Chapter 3

Materials and methods

MATERIALS AND METHODS

3.1 Isolation and identification of phosphate-solubilizing microorganisms for alkaline soils

3.1.1 Collection of soil samples

To isolate the phosphate-solubilizing microorganisms, soil samples were collected from different agricultural soil niches. These niches were rhizosphere of bamboo (*Bambusa vulgaris*) plants (multipurpose agroforestry crop) grown in experimental field of CORE, Thapar university, Patiala (30.30° N, 76.38° E); surface soil of potato (*Solanum tuberosum*) (tuberous crop) field of CORE, Thapar University, Patiala; surface soil of CORE (no vegetation, open area); rhizosphere soil of mustard (oil-seed crop) (*Brassica campestris*); rhizosphere of mature maize (cereal crop) (*Zea mays*) plants, rhizosphere soil of small maize plants, surface soil of berseem crop (fodder crop) (*Trifolium alexandrinum*) field; rhizosphere of sunflower (oil-seed crop) (*Helianthus annuus*) grown in agriculture field of Balachaur (31.07° N, 76.32° E), Punjab; and rhizosphere of *Stevia rebaudiana* (medicinal plant) grown in organic field of Pojewal (31.65° N, 76.26° E), Punjab, India. Soil samples to the depth of 5-10 cm were drawn carefully and were collected in sterile plastic bags and brought to the laboratory and stored at 4 ºC until used for the isolation of phosphate-solubilizing microorganisms.

3.1.2 Isolation of phosphate-solubilizing microorganisms

Isolation of phosphate-solubilizing microorganisms was done by serial dilution method on Pikovskaya's (PKV) agar plates (Pikovskaya 1948).

Composition of Pikovskaya's agar medium (Pikovskaya 1948)

Ingredients	**gms/liter**
Yeast Extract	0.50
Dextrose	10.0
Tri-calcium phosphate	5.00
Ammonium Sulphate	0.50
Potassium Chloride	0.20
Magnesium Sulphate	0.10
Manganese Sulphate	0.001
Ferrous Sulphate	0.001
Agar	15.00
Distilled Water	1000 ml
pH	7.2 ± 0.2

All ingredients except tri-calcium phosphate $Ca_3(PO_4)_2$ were dissolved in 1000 ml of distilled water, pH adjusted to 7.2 and autoclaved at 15 Psi for 15 minutes. Tri-calcium phosphate was autoclaved separately at 15 Psi for 15 minutes. Later these were mixed together and poured in to sterilized Petri plates. After solidification of medium, these Petri-plates were incubated at 30 ºC for overnight to check contamination.

3.1.2.1 Phosphate-solubilizing bacteria

For the isolation of phosphate-solubilizing bacteria 10 gram of soil sample was transferred to 90 ml of sterile physiological saline solution (0.85 % NaCl in distilled water) and mixed thoroughly. Serial dilutions were made up to 10^{-7} and from each dilution 100 µl were spread on Pikovskaya's agar plates supplemented with 0.5 % tri-calcium phosphate (TCP) and incubated at 30 ºC. Colonies showing the zone of solubilization were streaked on PKV agar plates to check their purity and stored in 40 % glycerol at -80 ºC for further use.

3.1.2.2 Phosphate-solubilizing fungi

For the isolation of phosphate-solubilizing fungi, soil samples were serially diluted up to 10^{-5} in sterile physiological saline solution (0.85 % NaCl in distilled water) and pour plating was done on PKV agar plates supplemented with 0.5 % TCP, having 0.003 % w/v of Rose Bengal powder to selectively inhibit the 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 zone were selected, purified by repeated culturing and maintained on PKV agar slants at 4 ºC.

3.1.3 Qualitative screening of phosphate-solubilizing bacteria and fungi

Pure culture of phosphate-solubilizing bacteria and fungi were spot inoculated on PVK agar plates and incubated at 30 ºC. For this PKV agar plates were formally divided into four parts. Ten µl suspensions of two days grown culture of bacterial isolates was used to inoculate centre of each quarter part of the Petri plate. Each isolate was inoculated in duplicate. In case of fungal isolates, 5 mm diameter disc, cut from the periphery of the actively growing colonies of fungal isolates, and were inoculated on PKV agar plates. The halo zone of phosphate solubilization around growth was recorded (in mm) after every 24 hours of incubation. The colonies forming more than 5.0 mm zone of solubilization were selected as efficient strains. Results were recorded up to five days. Solubilization index was evaluated according to the ratio of the total diameter (colony + halo zone) and the colony diameter (Edi-Premono et al. 1996).

3.1.4 Test of purity and stability (Fankem 2006)

To estimate the capability of the obtained isolates in solubilizing sparingly soluble phosphate ($Ca_3(PO_4)_2$), PVK agar medium was used along with Bromo Cresol Green (BCG) dye as an

indicator. 5.0 ml of 0.5 % Bromo Cresol Green was added to 1000 ml of PVK agar medium. In this medium the BCG was used as pH indicator dye which, in pH greater than 6 is green but changes into yellow color when the pH falls within 3.8-5.4.

Preparation of Bromo Cresol Green (Gadagi and Tongmin 2002)

A stock solution of 0.5% dye was prepared by dissolving 0.5 g BCG in 100 ml 70 % ethanol. The final pH was adjusted to 6.5 with KOH. BCG was sterilized by Glass Fibric Circle filter. Five ml of this stock solution was added aseptically to 1000 ml of autoclaved PVK agar medium.

Procedure

A five ml aliquot of the sterilized stock solution of BCG was added to 1000 ml of autoclaved PVK agar medium, and were mixed together, and poured in Petri plates. After solidification, these Petri plates were incubated at 30 °C for overnight to check the contamination. Phosphate-solubilizing bacterial culture of 0.8 $O.D._{600\ nm}$ was point inoculated on the Petri plates. In case of fungal isolates 5 mm diameter disc, cut from the periphery of the actively growing colonies of fungal isolates were inoculated on PKV agar plates supplemented with BCG dye. Plates were then incubated at 30 °C. Results were recorded after three to five days of incubation.

3.1.5 Quantitative Assay for P-solubilization in liquid medium (Jackson 1973)

These bacterial and fungal isolates were further tested for solubilization of tri-calcium phosphate and Rajasthan rock phosphate (RP) in quantitative terms.

Reagents

1. **Chloromolybdic acid:** 15.0 g ammonium molybdate was dissolved in about 400 ml of distilled water. Filtered and then 400 ml of 10 N HCl was added slowly with rapid stirring. Volume was made to 1000 ml with distilled water and stored in amber glass bottle.
2. **Chlorostannous acid:**

   ***Stock Solution*:**

$SnCl_2 .2H_2O$	10.0 g
Conc. HCl	25.0 ml

   $SnCl_2$ crystals were dissolved in conc. HCl and solution was kept in glass under airtight stopper.

   ***Working Solution*:** Fresh working solution was prepared by adding 1.0 ml of the above solution to 132.0 ml of distilled water.

Assay procedure for quantitative P solubilization in liquid medium

The PSB were grown in 50 ml nutrient broth for 24 hours at 30 °C in incubator shaker. 0.1 ml of each PSB was aseptically transferred to 100.0 ml PVK broth contained in 250 ml conical flasks. Similarly 5 mm diameter disc, cut from the periphery of the actively growing colonies of fungal isolates were inoculated in 100 ml PKV broth in 250 ml conical flask. The flasks were incubated at 30 °C in a rotary shaker at 130 rpm. Five ml of culture suspension was taken out in sterile conditions at regular interval of 2 days from third day onward and centrifuged at 10,000 rpm for 10 min. Then 500 µl aliquot of each supernatant was transferred to 50.0 ml volumetric flask. This was followed by addition of 10.0 ml chloromolybdic acid. The contents of the flasks were diluted to 40.0 ml with distilled water. Then 1 ml of chlorostannous acid was added. After mixing, the volume was made up to 50.0

ml with distilled water. The blue color intensity of the solution was measured in a spectrophotometer at O.D. 660 nm. The soluble 'P' was estimated from standard curve of KH_2PO_4 (100 ppm) drawn against O.D. 600 nm. In case of fungi at the end of incubation, the contents of the flasks were filtered through Whatman no. 42 filter paper, washed repeatedly with distilled water and the mycelial mass was dried at 70 ºC for 48 hours. Dry weight of the mycelium represents growth.

pH of the culture filtrates was also recorded. Isolates showed maximum P-solubilization in the TCP were tested for acid phosphatase and alkaline phosphatase enzyme production (Tabatabai and Bremner 1969), phytase enzyme production (Heinonen and Lahti 1981) and for organic acid exudation in culture supernatant by using an HPLC method as described by Relwani et al (2008).

Both fungal and bacterial isolates showed maximum P-solubilization in TCP were further screened for rock phosphate solubilization by supplementing RP equivalent to 100 mg P_2O_5 100 ml^{-1}. Rock phosphate used in this study was obtained from Rajasthan State Mines and Minerals, Ltd., Udaipur, India. The chemical constituents of the RP were: 31.5 % P_2O_5, 45.4 % CaO, 3.4 % MgO, 8.4 % Al_2O_3, 3.1 % fluoride, 0.044 % organic carbon and 0.003 % available P. Acid phosphatase and alkaline phosphatase activities, phytase activity and organic acids production was determined in culture supernatant.

3.1.6 Acid and alkaline phosphatase activity (Tabatabai and Bremner 1969)

Reagents

1. **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
1 N NaOH	488 ml

Final volume was made up to 1000 ml with distilled water and stored at 4 ºC.

Before use, the pH of 200 ml of MUB was adjusted to 5.5 with 0.5 N HCl for the assay of acid phosphatase or with 0.5 N NaOH to pH 9.0 for the alkaline phosphatase. The volume of pH modified buffer was made up to 1 liter with distilled water.

2. **p-nitrophenyl phosphate solution (0.115 M):** Dissolved 4.268 g disodium p-nitrophenyl phosphate hexahydrate in 100 ml of appropriate pH adjusted, diluted MUB (pH 5.5) for acid phosphatase and (pH 9.0) for alkaline phosphatase. The p-nitrophenyl phosphate substrate was stored at 4 ºC but no longer than 10 days.
3. **NaOH (0.5N):** 20 g of NaOH was dissolved in 70 ml distilled water and the volume was made up to 1 litre.
4. **p-nitrophenol standard:** 1 mg per 1 ml p.nitrophenol solution was prepared in modified universal buffer. The solution was stored in dark bottle at 4 ºC for no longer than 21 days.

Procedure

1. 0.5 ml aliquots of enzyme sample was taken (here culture filtrate).
2. Added 4.0 ml of the diluted MUB (pH 5.5 for acid phosphatase and pH 9.0 for alkaline phosphatase).
3. 1 ml of filter sterilized 0.115 M disodium p-nitro phenyl phosphate solution was added (of the same pH as of MUB).
4. The content was incubated at 37 ºC for one hour in dark, after one hour of incubation add 5 ml of 0.5 N NaOH solution to stop the reaction.
5. The content was then checked for phosphatase activity by measured the yellow color intensity with UV-Vis spectrophotometer at 410 nm.
6. Phosphatase enzyme activity was indicated as the amount of p-nitro phenol released in the filtrate from the p-nitro phenyl phosphate substrate as per ml of supernatant. The p-nitro phenol content was calculated with reference to a calibration graph plotted from the results obtained by standards containing 10-100 µg ml^{-1} of p-nitro phenol.
7. Controls were analyzed in the similar manner, except the p-nitro phenyl phosphate substrate was added after the NaOH solution.

3.1.7 Phytase enzyme activity (Heinonen and Lahti 1981)

Reagents

1. **Substrate solution:** Dissolved 2.5 mM dodecasodium phytate in 0.2 M sodium acetate buffer of pH 5.5.
2. **Color stop solution:** (10 mM ammonium molybdate : 5 N sulphuric acid: acetone, 1:1:2 ratio)
3. **1 M citric acid**
4. **Standard KH_2PO_4 solution:** mg/ml stock.

Procedure

1. 0.5 ml aliquots of enzyme sample was taken (here culture filtrate).
2. Added 0.5 ml substrate solution (2.5 mM dodecasodium phytate in 0.2 M sodium acetate buffer of pH 5.5) and incubated for 10 min at 37 ºC.
3. The reaction was stopped by addition of 2 ml ice cold color stop solution (10 mM ammonium molybdate : 5 N sulphuric acid: acetone, 1:1:2 ratio).
4. Added 100 µl 1 M citric acid and O.D. was taken at 380 nm.
5. Un-inoculated medium of experiment was taken as control.
6. Calculated the phytase activity per ml of sample. Enzyme activity was expressed as micromoles of inorganic P released per hour per milliliter of culture filtrate from sodium phytate at 37 °C.

3.1.8 Organic acid estimation

Organic acids produced by P-solubilizing microorganisms were determined using 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 5 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).

3.1.9 Biochemical characterization of P solubilizing microorganisms

3.1.9.1 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.

3.1.9.1.1 Gram staining

A thin smear of actively growing bacterial cells was made on separate glass slides, air dried and heat fixed the smears. Hold the slides using slide racks. Cover each smear with crystal violet for 30 sec and washed each slide with distilled water for a few seconds, using wash bottle. Each smear was covered with Grams iodine solution for 60 seconds. Iodine solution was washed off with 95% ethyl alcohol. Ethyl alcohol was added drop by drop, until no more colour flows from the smear. Slides were washed with distilled water and Safranin was applied to smear for 30 seconds. Slides were washed with distilled water and blot dried with absorbent paper. Let the stained slides air dry. Slides were examined microscopically using oil-immersion objective. Those bacteria that appeared purple were referred to as Gram positive and those appeared pink were described as Gram negative bacteria.

3.1.9.1.2 Hanging drop method

Hanging drop preparation is useful for microscopic examination of living microorganisms, especially bacteria without staining them and to see their motility due to flagella.

Cleaned and flamed a hanging drop slide and placed it on the table with the depression uppermost. A little Vaseline was spread around the cavity of the slides. A cover slip was cleaned and Vaseline was applied on each of the four corners of the cover slip. One loopful of culture was transferred in the centre of the cover slip. Depression slide was placed on the cover slip, with the cavity facing down so that the depression covers the suspension.

Preparation was examined under low-power objective with reduced light and a drop of oil was placed on the cover slip and the preparation was examined under oil-immersion objective. True motility was shown by bacteria that were moved swiftly across the microscope.

3.1.9.1.3 Catalase test

1. Small amount of bacterial cells were placed onto a clean microscope slide.
2. A few drops of H_2O_2 (3%) was added onto the smear.
3. A positive result was the rapid evolution of O_2 as evidenced by bubbling.
4. A negative result was no bubbles or only a few scattered bubbles.

3.1.9.1.4 Oxidase test

1. Small amount of organism from an agar slant/ plate was obtained with a sterile swab.
2. One drop of reagent (N,N,N‘,N‘-tetramethyl phenylenediamine dihydrochloride) was placed onto the culture on the swab.
3. Positive reactions turned the bacteria violet to purple immediately or within 10 to 30 seconds. Delayed reactions were ignored.

3.1.9.1.5 Nitrate reduction test

Nitrate broth composition

Ingredients	**gms / liter**
Peptic digest of animal tissue	5.000
Meat extract	3.000
Potassium nitrate	1.000
Sodium chloride	30.000
Final pH	7.0 ± 0.2

Distilled water 1000 ml

Dispensed the medium in tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Preparation of Nitrate test reagents:

1. **Sulfanilic Acid:** Dissolved 8 grams of sulfanilic acid in 1 liter 5 N acetic acid.
2. **Alpha-Naphthylamine reagent:** Dissolved 5 grams of alpha-naphthylamine in 1 liter 5 N acetic acid.

Procedure

Nitrate media 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. If the nitrate has not been reduced or if it has been reduced to free nitrogen gas the broth remains colourless. To differentiate between these two, zink dust was added. This catalysis the reduction of nitrate in the broth to nitrite which then allows the formation of diazonium compound which resulting red color. If the organism has reduced the nitrate to free nitrogen gas then the medium will remain colorless. There are positive results, if nitrate reduced either to nitrite or nitrogen gas. Negative results were obtained if nitrate has not been reduced.

3.1.9.1.6 Starch hydrolysis test

Requirements

1. **Starch agar**

Ingredients	**g/l**
Starch (soluble)	20.0
Peptone	5.0
Beef extract	3.0
Agar	15.0
pH	7.0
Distilled water	1000.0 ml

2. **Iodine solution**

Iodine	1.0 g
Potassium iodide	2.9 g
Distilled water	300.0 ml

A homogeneous preparation of the iodine and iodide was made by using mortar and pestle. Transferred the content to a reagent bottle and water was added to make a total volume of 300 ml. store the solution in a glass-stopper brown bottle.

Procedure

1. Using sterile technique, a single streak inoculation of each test organism was made into the centre of its appropriately labeled starch agar plate.
2. Incubated bacterial inoculated plates for 48 hours at 37 ºC and fungal inoculated plates for 72-96 hours at 25 ºC in an inverted position.
3. Surface of the plates was flooded with iodine solution with a dropper for 30 seconds.

4. Poured off the excess iodine solution.
5. The plates were examined for the starch hydrolysis around the line of growth of each organism, i.e. the color change of the medium.
6. Starch in the presence of iodine produced a dark blue coloration of the medium, and a yellow zone around the colony in an otherwise blue medium indicated amylolytic activity.

3.1.9.1.7 Cellulose production test

Evidence for the microbial utilization of cellulose can be detected using hexadecyltrimethyl ammonium bromide. This reagent precipitates intact carboxymethyl cellulose (CMC) in the medium and thus clear zones around a colony in an otherwise opaque medium indicating degradation of CMC.

Requirements

1. **Modified Czapek-mineral salt medium**

Ingredients	**g/l**
Sodium nitrate	2.0
Potassium phosphate	1.0
Magnesium sulphate	0.5
Potassium chloride	0.5
Carboxymethyl cellulose	5.0
Peptone	2.0
Agar	20.0
Distilled water	1000 ml

2. **Carboxymethyl cellulose (CMC)**
3. **Hexadecyltrimethyl ammonium bromide (1 % solution)**

Procedure

1. Dissolved the agar in 400 ml of hot distilled water by added in small amounts and stirring with a glass rod.
2. Dissolved the magnesium sulphate, potassium chloride, peptone, sodium nitrate in 200 ml of water.
3. Dissolved potassium phosphate in 100 ml of water.
4. Dissolved CMC in 200 ml of water with heat and mix.
5. Mixed all the solutions and made up to 1000 ml volume.
6. The pH of the medium was adjusted to 6.5 with the addition of acid or alkali and autoclaved at 15 lb/in^2 pressure (121 ºC) for 15 minutes.
7. Poured the autoclaved medium cooled to 45-50 ºC into sterile Petri plates and allow the media to solidify.
8. The appropriately labeled plates were inoculated with the respective organism.
9. Incubated the inoculated plates at 35 ºC for 2-5 days.
10. The plates were flooded with 1 % aqueous solution of hexadecyltrimethyl ammonium bromide and the plates were observed for the formation of a zone around the growth.

3.1.9.1.8 Urease test

Requirements

1. **Urea agar medium**

Ingredients	**gms/litre**
Peptic digest of animal tissue	1.000
Dextrose	1.000
Sodium chloride	5.000

Disodium phosphate	1.200
Monopotassium phosphate	0.800
Phenol red	0.012
Agar	15.000
Final pH	6.8 ± 0.2

Medium was sterilized by autoclaving at 10 lbs pressure (115 °C) for 20 minutes. Allowed to cool to 50 °C and aseptically added 50 ml of sterile 40 % urea solution (FD048) and mix well. Dispensed into sterile tubes and allowed to set in the slanting position.

Procedure

1. Inoculated the urea agar slants with test organism.
2. Incubated, inoculated slants for 24-48 hours at 37 °C.
3. Examined the slants as to their color for the presence of urease (red or cerise color) and for no urease (yellow color).

3.1.9.1.9 Fermentation of carbohydrates

The fermentation broth contains ingredients of nutrient broth, a specific carbohydrate and a pH indicator (phenol red), which is red at a neutral pH-7 and turns yellow at or below a pH of 6.8 due to the production of an organic acid.

Procedure

1. Preparation of fermentation medium

Peptone	10.0 g
Carbohydrates	5.0 g
Sodium chloride	15.0 g

Phenol red	0.018 g
Distilled water	1000.0 ml
pH	7.3

(Carbohydrates used were glucose, fructose, lactose and sucrose)

2. Broth was taken into fermentation tubes and Durham tubes were added in inverted position, was autoclaved at 12 lb pressure for 15 minutes.
3. Inoculated the four types of sugar fermentation broth with tested organism and kept one uninoculated tube of each fermentation broth as a comparative control. Incubated all the inoculated and uninoculated tubes at 35 °C for 24-48 hours.
4. Observed the reaction that developed in the inoculated tubes compared to un-inoculated tubes i.e. changed in color (due to production of acid) or changed in color and appearance of bubbles (due to production of acid and gas).

3.1.9.1.10 IMViC tests

The IMViC test consists of four different tests (1) indole production (2) methyl-red (3) Voges-Proskauer and (4) citrate utilization.

3.1.9.1.10.1 Indole production test

1. Preparation of (1 %) tryptone broth: Dissolved 10 g of tryptophan in one litre of distilled water. Sterilized in the autoclave at 15 psi (121 °C) for 15 minutes. Inoculated the broth with test organism and incubated at 35 ºC for 48 hours.
2. After 48 hours incubation, 1 ml of Kovacs reagent was added to each tube including control.
3. The tubes were shacked gently after intervals of 10-15 minutes.
4. The tubes were allowed to stand to permit the reagent to come to the top.

5. Development of a cherry (deep) red color in the top layer of the tube was a positive test for indole production. Absence of red coloration was indole negative.

3.1.9.1.10.2 Methyl-Red and Voges-Proskauer testes

Requirements

1. **MR-VP broth**
2. **Methyl red pH indicator**
3. **VP reagent I (napthol solution)**
4. **VP reagent II (40% potassium hydroxide)**

Procedure

1. Preparation of MR-VP broth (pH 6.9) tubes

Peptone	7.0 g
Dextrose	5.0 g
Potassium phosphate	5.0 g
Distilled water	1000.0 ml

   Poured the 5 ml broth in each tube and sterilized by autoclaving at 15 lb pressure for 15 minutes.
2. Inoculated the MRVP tubes with test organism and kept one tube as uninoculated comparative control.
3. Incubated all the tubes at 35 ºC for 48 hours.
4. Added 5 drops of methyl red indicator to the tubes of each set.
5. Observed the change in color of methyl red for MR test.
6. Added 12 drops of V-P reagent and 2-3 drops of V-P reagent II to the other set of tubes as well as to un-inoculated control tubes.

7. The tubes were shacked gently for 30 seconds with the caps off to exposed the media to oxygen.
8. The reaction was allowed to complete for 15-30 minute. Observed the tubes for changed in color for the VP test.
9. In MR test, the methyl red indicator in the pH range of 4 was remained red which was indicative of positive test, while turning of methyl red to yellow was a negative test.
10. In VP test, the development of crimson to ruby pink color was indicative of positive VP test while no change in coloration was a negative test.

3.1.9.1.10.3 Citrate utilization test

1. Preparation of Simmons citrate agar (pH 6.9) slants

Ammonium dihydrogen phosphate	1.0 g
Dipotassium phosphate	1.0 g
Sodium chloride	5.0 g
Sodium citrate	2.0 g
Magnesium sulphate	0.2 g
Agar	15.0 g
Bromothymol blue	0.8 g
Distilled water	1000.0 ml

Poured the medium in culture tubes and sterilized by autoclaving at 15 lb pressure for 15 minutes and the slants were prepared.

2. Inoculated the Simmons citrate agar slants, with test organisms, and incubated at 37 ºC for 48 hours.
3. After incubation, growth was visible on the surface and the medium color was blue showed citrate positive results, there was no growth and no change in the color of the medium showed citrate negative results.

3.1.9.1.11 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 12 antibiotic discs (HK001-1PK, Himedia, Mumbai, India) were used to test the sensitivity of the bacterial isolates. These were: Penicillin G (10 U), Cephalothin (30 mcg), Clindamycin (2 mcg), Erythromycin (15 mcg), Amoxyclav (30 mcg), Vancomycin (30 mcg), Ofloxacin (5 mcg), Teicoplanin (30 mcg), Ceftazidime (30 mcg), Gentamicin (10 mcg), Cephoxithin (30 mcg), Oxacillin (1 mcg).

3.1.9.2 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 2006) and further confirmed by ITS region sequence analysis of the ribosomal RNA gene.

3.1.10 Molecular methods for identification of P-solubilizing microorganisms

3.1.10.1 Isolation of genomic DNA from P-solubilizing bacteria

1. 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).
2. 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.

3. Resuspended each cell pellet in approximately 0.8 ml saline-EDTA buffer thoroughly. 50 µl of freshly prepared lysozyme solution was added and mixed well. Incubated at 37 ºC for 20 minutes.
4. Added 0.2 ml 10 % SDS, mixed well by inversion, and incubated in water bath at 60 ºC for 15 minutes.
5. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed well by inversion, and centrifuged at 10000 rpm at 4 °C for 10 min.
6. Upper aqueous phase was transferred to the sterile microfuge tube. 0.6 volume of ice cold isopropanol was added and gently mixed to precipitate DNA.
7. After centrifugation at 13000 rpm for 10 min., the supernatant was discarded and the pellet was washed twice with 70 % ethanol to remove salts.
8. 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.

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

1. All the fungal isolates were grown in the Pikovskaya's broth at 30 °C using incubator shaker at 130 rpm**.**
2. After 5 days of growth, the culture was washed with sterile distilled water through Whatman filter paper no. 42 up to the medium was removed from the mycelium.
3. The mycelia culture was pressed with the help of filter paper to remove the water and kept it at -80 °C for further use.
4. Mycelium was taken into the motor, added liquid nitrogen and crushed with the help of pestle.

5. Transferred the powder in sterilled eppendorf. Kept it at -20 °C or -80 °C.
6. A 1.5 ml of centrifuge tube was filled 1/3rd with freeze-dried mycelium powder.
7. Then 0.5 ml extraction buffer (Appendix I) (preheated at 65 °C) was added and mixed well and let stand for 15-20 minutes at 65 °C in water bath. (Vortex, if necessary added 4 µl of RNase (200 µg/ml).
8. Then 0.5 ml of equilibrated phenol was added and mixed well and left for 15 minutes at room temperature.
9. It was added with 0.5 ml of Sevag (chloroform: isoamyl alcohol; 24:1) followed by mixing. Then centrifuged tubes were incubated at room temperature for 15 minutes and centrifuged at 10,000 rpm for 10 minutes.
10. The upper aqueous layer was removed and transferred to new centrifuged tube.
11. Then 400 µl of Sevag was added and mixed by inverting gently and centrifuged for 10 minutes at 10,000 rpm and transferred supernatant to a new centrifuged tube.
12. Then 0.54 volumes of isopropanol was added to precipitate DNA and incubated at -20 °C for 20 minutes and centrifuged at 10,000 rpm for 10 minutes.
13. The pellet was washed with 100 µl of 70 % ethanol.
14. 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).Centrifuged at 10000 rpm at 4 ºC for 10 minutes and transferred the supernatant to fresh eppendorf.
15. Added 0.55 volume of isopropanol to precipitate the DNA. Immediately centrifuged at 10,000 rpm for 5minutes at 4 ºC.
16. Decanted the supernatant and washed the pallet twice with ice cold 70% ethanol to remove salts. Air dried the pellet and dissolved in 30 µl of Tris-EDTA (pH 8.0) buffer. Stored at 4°C (or -20°C) for further use.

3.1.10.3 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.

The concentration of DNA was determined using a Nano Drop 1000 spectrophotometer (Thrmo scientific, Wilmington, DE) and quality of DNA was evaluated by measurement of the A260 and A280 and the A230/A260 ratios. Ideally, the A260/A280 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.

3.1.10.4 Amplification of 16S rRNA

For amplification of 1.5 kb gene of 16S rRNA, the primers used were: (Forward primer 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer 5'-ACG GGC GGT GTG TTC-3' (Weisberg et al., 1991). DNA amplification was performed with GenAmp 2700 thermocycler (Applied Biosystem, USA). Reaction mixture for the PCR contained 1x PCR buffer (Fermentas, USA), 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 50 µl. PCR conditions for amplification of 1.5 kb fragment of 16S rRNA were as follows: Preheating at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 55 °C for 0.30 min and 72 °C for 0.30 min and final extension 72 °C for 7 min. Amplified DNA was verified by electrophoresis of aliquots of PCR product (5µl) on a 1.2% agarose gel in 0.5 % TBE buffer.

3.1.10.5 Amplification of ITS region

Fragments of the ITS1-5.8S-ITS2 of the rRNA from genomic DNA were amplified by the PCR using the primers ITS1 5'-TCC GTA GGT GAA CCT GCG G-3' and ITS4 5'-TCC TCC GCT TAT TGA TAT GC-3' as described by White et al. (1990). The 50 µl reaction mixture for PCR amplification contained the following: 40 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 2700 thermocycler (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.

3.1.10.6 Restriction analysis of DNA samples by agarose gel electrophoresis

1. The 10 µl DNA solution (0.2-1.0 µg of given DNA in a reaction volume of 20 µl) was taken in a sterile microfuge tube and added sterile MQ water to make up the final volume of 17 µl.
2. The 2 µl of appropriate 10x restriction enzyme assay buffer was added and mixed thoroughly by tapping the tube.
3. 1 µl (2-5 units) of the restriction enzyme was added, mixed by tapping the tube.
4. The mixture was incubated at the appropriate temperature for 2-3 h.
5. 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).

6. ITS-PCR products were digested with the restriction enzyme *RsaI* and 16S rRNA products were digested with the restriction enzymes *Alu1, Mbo1, Rsa1* and *Taq1* as per manufacturer‘s instructions.
7. The digested DNA was run through 1.5 % (w/v) agarose gels containing ethidium bromide and visualized and photographed using Gel Doc system.

3.1.10.7 Purification of PCR products

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 matrix material, using the QIAquick gel extraction kit (Qiagen Inc., USA) as per manufacturer‘s instructions. Purified PCR products were eluted with 30 µl TE buffer (pH 8.0) and used for the cloning

3.1.10.8 Ligation of 16S rRNA and ITS in TA cloning vector pTZ57R/T

The 16S rRNA and ITS PCR products were cloned using the restriction independent InsTA Cloning Kit, following the manufacturer’s protocol (Fermentas, USA). The 16S rRNA or ITS amplicon was ligated into pTZ57R/T vector. The reaction mixture was prepared as described below and incubated overnight at 4 °C.

Plasmid pTZ57R/T (55 ng/µl)	3 µl
Insert (75 ng/µl)	4 µl
Buffer (5x)	6 µl
T4 Ligase	1 µl
MQ water	16 µl

3.1.10.9 Genetic transformation using $CaCl_2$ method

1. A single colony of *E. coli* DH5α was picked from a freshly grown plate and transferred into 20 ml of Luria 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).
2. 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 $OD_{590\ nm}$ every one-hour (The $OD_{590\ nm}$ should be ~ 0.5).
3. 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.
4. The cells were recovered by centrifugation at 5000 rpm for 10 min at 4 °C. Decant the media from the cell pellet. 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. Decant the fluid from the cell pellet, the tube was kept in an inverted position for 1 min to allow the last traces of fluid to drain away.
5. Cell pellet was resuspended the in 1 ml of ice-cold 0.1 M $CaCl_2$. The cells in this stage may be stored on ice for 12-24 hours. $CaCl_2$ was mixed very gently with the pellet, do not vortex and placed in the ice for 10-15 min, centrifuged at 5000 rpm for 10 minutes, discard the supernatant.
6. One ml of ice chilled $CaCl_2$ was added to the pellet, mixed properly and gently. $CaCl_2$ treatment for 2½ hours induced considerably a transient state of competence in the *E. coli* cells.

7. 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.). The content of the tubes were mixed gently and stored in ice for 30 minutes.
8. Transferred the tubes to a floater and were placed in a water bath that was preheated to 42 °C. The tubes were left for exactly 2 min without shaking. Rapidly transferred the tubes to an ice bath and cells were chilled for 2 minutes.
9. Now added 1 ml of LB broth to each tube and incubated the cultures for 45 minutes in incubator shaker at 37 °C. This will allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.
10. Transferred the appropriate volume (100 µl) of transformed competent cells onto LB supplemented ampicillin (50 µg/ml) agar plates, which were spread with 20 µl of 100 mM IPTG (isopropyl beta-D-thiogalactopyranoside) and 40 µl of 20 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.

3.1.10.10 Blue/white screening for recombinant plasmids

After transformation of the ligated product, the *E. coli* DH5α (LacZ) bacterial host cells were plated on Luria agar 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 was done using vector's promoter specific sequences.

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

1. A single transformed *E. coli* white 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.
2. 1.5-2.0 ml of the above-saturated culture was transferred into a microfuge tube and cells were harvested by centrifugation at 8000 rpm for 1 min in a microfuge.
3. The medium was removed, leaving the bacterial pellet as dry as possible.
4. The bacterial pellet was resuspended in 200 µl of ice-cold Solution I (Appendix I) by vortexing to ensure that the bacterial pellet is completely dispersed in this solution. Placed as such at room temperature for 3 min.
5. Further 200 µl of freshly prepared solution II (Appendix I) was added. Tubes were closed tightly and mixed the contents by gently inverting the tubes ten to twenty times for 30 seconds. Store the tubes on ice for 3 minutes.
6. Finally 300 µl of ice cold Solution III (Appendix I) added and mixed by inversion to disperse Solution III through the viscous bacterial lysate. The tubes 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.
7. Added 400 µl chilled phenol:chloroform:isoamylalcohol mixture (25:24:1) for protein denaturation and centrifuged it for 10 minutes at 10000 rpm.

8. Took the supernatant in another tube and DNA was precipitated by adding equal volume of isopropanol.
9. 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.
10. 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.
11. The pellet was washed with 70 % ethanol, centrifuged at 8000 rpm for 5 min.
12. Air-dried the pellet and dissolved in 30 µl of TE buffer (pH 8.0). The DNA was stored at -20 °C for further use.

3.1.10.12 Size screening for recombinant plasmids

Clones containing approximately 1.5 kb 16S rRNA or 600 bp ITS inserts were identified by PCR screening using the rapid protocol for preparation of template DNA from single bacterial colonies and PCR amplification was done by using 16S rRNA and ITS primers. The amplification products were checked by agarose gel (1.0 % w/v) electrophoresis.

3.1.10.13 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).

3.1.10.14 DNA sequence analysis

The bacterial 16S rRNA sequences were compared against the available DNA sequences in Ez Taxon-e database (http://eztaxon-e-ezbiocloud.net) (Kim et al. 2012). In case of bacteria, the phylogenetic tree (Neighbor Joining) was constructed using the MEGA 5.1 (Tamura et al.

2011) software and the bootstrap values were inferred from 1000 replicates. In fungi, Nucleotide sequence comparisons were performed using the BLAST database (Altschul et al. 1997) and phylogenetic tree (Maximum Parsimony analysis) was constructed using the MEGA 5.1.

3.2 Physiological characterization phosphate-solubilizing microorganisms

3.2.1 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 fructose or arabinose or galactose or mannitol or maltose or lactose or sucrose or xylose sterilized separately and added to the Pikovskaya's broth [C source, 10.0 g; $(NH)_2SO_4$, 0.5 g; NaCl, 0.2 g; $MgSO_4.7H_2O$, 0.1 g; KCl, 0.2 g; Yeast extract, 0.5 g; $MnSO_4$, 0.1 mg; $FeSO_4.7H_2O$, 0.1 mg; tri-calcium phosphate (TCP), 2.19 g (equivalent to 100 mg P_2O_5/100 ml); water, 1000 ml; pH 7.2 ± 0.2]. Nitrogen sources were evaluated similarly by replacing ammonium sulfate with 0.5 gl^{-1}; $NaNO_3$ or NH_4NO_3 or $NaNO_2$ or KNO_3 or tryptophan or NH_4Cl or Urea. The flasks were incubated at 30 °C under shaking conditions 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 100 ml of medium. Acid phosphatase, alkaline phosphatase, phytase enzyme activity and organic acid were also estimated in the culture supernatant of bacteria and fungi.

3.2.2 Plant growth promotion activities

3.2.2.1 Indole acetic acid production

All the selected bacterial and fungal isolates were screened for indole acetic acid (IAA) production. Gorden and Paleg (1957) colorimeteric method was used for quantitative measurement of IAA.

Reagents

1. **0.5 M $FeCl_3$:** Dissolved 810 mg $FeCl_3$ in 10 ml distilled water
2. **35 % $HClO_4$:** 50 ml $HClO_4$ (70%) was mixed with 50 ml of distilled water
3. **Tryptophan:** 0.1 % w/v
4. **Salpar's reagent:** Mixed 1 ml of 0.5 M $FeCl_3$ with 50 ml of 35 % v/v $HClO_4$. This reagent should be freshly prepared.
5. **Czapek's Dox broth**

Components	**g/l**
$NaNO_3$	3.0
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.5
KCl	0.5
$FeCl_2$	Trace
Sucrose	30.0
pH	7.3

Tryptophan medium was prepared by omission of sodium nitrate from the Czapek Dox broth and substitution of 0.1 % L. tryptophan as a sole source of nitrogen.

Procedure

1. 100 µl of the bacterial inoculum ($O.D._{600}$ =0.8) was inoculated in 20 ml nutrient broth, both with and without tryptophan (0.1%) and incubated at 30 °C for 5 days at 130 rpm.
2. 2 ml of the culture was taken and centrifuged at 10,000 rpm for10 min.
3. In case of fungi 5 mm mycelia disc of each fungal isolate was cut aseptically and transferred to 50 ml of Czapek Dox broth contained in 250 ml conical flask. Flasks were incubated at 30 °C for 5 days on incubator shaker at 130 rpm.
4. Culture was centrifuged at 10000 rpm for 10 min and supernatant was filtered through whatman no. 1 filter paper.
5. 1 ml of supernatant of each isolate was taken in separate test tubes and 2 ml salper's reagent was added drop wise but rapidly with continuous mixing in each tube.
6. The samples were placed in dark for 30 minutes.
7. Development of pink color was assayed with spectrophotometer at 535 nm.
8. The un-inoculated medium was used as blank and amount of IAA production was estimated from the standard curve of IAA (0-50 ppm) drawn against O.D.

3.2.2.2 HCN production activity

HCN production was tested by the method of Bakker and Schippers (1987).

Procedure

1. Bacterial cultures were inoculated on Nutrient agar plates and fungal cultures were inoculated on Czapek Dox agar plates supplemented with 4.4 g/l Glycine.
2. A whatman filter paper soaked in 2 % w/v sodium carbonate in 0.5% (w/v) picric acid solution was plated inside the lid of a petriplates.
3. The plates were then sealed with parafilm and incubated at 30 °C for 3 days.

4. A change in the filter paper color from yellow to reddish brown was considered to be an indicator of HCN production.

3.2.2.3 Siderophore production

Siderophore production ability of isolates was detected by using Chrome-Azurol 'S' (CAS) Agar medium (Schwyn and Neilands 1987).

Reagents

1. **Preparation of CAS Dye**

- Dissolved 60.5 mg of Chrome-Azurol 'S' dye in 50 ml distilled water and constantly mix 1mM $FeCl_3.6H_2O$ solution prepared in 10 ml 10 mM HCl. It results dark reddish color.
- Dissolved 72.9 mg of Cetyl trimethyl ammonium bromide into 40 ml of distilled water and mix slowly into CAS dye solution under constant stirring before autoclave. This results in dark blue color.

2. **Preparation of CAS Agar Medium**

- Mix 100 ml CAS dye in 300 ml sterile nutrient agar before pouring check pH, if below 7.0 maintain with NaOH (autoclaved).

Procedure

1. PSB isolates and fungal isolates were spot inculcated on CAS agar plates.
2. Incubated the plates at 28 °C for 3 days.
3. Isolates exhibiting an orange/yellow halo zone were considered as siderophore producers.
4. Their diameter of zone was measured.

3.2.2.4 Detection of type of siderophore (Neilands 1981)

Reagents

1. **$FeCl_2$ (2 %)**
2. **CAS broth:**

Components	**g/l**
Succinic acid	4.0
K_2HPO_4	3.0
$(NH_4)_2SO_4.H_20$	0.2
CAS dye	100 ml
pH	7.0

Procedure

1. 100 µl of the bacterial culture ($O.D._{600\ nm}$ = 0.8) was inoculated in 20 ml CAS both and incubated at 30 °C for 5 days at 130 rpm.
2. In case of fungi 5 mm mycelia disc of each fungal isolate was cut aseptically and transferred to 20 ml CAS broth contained in 250 ml conical flask. Flasks were incubated at 30 °C for 5 days on incubator shaker at 130 rpm.
3. After five days of incubation culture was centrifuged at 10,000 rpm for 10 min.
4. I ml of culture supernatant was added to 3 ml freshly prepared 2 % aqueous $FeCl_3$ solution and absorbance between 200 – 600 nm was recorded.
5. A peak at 420-450 nm indicated the presence of ferrate hydroxamate siderophores and a peak at 495 nm indicated the catecholate nature of siderophores.

3.2.2.5 Nitrogen fixation ability

The ability of the bacteria to fix di-nitrogen was measured based on acetylene reduction activity as described in Hardy et al (1973).

Jensen broth (Jensen 1942)

Ingredients	**gms/litre**
Sucrose	20.00
Dipotassium phosphate	1.000
Magnesium sulphate	0.500
Sodium chloride	0.500
Ferrous sulphate	0.100
Sodium molybdate	0.005
Calcium carbonate	2.000
Distilled water	1000 ml

Procedure

1. The bacterial culture (100 µl of $O.D._{600\ nm}$ = 1.0) was inoculated in 10 ml of Jensen broth in test tubes and incubated at 30 °C at 130 rpm on rotary shaker.
2. After three days of incubation, the cotton plugs were replaced with a rubber stopper.
3. One ml of air was removed from the tube with a syringe and one ml of acetylene (0.1 atm) was injected into the tubes which were then incubated for 24 hours.
4. One ml of the gas was sampled and the ethylene concentration was measured by gas chromatography.
5. Ethylene analysis was accomplished by gas chromatography (Nucon GC-FID, model no 5900) equipped with a hydrogen flame ionization detector (FID). Instrument

operating conditions were as follows: a stainless-steel column 150×0.2 cm packed with Porapak N, 80/100, a column temperature of 60 °C, an injector temperature of 50 °C, a detector temperature of 200 °C, N_2 carrier gas and H_2 at a flow rate of 25 ml/min, and air flow rate of 300 ml/min.

3.2.3 Effect of buffering of media on phosphate solubilization

To study the effect of buffering condition on P-solubilization by phosphate-solubilizing bacteria, bacterial isolates were grown in PKV broth supplemented with 100 mg P_2O_5 per 100 ml of medium. For the buffering the media 100 mM Tris-HCl of pH 7.0, 8.0 and 9.0 was used. In another set pH of this medium was adjusted to 7.0, 8.0 and 9.0 with 100 mM NaOH. The flasks were incubated at 30 °C under shaking conditions for fifteen days. The media was analyzed for soluble P and pH reduction. Acid phosphatase, alkaline phosphatase and phytase enzyme activities were also estimated in the culture supernatant.

3.3 Inoculum formulations for field applications with respect to phosphate solubilization efficiency and shelf life

3.3.1 Field experiment

To check the effect of selected isolates on crop production and soil fertility, field experiments of maize and wheat crop were conducted at three different agroclimatic regions of Punjab, India.

One experiment was conducted at Thapar University Patiala, Punjab. This site was situated at 30.30° N latitude and 76.38° E longitude. The site falls under central plain agroclimatic region of Punjab. Soil was low in organic carbon level and in available P level. The region possessed tropical hot and dry climatic conditions characterized by very hot and dry in summer and very cold in winter. The soil in experimental site belongs to typic Ustifluvents,

sandy loam in texture with the following characteristics: pH - 8.27, electric conductivity - 0.17 $mScm^{-1}$, organic carbon - 0.33%, available P - 3.90 mg kg^{-1}, total P - 251 mg kg^{-1}, organic P - 138 mg kg^{-1} and total nitrogen - 0.020 %.

Second site was an agricultural field of Balachaur, Punjab, situated at 31.07° N latitude and 76.32° E longitude. The region falls under sub-mountain undulating central agroclimatic region of Punjab state and possessed dry sub humid type of climate. Soil was low in organic carbon level and in available P level. The soil in experimental site belongs to typic Ustifluvents, sandy loam in texture with the following characteristics: pH - 8.18, electric conductivity - 0.14 $mScm^{-1}$, organic carbon - 0.31 %, available P - 3.6 mg kg^{-1}, total P - 237 mg kg^{-1}, organic P - 124 mg kg^{-1} and total nitrogen - 0.019 %.

Third site an organic field located at Pojewal, Punjab. Organic field used in this study was a field where from last ten years no chemical fertilizer was used. Mainly animal manure, vermi-compost and green manure were used to maintain the soil fertility. The site is situated at 31.65° N latitude and 76.26° E longitude and falls under sub-mountain undulating agroclimatic region of Punjab state. The region possessed dry sub humid type of climate. Soil was medium in organic carbon level and low in available P level. The soil in experimental site belongs to typic Ustorthents, loamy sand in texture with the following characteristics: pH - 8.37, electric conductivity - 0.18 $mScm^{-1}$, organic carbon - 0.42 %, available P – 4.3 mg kg^{-1}, total P - 245 mg kg^{-1}, organic P – 207 mg kg^{-1} and total nitrogen - 0.035 %. All three sites used in this study were comes under different agroclimatic regions and were different in physiochemical properties of soil like in comparison to soil fertility Balachaur come under less fertile soil in comparison to Patiala and Pojewal (organic farm). Bio inoculation was done as seed inoculation.

3.3.2 Seed inoculation

Seed inoculation of phosphate-solubilizing bacteria and fungi was done by mixing the bacterial culture and fungal spores in 10 percent sugar and 40 percent gum arabic to form slurry to which seeds were added. A uniform coat of inoculum was formed around the seeds. The inoculated seeds were dried in shade and used for sowing.

3.3.2.1 Methodology for preparation of inoculum

1. Phosphate-solubilizing bacterial culture was grown in 500 ml PKV broth and incubated at 30 °C for three days at 130 rpm.
2. Centrifuged the culture ($O.D._{600\ nm}$ 1.0) at 8000 rpm for 10 min and pellet was washed three time with sterile distilled water and finally suspended in sterile distilled water to make the suspension.
3. Fungal culture was grown in PKV agar plates for five days at 30 °C.
4. Using a scalpel fungal growth was scraped from the plates and suspended in sterile distilled water.
5. For fungal consortium (FC) the scraped spores of both the fungi which were of equal quantity, were mixed in sterile distilled water. To prepare bacterial consortium (BC) both the selected bacterial isolates were grown separately to a 1.0 $OD_{600\ nm}$, centrifuged at 8000 rpm for 10 minutes and cell pellets were mixed in sterile distilled water.
6. 50 g sugar was taken in 500 ml water and heat the sugar solution for 15 min.
7. 200 g gum arabic was added to hot sugar solution and the solution was allowed to cool at room temperature.
8. Mix the fungal and bacterial inoculum separately in above solution to form a slurry.

9. For seed inoculation, surface sterilization of seeds was performed by dipping them in 95 % ethanol for 3 min followed by 3 % sodium hypochlorite for five min and subsequently washing with sterile distilled water followed by treatment with slurry.
10. Seeds were added to the inoculums slurry and mixed properly to make a uniform coat of inoculums on the seeds.
11. Surface sterilized seeds treated with 40 % gum arabic and 10 % sugar solution that did not contain inoculum served as a control.
12. Seeds were dried in shade for two hours and were sown in the field.
13. Check the seeds for inoculation density by serial dilution method on PKV agar plates.

3.3.3 Experimental field preparation and cultivation practices

Field trials were conducted at three different sites comes under different agroclimatic regions. Field trials were conducted in a completely randomized block design; each plot size was 4 m × 4 m (16 m^2) and included 15 treatments each with three replicates.

Treatments consisted of:

Soil;

Soil + *Pantoea cypripedii* (PSB-3);

Soil + *Pseudomonas plecoglossicida* (PSB-5);

Soil + *Aspergillus tubingensis* (PSF-4);

Soil + *Aspergillus niger* (PSF-7);

Soil + BC (*Pantoea cypripedii* + *Pseudomonas plecoglossicida*);

Soil + FC (*Aspergillus tubingensis* + *Aspergillus niger*);

Soil + RP;

Soil + RP + *Pantoea cypripedii* (PSB-3);

Soil + RP + *Pseudomonas plecoglossicida* (PSB-5);

Soil + RP + *Aspergillus tubingensis* (PSF-4);

Soil + RP + *Aspergillus niger* (PSF-7);

Soil + RP + BC (*Pantoea cypripedii* + *Pseudomonas plecoglossicida*);

Soil + RP + FC (*Aspergillus tubingensis* + *Aspergillus niger*);

Soil + Di-ammonium phosphate (DAP) (chemical P fertilizer).

During first year of field study, maize variety DKC-9106 (20 kg/ha) was cultivated in the rainy season. Rock phosphate was amended in respective plots at the rate of 59 kg P_2O_5 ha^{-1} before seeding only once during maize cropping. Di-ammonium phosphate (DAP) at a rate of 59 kg P_2O_5 ha^{-1} was added in chemical fertilizer treatment at the time of sowing of each crop. Inoculum was added as a seed treatment. At the time of seeding, size of bacterial inoculum per maize seed was 2.5- 3.0×10^5 cfu per seed and size of fungal inoculums per maize seed was 2.3-2.9 $\times10^5$ cfu per seed. All the plots were irrigated once before the sowing to ensure proper germination of seeds and then regularly during crop growth as per agronomic practices. Nitrogen fertilizer of 272 kg/ha was used in the form of urea as per agronomic practices. No other chemical fertilizer was applied to the crop.

Wheat variety PBW-621 (99 kg/ha) was sown in the same field after one year to check the effect of inoculum on wheat crop in second year. The field was tilled without disturbing the experimental design. At the time of sowing a booster dose of bio-inoculant was added as seed treatment, size of the bacterial inoculum per wheat seed was 1.7-2.0×10^5 cfu and size of fungal inoculums per seed was 1.5-2.0 cfu. Urea was added at the rate of 272 kg/ha as per agronomic practices. DAP was added at a rate of 59 kg P_2O_5/ha in chemical fertilizer treatment at the time of sowing and no rock phosphate was added in this crop to check the effects of previously added (in maize field) RP on wheat crop growth in second year of field study. In the both maize and wheat crops field trails in chemical fertilizer treatments where

DAP was used at the rate of 129 kg/ha (59 kg P_2O_5/ha) reduced the urea dose by 47 kg/ha because DAP already contained 18 % nitrogen (N).

3.3.4 Plant and soil analysis after harvesting

At the time of harvesting of both the crops, from each plot, 10 randomly selected plants were uprooted and the root surface was cleaned several times with distilled water. Growth parameters such as shoot height, shoot and root dry biomass were measured and recorded. During each crop harvest, entire observation plot (4 m × 4 m (16 m^2)) of each treatment was harvested to record the grain yield. After harvesting root, shoot and seed samples of ten randomly selected plants of each plot were oven dried at 65 °C for 72 hours and were grounded to pass through a 0.5 mm sieve and analyzed for total P content. Total phosphorus content in plant roots, shoots and seeds were determined by vanado-molybdophosphoric yellow color method described by Kitson and Mellon (1944). After harvesting in order to assess the effect of different treatments on physiochemical properties of rhizospheric soil, soil samples to the depth of 5-10 cm were drawn carefully from rhizosphere of 10 randomly selected plants from each plot and homogeneous composite sample was prepared for each plot. Rhizospheric soil samples were stored at 4 ºC and analyzed within week for soil enzyme activities and for phosphate solubilizing bacterial and fungal population. For physiochemical analysis soil samples were air dried under shade and then passed through 2.0 mm sieve and stored in labeled polythene bag before analysis. Soil samples of each plot were analyzed for its pH, electric conductivity, TDS, organic carbon (Walkley and Black 1934), available P (Olsen 1954), total phosphorus (Kitson and Mellon 1944), Total nitrogen (Piper 1966), acid and alkaline phosphatase activity (Tabatabai and Bremner 1969), phytase activity (Heinonen and Lahti 1981), dehydrogenase enzyme activity (Casida 1977) and phosphate-solubilizing bacterial and fungal population in respective plots by serial dilution method on Pikovskaya's agar plates.

3.3.5 Plant and soil analytical procedures

3.3.5.1 Determination of soil pH

Procedure

1. 25g of air dried soil samples were weighed and taken in a 100 ml beaker.
2. Added 50 ml of distilled water and thoroughly stirred for 2-3 min using a glass rod.
3. Further, it was kept in shaking condition (130 rpm) for 2 hours.
4. Suspension was allowed to settle down for 30 min.
5. Mean while, pH meter was switched on and checked with two buffer solutions of known pH (pH 9.0 and pH 4.0) with the help of standardization knob.
6. The pH of sample was measured by immersing the electrode in supernatant solution and recorded when the reading was stabilized (usually after 30 sec).
7. The electrode was rinsed with distilled water and carefully wiped with filter paper for every sample.

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

The TDS and electric conductivity (EC) was determined in 1:2 (w/v) soil: water suspension using Deluxe Water and Soil Analysis Kit (Model 191 E).

Reagent

1. **KCl solution:** Dissolved 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 $mScm^{-1}$ at 25 °C. ($mScm^{-1}$, which is equal to mmho cm^{-1} (1 mho = 1 Siemen). The TDS of this solution is 650 ppm.

Procedure

1. Weighed 25 g soil and transferred it to a 100 ml beaker.
2. Added 50 ml distilled water to it.
3. 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/TDS measurement).
4. In the meanwhile, switch on the EC/TDS meter and allowed it to warm for 20 minutes.
5. KCl solution (EC=1mS cm^{-1}) was used to calibrate the meter.
6. The electrode was dipped in the supernatant solution and recorded the reading displayed for EC and TDS.

3.3.5.3 Available phosphorus (P)

Available phosphorus in the alkaline soil was estimated as per the method given by Olsen et al. 1954.

Reagents

1. **0.5 M $NaHCO_3$ extracting solution:** 84 g of sodium bicarbonate was added in distilled water and volume was made up to 2 liter. The pH was adjusted to 8.5 with 1M or 1N NaOH.
2. **Reagent A:** 12.0 g ammonium molybdate in 250 ml distilled water and 0.2908 g antimony potassium tartarate in 100 ml distilled water was added to 1000 ml of 2.5 M H_2SO_4, mixed thoroughly and volume was made up to 2 liter with distilled water.
3. **Reagent B (freshly prepared):** 1.058 g of ascorbic acid was added in 200 ml of reagent A and mixed.
4. **Sulphuric acid (2.5 M):** 140 ml of conc. H_2SO_4 was diluted to 1 liter.

5. **Stock standard P solution (50 ppm):** 0.2917 KH_2PO_4 was dissolved in distilled water to a final volume of 1 liter.
6. **Working standard P solution (1 ppm):** 20 ml of 50 ppm solution was diluted to 1 liter.

Procedure

1. 2.5 g soil was weighed and 50 ml of extracting solution was added to it.
2. Kept on a shaker for 30 minutes and was filtered through whatman filter paper no. 42.
3. 10 ml aliquot of filtrate was transferred to a 100 ml beaker.
4. 1 ml of 2.5 M H_2SO_4, 15.5 ml distilled water, 8 ml reagent B and again 15.5 ml of distilled water was added.
5. After 10 minutes, the intensity of the color was measured at 882 nm against blank.
6. Blank was prepared as above without the soil.
7. To prepare standard curve, 0, 2, 5, 10, 15 and 20 ml of 1 ppm working standard solution was added in 50 ml volumetric flasks separately. Added 10 ml of extracting solution, 1.0 ml of 2.5 ml H_2SO_4, 8 ml Reagent B and final volume was made up to 50 ml. The P concentrations of these solutions were 0.04 ppm, 0.1 ppm, 0.2 ppm, 0.3 ppm and 0.4 ppm respectively. After 10 min read the P concentration at 882 nm.

Calculation

Available P in soil (ppm): P in extract (ppm) × 20 (standard soil to solution ratio)

3.3.5.4 Sample preparation for elemental analysis

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

3.3.5.4.1 HNO_3/ $HCLO_4$ digestion

1. 1 g sample of air dried soil was weighed in digestion tube and added 10 ml concentrated HNO_3 digest on electric heater for 1hr at 145 °C in acid proof digestion chamber having fume exhaust system.
2. Allowed to cool it and 10 ml concentrated HNO_3 and 5 ml $HClO_4$ was added and heated at about 100 °C for the first one hour and then raised the temperature to about 200 °C.
3. Continued the digestion until the contents become colorless and only white fumes appeared.
4. Reduced the acid contents till white matter remains left in the digestion tube.
5. After it removed from the heating mental and cooled and added 50 % diluted HCl and filtered through whatman filter paper no. 42.
6. 2 or 3 washings with 50 % diluted HCl was given and final volume made was 50 ml with diluted 50 % HCl.
7. This was used to determine total phosphorus.

3.3.5.4.2 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 color is proportional to phosphate concentration.

Reagents

1. **Vanadomolybdate solution:**

   Solution A - 25 g ammonium molybdate [$(NH_4)_6 Mo_7O_{24}.4H_2O$] was dissolved 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. Solution A was added to solution B and was made up to 1000 ml in a volumetric flask.

2. **Phosphorus stock standard solution (50 mg/l 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 1 liter to get 50 mg/ml P solution. 4 to 5 drops of toluene were added to prevent microbial activity. (KH_2PO_4 was dried to 100 °C for 1 hour and cooled in desiccators before weighing)

Procedure

1. 10 ml of acid digests of soil or plant sample was placed in 50 ml volumetric flask, 10 ml of the vanado molybdate reagent was added and diluted to 50 ml.
2. Mixed well and read the phosphorus concentration after 10 minutes using spectrophotometer at 420 nm.
3. Took 0, 1, 2, 3, 4 and 5 ml of 50 mg per liter stock phosphorus solution in 50 ml volumetric flasks and developed the color as mentioned above.
4. Calibrated the spectrophotometer with known phosphorus concentration and read the concentration of the sample.

Calculation

$$P\ (mg/kg) = \frac{vol.make\ up\ after\ digestion\ (ml)}{weight\ of\ sample\ (g)} \times \frac{50}{vol.of\ the\ digest\ used\ to\ develop\ color\ (ml)} \times P(mg/l)$$

3.3.5.5 Organic carbon

The total organic carbon in (%) was estimated as per the method given by Walkley and Black 1934.

Reagents

1. **1 N potassium dichromate:** Dissolved 49.04 g $K_2Cr_2O_7$ in distilled water and volume was made up to 1 liter.
2. **0.5 N ferrous ammonium sulphate:** Dissolved 198 g ferrous ammonium sulphate in distilled water and volume was made up to 1 liter.
3. **Di phenyl amine indicator:** 0.5 g of di phenyl amine indicator (DPA) was dissolved in a mixture of 20 ml water and 100 ml concentrated H_2SO_4.
4. **Concentrated Sulphuric acid**
5. **Orthophosphoric acid (85 %)**
6. **Sodium fluoride**

Procedure

1. 1 g of soil was taken in 500 ml conical flask and 10 ml of 1 N $K_2Cr_2O_7$ was added.
2. The flask was swirled for mixing the soil and reagent.
3. 20 ml of concentrated H_2SO_4 was added and the flask was allowed to stand undisturbed for 30 minutes after which 200 ml of distilled water was added.
4. 1 ml of diphenylamine indicator was then added.
5. Ultimately the contents were titrated with freshly prepared 0.5 N ferrous ammonium sulphate till the end point is observed from blue violet to green.
6. A blank was run without soil samples.

Calculation

$$\textit{Organic carbon (\%)} = \frac{10(B-T)\times 0.003\times 100}{B\times \textit{weight of soil}(g)}$$

Where,

B is volume of ferrous ammonium sulphate solution required for blank titration

T is volume of ferrous ammonium sulphate solution required for soil sample

Because organic matter contains 58 % carbon, so

Organic matter (%): Organic carbon (%) × 1.724 (van Bemnelen factor)

3.3.5.6 Total nitrogen

Total nitrogen was estimated as per the Kjeldahl method given by Piper (1960).

Reagents

1. **Concentrated H_2SO_4**
2. **0.02 N H_2SO_4**
3. **Sulphuric salicylic acid:** 1g salicylic acid was mixed with 30 ml sulphuric acid.
4. **Sodium thiosulphate**
5. **4% boric acid:** 4 g of boric acid was dissolved in 100 ml of distilled water.
6. **Mixed indicator:** 0.066 g of methyl red and 0.099 g of bromo cresol green was dissolved in 100 ml of ethyl alcohol.
7. **50 % NaOH**
8. **Digestion mixture:** 10 g HgO , 5 g $CuSO_4$ and 100 g K_2SO_4 (2:1:20).

Procedure

1. 5 g soil was mixed thoroughly with sulphuric salicylic acid and followed by 5 g of sodium thiosulphate. Heating was carried out for 5 minutes followed by cooling and addition of 10 g of digestion mixture. The contents were mixed well in a kjeldahl flask.
2. The flask was kept in a digestion chamber at 100°C for two hours.
3. The color change was monitored from dark brown to greenish white after that the contents were cooled and 300 ml distilled water was added.
4. 20 ml of the digested sample, 15-20 ml NaOH and glass beads were added to the distillation flasks through the open end of the condenser attachment and stoppered. Water flow was maintained through the condenser.
5. The distillate was collected through a receiver tube in a beaker containing 15 ml boric acid and 2 drops of mixed indicator was added till the end point color changes from pink to green.
6. The distillate was titrated against 0.02 N H_2SO_4 until the color changed from green to pink.

Calculation

$$\textit{Total N (\%):} \quad \frac{(T-B)\times normality\ of\ H_2SO_4 \times 1.4 \times 300}{Weight\ of\ sample(g)}$$

T is titer value for sample, B is for blank

3.3.5.7 Enzyme activities of soil

3.3.5.7.1 Acid and phosphatase activity in soil (Tabatabai and Bremner 1969)

Reagents

1. **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
1 N NaOH	488 ml

Final volume was made up to 1000 ml with distilled water and stored at 4 ºC.

Before use adjust the pH of 200 ml of solution with 0.5 N HCl to pH 5.5 for the assay of acid phosphatase or with 0.5 N NaOH to pH 9.0 for the alkaline phosphatase. Adjust the volume of the pH modified buffer to 1 liter with distilled water.

2. **p–nitrophenyl phosphate solution (0.115 M):** Dissolve 4.268 gm disodium p-nitrophenyl phosphate hexahydrate in 100 ml of appropriate pH adjusted, diluted MUB (pH 5.5) for acid phosphatase and (pH 9.0) for alkaline phosphatase. Store the p-nitrophenyl phosphate substrate at 4 ºC for no longer than 10 days.
3. **NaOH (0.5N):** 20 g of NaOH was dissolved in distilled water and the volume was made up to 1 litre.
4. **p-nitrophenol:** 1 mg per ml solution in modified universal buffer (pH 5.5) for acid phosphatase and (pH 9.0) for alkaline phosphatase.

Procedure

1. Weighed 1 g of soil sample (air dry) and transferred to flask.
2. Added 4.0 ml of the diluted MUB (pH 5.5 for acid phosphatase and pH 9.0 for alkaline phosphatase).
3. 1 ml of filter sterilized 0.115M p- nitrophenyl phosphate solution was added to the flask.
4. The flasks were swirled for few seconds to mix the contents.
5. The flasks were stoppered and incubated at 37 °C for 1hour in dark.
6. 4 ml of 0.5M NaOH was added to stop the reaction.
7. Mixture was swirled and filtered through whatman filter paper no.2.
8. Transferred the filtrate to glass cuvette and measured the yellow color at 410 nm.
9. Phosphatase activity was indicated as the amount of p-nitrophenol released in the filtrate from the p-nitrophenyl phosphate substrate per gram of soil. The p-nitrophenol content was calculated with reference to a calibration graph plotted from the results obtained by standard containing 0, 10, 20, 30, 40, 50 µg of p-nitrophenol.
10. To perform control, followed the procedure described for the assay but made the addition of 1 ml of p-nitrophenyl phosphate after the addition of 0.5 N NaOH (i.e. immediately before filtration).

3.3.5.7.2 Phytase activity (Heinonen and Lahti 1981)

Reagents

1. **Substrate solution:** 2.5 mM dodecasodium phytate in 0.2 M sodium acetate buffer of pH 5.5.
2. **Color stop solution:** (10 mM ammonium molybdate : 5 N sulphuric acid: acetone, 1:1:2 ratio).

3. **0.5 M $NaHCO_3$ extracting solution:** 84 g of sodium bicarbonate was added in distilled water and volume was made up to 2 liter. The pH was adjusted to 8.5 with 1M or 1N NaOH.
4. **1 M citric acid**
5. **Standard KH_2PO_4 solution:** mg/ml stock.

Procedure

A. Extraction

1. Weighed 5 g of soil sample into a 250 ml shaking bottle and 10 ml of sodium bicarbonate (0.5 M) extractant was added.
2. Shook for 30 minutes and centrifuged for 5 min at 12000 rpm.
3. Took supernatant in aliquots of 0.2 ml and determined the phytase activity by the method given by (Heinonen and Lahti 1981).

B. Determination

1. 0.5 ml aliquots of enzyme sample was taken (here soil extract).
2. 0.5 ml substrate solution (2.5 mM dodecasodium phytate in 0.2 M sodium acetate buffer of pH 5.5) was added and incubated for 10 min at 37 ºC.
3. The reaction was stopped by addition of 2 ml ice cold color stop solution (10 mM ammonium molybdate : 5 N sulphuric acid: acetone, 1:1:2 ratio).
4. Added 100 µl 1 M citric acid.
5. O.D. was taken at 380 nm.
6. In the case of blank substrate solution was added after adding the coloring reagent.
7. Calculated the phytase activity per g of soil sample. Enzyme activity was expressed as micromoles of inorganic P released per hour per milliliter of culture filtrate from sodium phytate at 37 °C.

3.3.5.7.3 Soil dehydrogenase activity (Cassida 1977)

Reagents

1. **1, 3, 5 triphenyl tetrazolium formazan stock solution (1mg/ml):** 1 mg of tetrphenyl tetrazolium formazan (TPF) was dissolved in 1 ml of methanol.
2. **1, 3, 5 triphenyl tetrazolium formazan working solution (100 µg/ml):** added 2 ml of stock solution and volume was made up to 20 ml with methanol.
3. **2, 3, 5 triphenyl tetrazolim chloride (3 %):** 3 g of triphenyl tetrazolium chloride was dissolved in 100 ml of distilled water.
4. **0.1 % and 0.2 % yeast extract**: 0.1 g and 0.2 g of yeast extract was dissolved in 100 ml distilled water respectively.

Procedure

1. 10 g of soil sample was weighed and calcium carbonate was mixed in the ratio of 100:1.
2. 3 g of each sample was dispensed in screw cap glass vials and 0.5 ml sterile water was added followed by 1 hour incubation at 28 °C.
3. 0.25 ml of single strength substrate solution was added (0.1 % yeast extract) followed by 0.25 ml distilled water. The vials were incubated for 8 hours at 28 °C.
4. 0.5 ml of 3 % aqueous TTC (2, 3, 5 triphenyl tetrazolium chloride) and 0.25 ml double strength (0.2 %) yeast extract was added and mixed thoroughly with sterile glass rod.
5. This was followed by 6 hours incubation at 37 °C followed by immediate extraction with 25 ml methanol and subsequent filtration through whatman no. 1 filter paper.
6. The methanol extract containing red coloured formazan was read at 480 nm.
7. In 100 ml volumetric flasks 2, 5, 10, 15 and 20 ml of working standard of 100 µg ml^{-1} 1, 3, 5 triphenyl tetrzolium formazan was added (diluted from stock solution of 1mg/ml TPF) and final volume was adjusted with methanol. The absorbance was read at 480 nm.

3.3.5.8 Population density of phosphate-solubilizing bacteria and fungi

At the end of each field trail, rhizospheric soil samples were tested for phosphate-solubilizing bacterial and fungal population in each respective plot. The rhizosphere soil adhere to the roots of harvested plants was separated by gentle tapping and stored in sterilized Petri plates at 4 °C. One gram soil of each replicate soil samples were serially diluted and plated on Pikovskaya's agar plates. The plates were incubated at 30 °C and, bacterial and fungal colonies showing a clear zone of phosphate solubilization were counted at the end of incubation.

3.3.6 Formulation development of phosphate-solubilizing bacteria and fungi

To develop the inoculum formulations rock phosphate, fly ash, charcoal and vermiculite were used as carrier materials in the present study. Rock phosphate used in this study was obtained from Rajasthan state mines and minerals limited, Udaipur, India. The chemical constituents of the RP were: 31.5% P_2O_5, 45.4% CaO, 3.4% MgO, 4.3% aluminium, 3.1% fluoride, 0.044% organic carbon and 0.003% available P, pH-7.04. Fly ash used in the present study was procured from Orissa, India. The chemical constituents of fly ash were: 0.58 % organic carbon, 2.30 mg kg^{-1} P, 53.8% SiO_2, 31.3% Al_2O_3, 6.4% CaO, 0.52% MgO, 5.8% Fe_2O_3, 0.13% MnO, 0.96% TiO_2, 0.12% Na_2O, 0.21% K_2O, pH-7.24. Vermiculite used in present study was procured from Trade Link Engineering Services, Sirhind Road Patiala. The chemical constituents of vermiculite were: 14.39% MgO, 43.48% Al_2O_3, 12.82 % FeO, 11.92% SiO_2, H_2O 17.87%, 0.57% organic carbon, pH-8.78. Charcoal was procured from E. Merck (India) Limited, Mumbai. The chemical constituents of charcoal were: 0.2% chloride, 0.2% sulfate, 0.1% iron, 32% CO_2, 1% Al_2O_3, 17% SiO_2, 0.1% CaO, 50% O, pH-3.69. Phosphate-solubilizing bacterial culture were grown in PKV broth and incubated at 30 °C for five days at 130 rpm. Centrifuged the culture ($O.D._{600}$ 1.0) at 8000 rpm for 10 min and pellet

was washed three time with sterile distilled water and made a suspension in sterile distilled water. Fungal cultures were grown in PKV agar plates for five days at 30 °C. Using a scalpel fungal growth was scraped from the plates and suspended in sterile distilled water. 100 g of each carrier material was transferred in poly-propylene bags and sterilized at 121°C for three hours on three alternate days. After cooling the contents to the bags were inoculated separately with 20 ml suspension of five day cultures of bacteria and fungi. The final moisture content in each bag was adjusted to 30 % with sterile distilled water. The content of the bags were mixed properly by shaking the bags and were sealed. The packets were incubated at 4 °C and 37 °C. Both the phosphate-solubilizing bacteria and fungi were enumerated periodically on 0^{th}, 15^{th} and 30^{th} day and after that on regular interval of 30 days by serial dilution method on Pikovskaya's agar plates. Quality of the final product was determined by check the viable count of each formulation on PKV agar plates by serial dilution method, inoculum formulations showed maximum viable count after 270 days of incubation were tested for P-solubilization, acid phosphatase, alkaline phosphatase, phytase enzyme activity and plant growth promotion activities and compared with fresh culture.

3.3.7 Analysis of maize for aflatoxins

Monitoring grain for the presence of aflatoxins is important to ensuring consumer safety. Maize 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 g of maize 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 di-chloromethane (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 was opened and 1g 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. 30 ml 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). Elute was evaporated to 5 ml on rotavapour then 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 trifluoroacetic acid (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.

3.4 Statistical analysis

Three replicates were used for each experiment. In field experiments, plots with different treatments were arranged in a randomized complete block design with three replicates per treatment. The data were analyzed by analysis of variance (ANOVA) and the means were compared with Tukey's test at $p < 0.05$. All the analysis was performed by using Graph Pad Prism 5.0 software.