce metabolites such as phytohormones, antibiotics and siderophores and aid in plant growth (Kloepper et al. 1989). Phosphate solubilizing and phytohormone producing *Azotobacter chroococcum* showed increase in grain and straw yield of wheat (Kumar et al. 2001).

Stimulation in growth and yield of maize by inoculation with *Rhizobium leguminosarum* or *Penicillium rugulosum* in glasshouse or field conditions is reported (Chabot et al. 1996; Reyes et al. 2002). Phosphate solubilizing *P. fluorescens* is reported to enhance growth, increase in yield, N and P contents in shoot and kernels of peanut (Dey et al. 2004).

Slight reduction in soil pH was also observed after final harvest of crop. Son et al. (2003) reported that, phosphate solubilization was mainly due to the acidification of the culture by bacteria; however, this high level of phosphate solubilization may not be achievable in soil because most soils have a great pH buffering capability. Moreover, the final pH did not reduce to the strongly acidic levels. It is known that the production of organic acids by soil microorganisms and commensurate pH decrease is the major mechanism of inorganic phosphate solubilization (Whitelaw et al. 1999). Since the final pH values in the present study were not as low as those in the study of Son et al. (2003), some mechanism other than those described above may be responsible for the phosphate solubilization. Production of growth regulators by phosphate solubilizing bacteria has been studied by Ponmurugan and Gopi (2006). There is increasing evidence that phosphobacteria improve plant growth due to biosynthesis of plant growth substances rather than their action in releasing available phosphorus.

The available P, organic C and total N increased in the soil having RRP amended bacterial consortium inoculated treatments. The microorganisms involved in P solubilization can enhance plant growth by increasing the efficiency of biological nitrogen fixation, enhancing the availability of plant growth promoting substances and other trace elements. Previous studies involving plants inoculated with PSMs showed growth enhancement and increased P contents but large variations were found (Kucey et al. 1989).

Acid phosphatase as well as phytase activity was higher in the soil having bacterial consortium and RRP fertilization than in the control soil. Mineralization of organic phosphorus is another mechanism of phosphate solubilization and several phosphatases (also called phosphohydrolases) are involved in the mineralization of various organic phosphorus compounds by bacteria (Rodríguez and Fraga, 1999). Phosphate solubilizing bacteria play an important role in plant nutrition through the increase in P uptake by the plant and their use with RP as fertilizer is an important contribution to biofertilization of agricultural crops.

Many reports are available on P solubilization by different microorganisms. However in the present study the PSMs were isolated from the mine landfills of rock phosphate which might have more adaptability to solubilize rock phosphate. As indicated in previous reports, rock phosphate is not plant available in soils with the pH greater than 5.5 to 6.0 (Vassilev et al. 2006); whereas in the present study PSMs increased the P levels when inoculated to RP amended alkaline soils where the pH of the soil was 8.1. Many studies in relation to crop improvement by P solubilizing microorganisms were carried out either in pot cultures or field conditions (Whitelaw et al. 1997; Omar 1998; Saber et al. 2009) for a particular crop. However in the present study, we have studied the growth and yield pattern of two consecutive crops and the soil properties after amending the rock phosphate into the soil. The results clearly showed that amending RP along with PSMs can improve the growth and sustainable release of P in the soil.

Malotfi and Prasad (1976) reported that rock phosphate was less efficient than superphosphate when applied on an equivalent P basis. However, when rock phosphate was applied at the rates double to those of superphosphate, the yields of cowpea were almost similar. Considering the effect of difficulties, environment unfriendly and costly treatment process needed for manufacturing superphosphate or other water-soluble phosphates, crops can be fertilized with rock phosphate as P source. Moreover it is clear in the present study that the efficiency of rock phosphate as P fertilizer can be increased using P solubilizing microorganisms. It could be concluded that crude rock phosphate (equivalent to 20 mg/kg soil P_2O_5) can be suitable phosphate fertilizer by direct application in alkaline soils when used along with P solubilizing microorganisms.

This may assist in solving problems encountered for crop production economy and could contribute to reversing the trend of soil degradation and actually encourage soil conservation during cultivation of the land. Thus, the application of PSMs is recommended as a sustainable way for increasing crop yield and also improving the physio-chemical properties of the soil.

Salient findings

- Efficient P solubilizing microorganisms were isolated from rhizosphere soil of *Jatropha curcas* plants growing in the landfills of rock phosphate mines of Rajasthan State Mines and Minerals Limited (RSMML), Jhamarkotra, Udaipur, India.
- After biochemical analysis of the efficient bacterial isolates (2C9, SC18, C19 and 4G2), molecular characterization was carried out on the basis of 16S rRNA. The

isolates were identified as *Bacillus pumilis* (2C9 and SC18), *Bacillus sp.* (C19) and *B. cereus* (4G2).

- Based on morphological analysis and molecular characterization, the fungal isolates were identified as *Aspergillus tubingensis (AT6), A. niger* (2D4) and *Penicillium oxalicum* (07).
- Physiological characterization of P solubilizing bacteria using different C and N sources showed that excep*t Bacillus C19* (sucrose) all the three bacterial isolates showed maximum P solubilization in presence of glucose as C source. Urea and asparagine were found to be best N source fo*r Bacillus* C19 an*d B. pumilus SC18* for P solubilization*. B. pumilus 2C9* an*d B. cereus 4G2* showed highest solubilization in presence of ammonium sulphate as N source.
- Solubilization of inorganic P by fungal isolates was tested by altering different C and N sources in the media. Mannitol and potassium nitrate respectively proved to be best C and N sources for solubilization of P by all the four fungal isolates.
- Malic acid and succinic acid may be responsible for maximum solubilization in presence of mannitol in *A. niger* and *P. oxalicum* whereas oxalic acid was dominating in *Aspergillus tubingensis (AT6)*. The high solubilization in the media having potassium nitrate as N source can be attributed to exudation of high amount of formic acid (AT6 and *A. niger*) and malic acid (*P. oxalicum*) in the media.
- All the fungal isolates efficiently solubilized different metal phosphates such as copper phosphate, ferric phosphate, zinc phosphate, aluminium phosphate and cobalt phosphate. Exudation of organic acids was mainly responsible for the

solubilization of metal phosphates, however, the type and amount of organic acid production varied in presence of different metal phosphate, fungal isolate and in different C and N sources.

- The yield of wheat and maize and the P content in plants significantly improved due to inoculation with P solubilizing bacterial consortium in the RP amended soil. The yield of maize increased to 1.5 times as compared to the yield in control soil. Inoculation of P solubilizing bacteria improved the available P levels in alkaline soil amended with RP. The available P in the soil after maize harvest was more than five times higher than control soil.
- P solubilizing fungi when inoculated with RP in alkaline soil showed significant increase in yield and growth parameters of wheat and maize and also improved the soil properties. For example AT6 and *P. oxalicum* when inoculated in RRP amended soil showed more than 45% and 82% increase in yield of wheat and maize respectively as compared to non-inoculated and non-amended control. The significant increase in available P was observed for example available P was more than ten folds in RRP amended soil inoculated with *P. oxalicum* than control soil.
- This may be the first report indicating the direct application of raw rock phosphate as fertilizer along with P solubilizing microorganisms in alkaline soils. The study on effect of PSMs in RP amended alkaline soil for two consecutive crops showed significant increase in yield of wheat and maize and the available P levels in the soil. It can be concluded from this study that the chemical fertilizers might be substituted by cheaply available RP along with P solubilizing microorganisms in alkaline soils.

Summary

Summary

Phosphorus is an important nutrient for plant growth and development. Despite its wide distribution in nature, it is a deficient nutrient in most soils. Therefore large quantity and soluble forms of phosphorus is added to soil in the form of phosphate fertilizer, part of which is utilized by plants and the remainder converts into insoluble fixed forms which leads to excess and frequent application of soluble forms of inorganic phosphate to maintain adequate P levels for plant growth.

In recent years, the possibility of practical use of rock phosphate (RRP) as fertilizer has received significant interest. In India, it is estimated that about 260 million tons of phosphatic rock deposits are available and this material should provide a cheap source of phosphate fertilizer for crop production. Unfortunately, rock phosphate is not plant available in soils with a pH greater than 5.5 - 6.0 and even when conditions are optimal, plant yields are lower than those obtained with soluble phosphate. For a sustainable agriculture system, microbial solubilization of rock phosphate and its use in agriculture is receiving greater attention. Phosphate solubilizing microorganisms (PSMs) can be useful to reverse the process of phosphate fixation. Many soil fungi and bacteria can solubilize inorganic phosphate into soluble forms through the process of acidification, chelation, exchange reactions and production of organic acids which not only compensates for higher cost of manufacturing fertilizers in industry but also mobilizes the fertilizers added to soil. Acid phosphatases and phytases secreted by these microorganisms also have an important role in phosphate solubilization.

The PSMs occur in soil, usually their number are not high enough to compete with other microorganisms commonly established in the rhizosphere. Thus the amount of P liberated by them is generally not sufficient for a substantial increase in plant growth. Therefore inoculation of plants by a target microorganism at a much higher concentration than the normally found in soil is necessary to take advantage of the property of phosphate solubilization for plant yield enhancement. The use of phosphate solubilizing microorganisms in agriculture can not only compensate for higher cost of manufacturing fertilizers in industries but also mobilizes the fertilizers added to soil. PSMs will be a boon for the farmers where the soil pH is high. The present study was focused on isolation of efficient phosphate solubilizing microorganisms from rock phosphate mines, their characterization and uses to increase the fertilizer value of rock phosphates.

Phosphate solubilizing microorganisms (PSMs)

To study the diversity of PSMs the rhizospheric soil from phosphate rock mine landfills of Rajasthan state Mines and Minerals Ltd, Jhamarkotra, Udaipur, Rajasthan, was collected. The PSMs were isolated on Pikovskaya's agar (Pikovskaya 1948) plates containing Rajasthan rock phosphate (RRP)/tricalcium phosphate (TCP) as phosphate source. A total of 188 bacterial colonies and 52 fungal colonies were isolated out of which only 64 bacterial colonies and 14 fungal colonies showed solubilization zone and were further tested for solubilization of phosphate in quantitative terms.

The results showed that only four bacterial isolates namely C19, 2C9, SC18 and 4G2 solubilized TCP as well as rock phosphate (RRP) to greater extent as compared to other bacterial isolates. Therefore these four isolates were selected for further analysis and characterization. The results showed that at higher concentration of TCP, the bacterial isolates did not respond well. The solubilization of RRP was also not significant when compared to TCP. The maximum solubilization of TCP (~1% P_2O_5) was observed in 4G2 i.e. 276 µg/ml followed by 2C9 which solubilized 249 µg/ml of P in fifteen days whereas the solubilization of RRP (~1% P_2O_5) by 4G2 and 2C9 was 88 µg/ml and 65 µg/ml respectively.

The fungal isolates were tested for quantitative solubilization in Pikovskaya's broth amended with desired quantity of TCP at 37° C for fifteen days. All the 14 isolates showed maximum solubilization in seven days. The solubilization was highest i.e. 463 µg/ml by fungal isolate 07 followed by 2D4 which showed 430 µg/ml soluble P. The solubilization of RRP by all the fungal isolates was significantly high. The soluble P was maximum in case of fungal isolate 07 i.e. 458 µg/ml; whereas it was 398 µg/ml by isolate 2D4 in seven days. The study shows that the fungal isolates have the more P solubilizing ability than bacterial isolates. The same pattern was observed in the study done by Rajankar et al. (2007).

Microbial diversity

To further characterize the bacterial and fungal isolates, 16S rDNA sequencing and biochemical characterization were carried out for bacterial isolates and ITS sequence analysis was performed for fungal isolates.

The sequences of the 16S rRNA gene of the 2C9and SC18 strains showed 99 % similarity with *Bacillus pumilus*, C19 had 99% similarity with uncultured *Bacillus* spp. and the 4G2 strain showed 99% similarity with *Bacillus cereus*. These results were similar to the classification obtained with biochemical testing as they all were identified as *Bacillus* species.

The fungal isolates were also identified under the microscope based on morphological characteristics, which revealed that among fourteen fungal isolates, one was of genus *Penicillium* and others were of *Aspergillus*. Through ITS sequencing and RFLP analysis, out of twelve, two isolates were grouped together as *Aspergillus tubingensis* while other 11 were grouped as *Aspergillus niger*. One fungus from each group (06, 2D4 and 07) was taken as representative isolate based on maximum solubilization efficiency for further analysis. The organisms were identified on the basis of sequencing of ITS region. The sequences obtained were compared with existing databases using the NCBI program, which revealed that fungal isolate 06 showed similarity with *Aspergillus tubingensis* (AT6), 2D4 with *Aspergillus niger* and 07 with *Penicillium oxalicum*. One more strain of *Aspergillus tubingensis* (AT1), an efficient phosphate solubilizer, reported by Reddy et al. (2002), was also used for further studies.

Effect of carbon and nitrogen sources on P solubilization

Phosphate solubilization is a complex phenomenon, which depends on many factors such as the nutritional, physiological, and growth conditions of the cultures. All the isolates were grown with different carbon sources which include sucrose, mannitol, arabinose, fructose and xylose and nitrogen sources i.e. ammonium sulphate was also substituted with ammonium nitrate, sodium nitrate, potassium nitrate, asparagine and urea in Pikovskaya's media.

The best solubilization was observed in media with mannitol and potassium nitrate by fungi when grown at 30 °C. The same combination was then used to test the solubilization of RRP which showed significantly enhanced soluble P. The same experiments were then performed with bacterial cultures for fifteen days which revealed that sucrose and glucose were good carbon sources and ammonium sulphate may be the best N source for bacterial isolates followed by organic forms, urea and asparagine.

Solubilization of metal phosphates

To study the solubilization of different metal phosphates, fungal inoculum was added to each 250 ml conical flask containing 50 ml of Pikovskaya's broth amended with different metal phosphates (copper phosphate, ferric phosphate, zinc phosphate, aluminium phosphate and cobalt phosphate) equivalent to 5 mM P_2O_5 and grown for seven days at 30 °C. The culture filtrate was analyzed for soluble P, pH and enzyme activity. The organic acids produced during P solubilization were also determined in the culture filtrates using HPLC method. All the fungal isolates showed solubilization of metal phosphates. The increase in solubilization coincided with decrease in pH of the medium which may be due to microbial exudation of organic acids. The acid phosphatase and phytase activity were also increased in these isolates.

The pH of the media decreased to 2.0-3.5 after seven days of incubation in all the metal phosphates. Phosphate solubilization increased with pH decrease of the media. In case of $AlPO_4$, the highest solubilization was shown by *P. oxalicum* which directly can be correlated to highest amount of oxalic acid and succinic acid exudation. *P. oxalicum* showed highest solubilization of $Zn_3(PO_4)_2$ may be due to highest amount of phytase activity amongst the four isolates. It was followed by *A. niger* which had high level of formic acid production. In Cu_2OHPO_4 amended media *A. tubingensis* (AT6) showed maximum solubilization which directly relates to gluconic acid exudation in significant amount. *P. oxalicum* showed highest pH reduction in case of Cu_2OHPO_4 but it was not associated with high solubilization. In case of $FePO_4$ there was not much difference in pH reduction by all the fungal isolates though *A. niger* and *P. oxalicum* showed good solubilization which coincides which high oxalic acid production, whereas the solubilization was very poor in case of both the strains of *A. tubingensis*. *P. oxalicum* showed high pH reduction in $Co_3(PO_4)_2$ amended media along with significant solubilization but the highest solubilization was by *A. tubingensis* (AT1) where pH reduction was 3.77 and exudation of gluconic acid was maximum. The major organic acids produced by the four fungi to solubilize $Co_3(PO_4)_2$ were, gluconic acid, oxalic acid, citric acid and formic acid. Overall, there was a relationship between the lowering of the pH, exudation of organic acids and increase in soluble P. The isolates that were least effective at P solubilization had the highest pH and vice versa. In some instances, for example in $FePO_4$ in case of *A. tubingensis* (AT1), a substantial lowering of pH associated with high level of solubilization. The study is relevant to an understanding of fungal responses towards metal species as well as the mechanisms fungi use in affecting metal and phosphate bioavailability.

The ability of phosphate solubilizing fungi to alter the rate of solubilization in response to changing nutrients is important in the natural environment. The manipulation of laboratory media could be useful to optimize the production of organic acids, and this may have potential for biotechnological applications, such as recycling of metals from industrial waste and low-grade byproducts, and possibly in the bioremediation of polluted soils. The solubilization of all the five metal phosphate was tested using different C and N sources. In Pikovskaya media, TCP was substituted with different metal phosphates (copper phosphate, ferric phosphate, zinc phosphate, aluminium phosphate and cobalt phosphate) equivalent to 5 mM of P_2O_5. Glucose was replaced with different C sources (sucrose, mannitol, arabinose, fructose and xylose), ammonium sulphate with different N sources (ammonium nitrate, sodium nitrate, potassium nitrate, asparagine and urea) and the fungi was grown for seven days at 30 °C. The culture filtrate was analyzed for soluble P, pH and enzyme activity after seven days. In case of $AlPO_4$, fructose was the best source for *A. tubingensis* (AT6) and *A. niger* whereas *A. tubingensis* (AT1) and *P. oxalicum* showed best solubilization in presence of mannitol and glucose respectively. Ammonium sulphate was the best N source for all the isolates for solubilization of aluminium phosphate followed by asparagine. Both the strains of *A. tubingensis* showed tremendous increase in solubilization of $Zn_3(PO_4)_2$ when grown with fructose and asparagine as compared to other C and N sources. *P. oxalicum* showed significant increase in solubilization of Cu_2OHPO_4 in media having arabinose and sodium nitrate as compared to Pikovskaya media while for both the isolates of *A. tubingensis* sucrose and potassium nitrate was the best combination. In presence of xylose and sodium nitrate *A. tubingensis* (AT6) showed the highest solubilization of $FePO_{4;}$ whereas for all the other isolates, glucose and ammonium sulphate was the best combination for maximum solubilization of $FePO_{4.}$ Fructose was the best C source for *A. tubingensis* (AT1) and *A. niger* to solubilize $Co_3(PO_4)_{2.}$ *P. oxalicum*, if grown in presence of sucrose and asparagine, can solubilize quite high amount of $Co_3(PO_4)_{2.}$ Thus the results showed that the solubilization of metal phosphate is affected by different C and N sources in different manner by different micro organisms.

Enhancement of fertilizer value of rock phosphate

Inoculation of plants by target phosphate solubilizing microorganisms at a much higher concentration than that normally found in soil was necessary to take an advantage of property of phosphate solubilization for plant yield enhancement. Combined direct application of rock phosphate and phosphate solubilizing microorganisms (PSMs) has produced mixed results on plant growth responses, which were perhaps attributed to differences in microbial strains and/or soils being treated.

The results of present study showed that addition of rock phosphate as a phosphate fertilizer along with the phosphate solubilizing microorganisms greatly enhanced the plant growth and nutrient uptake of wheat and maize plants. When all the treatments involving rock phosphate are compared with non-inoculated controls, they showed significantly increased P accumulation in shoots, grain, seed cover and roots of wheat and maize. The yield of wheat was significantly high in case of RRP fertilized soil inoculated with *P. oxalicum* followed by *A. niger*.

The results showed that inoculation with bacterial consortium improves the shoot length of the wheat in RRP fertilized or/and non fertilized treatments to a greater extent than the fungal treatments. Consortium of the fungal strains, in RRP amended soil, significantly increased the height up to 63 cm as compared to non-inoculated control soil having shoot length of 43 cm. When all treatments involving RRP are compared with non-inoculated controls, significant increase in P accumulation was observed in shoots, grain, seed cover and roots of the wheat. The total P uptake by plant was ten folds higher in the RRP amended treatment when *P. oxalicum* was added compared to control soil.

To evaluate the effectiveness of the phosphate solubilizing microorganisms on the changes of soil characteristics, the soil was analyzed for available P, total P, organic carbon, pH and electric conductivity. The soil properties were improved after inoculation with the phosphate solubilizing microorganisms in RRP amended soils. This application caused a drop in pH values (0.2-0.4) of the soil in all the treatments as compared to the control soil however this reduction was not significant. A general reduction in EC values was observed after the experiment. There were significant differences in EC due to the treatment applications, although it was seen that rock phosphate plots retained higher salinity levels at the time of harvesting compared to control plots.

The P content of soil decreased in all the treatments as compared to initial values, however in the rock phosphate amended plants, the phosphorus content was higher as compared to non-fertilized treatments. The organic C was significantly higher at the final harvest stage in all the treatments, than at the preplanting stage however the increase in organic C was maximum in the RRP fertilized soil inoculated with *A. niger* and *P. oxalicum.* When all the treatments were compared with non-inoculated and non-fertilized soil, the available P was found to be increased many folds. Rock phosphate fertilization as well as PSM inoculum had significant positive effect on soil available P. The RRP fertilized plot inoculated with fungal consortium showed threefold increase in available P as compared to non fertilized control soil.

In maize experiment it was observed that the yield of maize was almost double in the RRP amended soil inoculated with *A. tubingensis* (AT1) when compared to control soil. The yield of maize increased to 1.5 times in the treatment with P solubilizing bacterial consortium in the RP amended soil as compared to the yield in control soil. Inoculation of P solubilizing bacteria improved the available P levels in alkaline soil amended with RP. The available P in the soil after maize harvest was more than five times higher than control soil.The inoculation with PSMs significantly increased shoot and root dry weight of RP fertilized plants when compared with the control soil. The P content in the different parts of plants was determined which reveals that the total P in plants increased on addition of inoculum along with RRP, it was found to be highest in the treatment where fungal consortium was added, followed by *A. tubingensis* (AT1) when compared to control soils. After maize harvest, there was a reduction in the pH of the soil having microbial inoculum and RRP fertilization when compared to control. This may be attributed to ability of such microorganisms to excrete organic acids, thereby decreasing the pH and increasing the concentration of phosphorus in soil by mechanisms involving chelation and exchange reactions. A general increase in organic carbon occurred in soil after harvesting the maize. The reduction in the total P content of the soil was observed after the two crops may be due to increased uptake of P by plants. The available P increased significantly in all the inoculated and RRP fertilized soils as compared to control. In case of soil, having fungal consortium and RRP, the available P after final harvest was seven times more than the control soil. Improved crop yield in this investigation could also have resulted due to the significant increase of the available P in all the treatments compared to non-inoculated and non-fertilized soil.

The increase in wheat and maize yield was the result of increase in soil fertility via fertilization with rock phosphate and improvement in soil characteristics by inoculated PSMs that too for two crops. It was found that these phosphate solubilizing strains along with RRP can substitute the chemical fertilizer, might be used to reduce the alkalinity of soil by neutralization phenomenon through organic acid exudation and can survive in the soil system to retain the phosphate solubilizing potential for long time.

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Appendix

APPENDIX - I

Pikovskaya's agar

Ingredients	**Quantity (g/l)**
Agar	15.0
Ammonium sulphate	0.5
Calcium phosphate	5.0
Dextrose	10.0
Ferrous sulphate	0.0001
Magnesium sulphate	0.1
Manganese sulphate	0.0001
Potassium chloride	0.2
Yeast extract	0.5

Suspended 31.3 g in 1000 ml distilled water. Boiled to dissolve the medium completely and sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Nitrate agar

Ingredients	**Quantity (g/l)**
Agar	12.0
Beef extract	3.0
Peptic digest of animal tissue	5.0
Potassium nitrate	1.0

Suspended 21 g of nitrate agar in 1000 ml distilled water. Boiled to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min.

LB broth (Luria-Bertani) (Sambrook and Russell 2001)

Bacto-tryptone 10 g/l

Bacto-yeast extract 5 g/l

NaCl 5 g/l

Adjust pH to 7.0 with 5 N NaOH and autoclave 20 minutes at 121°C.

LB/amp+ agar plates

Prepare LB broth as above. Add agar (18 g/l), autoclave, and cool to 50°C. Add ampicillin to 50 µg/ml. Pour plates and store at 4°C.

IPTG stock solution (0.1M)

1.2 g IPTG

Add water to 50 ml final volume. Filter sterilize and store at 4°C.

X-Gal (2ml)

100 mg 5-bromo-4-chloro-3-indolyl-Dgalactoside dissolve in 2 ml N,N´-dimethylformamide.

Cover with aluminum foil and store at 20°C.

LB plates with ampicillin/IPTG/X-Gal

Make the LB plates with ampicillin as above; 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal may be spread over the surface of an LB ampicillin plate and allowed to absorb for 30 minutes at 37°C prior to use.

Genomic DNA Extraction buffer

Sodium acetate 100 mM

Na2EDTA 50 mM

NaCl 500 mM

SDS 1%

TBE Buffer (10x)

Tris-HCl 0.09 M (pH 8)

Boric acid 0.9 M

EDTA 0.02 M (pH 8)

Plasmid extraction solution I (10X)

Tris-HCl 25 mM (pH 8.0)

Glucose 50 mM

Na2EDTA 10mM

Plasmid extraction solution II

NaOH 5M

SDS 10%

Plasmid extraction solution III

5.0 M Potassium acetate (pH 4.5)

Agarose gel loading dye (6X)

Bromophenol blue 0.25%

Xylene cyanol FF 0.25%

Glycerol in water 30.0%

TE buffer 10X

Tris-HCl 0.1 M (pH 8)

Na2EDTA 10 M (pH 8)

APPENDIX- II

Sequence of 16S rRNA of bacterial isolate SC18

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ORGANISM    Bacillus sp. SC18(2010b)
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 672)
AUTHORS     Reddy,S.M. and Singh,H.
TITLE       Diversity of bacteria isolated from rock-phosphate-mine
landfills
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 672)
  AUTHORS   Reddy,S.M. and Singh,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-AUG-2010) Department of Biotechnology, Thapar
            University, Bhadson Road, Patiala, Punjab 147004, India
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Sequence of 16S rRNA of bacterial isolate 2C9

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ORGANISM    Bacillus sp. SC18(2010b)
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REFERENCE   1  (bases 1 to 672)
AUTHORS     Reddy,S.M. and Singh,H.
TITLE       Diversity of bacteria isolated from rock-phosphate-mine
landfills
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 672)
  AUTHORS   Reddy,S.M. and Singh,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-AUG-2010) Department of Biotechnology, Thapar
            University, Bhadson Road, Patiala, Punjab 147004, India
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REFERENCE   1  (bases 1 to 699)
  AUTHORS   Reddy,S.M. and Singh,H.
  TITLE     Diversity of bacteria isolated from rock-phosphate-mine
landfills
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 699)
  AUTHORS   Reddy,S.M. and Singh,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-AUG-2010) Department of Biotechnology, Thapar
            University, Bhadson Road, Patiala, Punjab 147004, India
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  TITLE     Diversity of bacteria isolated from rock-phosphate-mine
landfills
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 666)
  AUTHORS   Reddy,S.M. and Singh,H.
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Sequence of ITS rDNA of fungal isolate 06

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            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Eurotiomycetes; Eurotiomycetidae; Eurotiales; Trichocomaceae;
            mitosporic Trichocomaceae; Aspergillus.
REFERENCE   1  (bases 1 to 638)
  AUTHORS   Reddy,S.M. and Singh,H.
  TITLE     Diversity of phosphate solubilizing fungi isolated from rock
            phosphate mine landfills
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 638)
  AUTHORS   Reddy,S.M. and Singh,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-JUL-2010) Department of Biotechnology, Thapar
            University, Bhadson Road, Patiala, Punjab 147004, India
FEATURES             Location/Qualifiers   source             1..638
                     /organism="Aspergillus sp. 06 SMR-2010"
                     /mol_type="genomic DNA"
                     /strain="06"
                     /db_xref="taxon:885751"
     rRNA            <1..108
                     /product="18S ribosomal RNA"
     misc RNA        109..298
                     /product="internal transcribed spacer 1"
     rRNA            299..458
                     /product="5.8S ribosomal RNA"
     misc RNA        459..623
                     /product="internal transcribed spacer 2"
     rRNA            624..>638
                     /product="28S ribosomal RNA"
ORIGIN
        1 tcttggtcat ttagaggaag taaaagtcgt aacaaggttt ccgtaggtga acctgcggaa
       61 ggatcattac cgagtgcggg tcctttgggc ccaacctccc atccgtgtct attataccct
      121 gttgcttcgg cgggcccgcc gcttgtcggc cgccgggggg gcgcctttgc cccccgggcc
      181 cgtgcccgcc ggagacccca acacgaacac tgtctgaaag cgtgcagtct gagttgattg
      241 aatgcaatca gttaaaactt tcaacaatgg atctcttggt tccggcatcg atgaagaacg
      301 cagcgaaatg cgataactaa tgtgaattgc agaattcagt gaatcatcga gtctttgaac
      361 gcacattgcg ccccctggta ttccgggggg catgcctgtc cgagcgtcat tgctgccctc
      421 aagcccggct tgtgtgttgg gtcgccgtcc ccctctccgg ggggacgggc ccgaaaggca
      481 gcggcggcac cgcgtccgat cctcgagcgt atggggcttt gtcacatgct ctgtaggatt
      541 ggccggcgcc tgccgacgtt ttccaaccat tttttccagg ttgacctcgg atcaggtagg
      601 gatacccgct gaacttaagc atatcaataa gcggagga
```

Sequence of ITS rDNA of fungal isolate 2D4

```
LOCUS       HM801882             600 bp    DNA     linear   PLN 21-SEP-
2010
DEFINITION  Aspergillus sp. 2D4 SMR-2010 18S ribosomal RNA gene, partial
           sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene,
           and internal transcribed spacer 2, complete sequence; and 28S
           ribosomal RNA gene, partial sequence.
ACCESSION   HM801882
VERSION     HM801882.1  GI:307088982
KEYWORDS
SOURCE      Aspergillus sp. 2D4 SMR-2010
ORGANISM    Aspergillus sp. 2D4 SMR-2010
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
           Eurotiomycetes; Eurotiomycetidae; Eurotiales; Trichocomaceae;
            mitosporic Trichocomaceae; Aspergillus.
REFERENCE   1  (bases 1 to 600)
  AUTHORS   Reddy,S.M. and Singh,H.
  TITLE     Diversity of phosphate solubilizing fungi isolated from
rock
            phosphate mine landfills
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 600)
  AUTHORS   Reddy,S.M. and Singh,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-JUL-2010) Department of Biotechnology, Thapar
            University, Bhadson Road, Patiala, Punjab 147004, India
FEATURES             Location/Qualifiers
  source              1..600  /organism="Aspergillus sp. 2D4 SMR-2010"
                     /mol_type="genomic DNA"
                     /strain="2D4"
                     /db_xref="taxon:885752"
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     misc_RNA        31..215
                     /product="internal transcribed spacer 1"
     rRNA            216..372
                     /product="5.8S ribosomal RNA"
     misc_RNA        373..541
                     /product="internal transcribed spacer 2"
     rRNA            542..>600
                     /product="28S ribosomal RNA"
ORIGIN
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       61 catccgtgtc tattgtaccc tgttgcttcg gcgggcccgc cgcttgtcgg ccgccggggg
      121 ggcgcctctg ccccccgggc ccgtgcccgc cggagacccc aacacgaaca ctgtctgaaa
      181 gcgtgcagtc tgagttgatt gaatgcaatc agttaaaact ttcaacaatg gatctcttgg
      241 ttccggcatc gatgaagaac gcagcgaaat gcgataacta atgtgaattg cagaattcag
      301 tgaatcatcg agtctttgaa cgcacattgc gccccctggt attccggggg gcatgcctgt
      361 ccgagcgtca ttgctgccct caagcccggc ttgtgtgttg ggtcgccgtc cccctctccg
      421 gggggacggg cccgaaaggc agcggcggca ccgcgtccga tcctcgagcg tatggggctt
      481 tgtcacatgc tctgtaggat tggccggcgc ctgccgacgt tttccaacca ttctttccag
      541 gttgacctcg gatcaggtag ggatacccgc tgaacttaag cgtatcaata agcggaggaa
```

Sequence of ITS rDNA of fungal isolate 07

```
LOCUS        HM801883         589 bp    DNA     linear   PLN 21-SEP-2010
DEFINITION  Penicillium sp. 07 SMR-2010 18S ribosomal RNA gene, partial
            sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene,
            and internal transcribed spacer 2, complete sequence; and 28S
            ribosomal RNA gene, partial sequence.
ACCESSION   HM801883
VERSION     HM801883.1  GI:307088983
KEYWORDS
SOURCE       Penicillium sp. 07 SMR-2010
ORGANISM     Penicillium sp. 07 SMR-2010
             Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Eurotiomycetes; Eurotiomycetidae; Eurotiales; Trichocomaceae;
             mitosporic Trichocomaceae; Penicillium.
REFERENCE    1  (bases 1 to 589)
  AUTHORS    Reddy,S.M. and Singh,H.
  TITLE      Diversity of phosphate solubilizing fungi isolated from
rock
             phosphate mine landfills
  JOURNAL    Unpublished
REFERENCE    2  (bases 1 to 589)
  AUTHORS    Reddy,S.M. and Singh,H.
  TITLE      Direct Submission
  JOURNAL    Submitted (20-JUL-2010) Department of Biotechnology, Thapar
             University, Bhadson Road, Patiala, Punjab 147004, India
FEATURES              Location/Qualifiers
  source              1..589 /organism="Penicillium sp. 07 SMR-2010"
                      /mol_type="genomic DNA"
                      /strain="07"
                      /db_xref="taxon:885757"
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                      /product="18S ribosomal RNA"
     misc_RNA         31..207
                      /product="internal transcribed spacer 1"
     rRNA             208..363
                      /product="5.8S ribosomal RNA"
     misc_RNA         364..542
                      /product="internal transcribed spacer 2"
     rRNA             543..>589
                      /product="28S ribosomal RNA"
ORIGIN
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       61 cacccgtgtt tatcgtacct tgttgcttcg gcgggcccgc ctcacggccg ccgggggggca
      121 tccgcccccg ggcccgcgcc cgcccgaagac acacaaacga actcttgtct gaagattgca
      181 gtctgagtac ttgactaaat cagttaaaac tttcaacaac ggatctcttg gttccggcat
      241 cgatgaagaa cgcagcgaaa tgcgataagt aatgtgaatt gcagaattca gtgaatcatc
      301 gagtctttga acgcacattg cgccccctgg tattccgggg ggcatgcctg tccgagcgtc
      361 attgctgccc tcaagcacgg cttgtgtgtt gggctctcgc cccccgcttc cgggggggcgg
      421 gcccgaaagg cagcggcggc accgcgtccg gtcctcgagc gtatggggct tcgtcacccg
      481 ctctgtaggc ccggccggcg cccgccggcg aacaccatca atcttaacca ggttgacctc
      541 ggatcaggta gggatacccg ctgaacttaa gcatatcaat aagcggagg
```