MANUAL FOR PHOSPHATE SOLUBILIZING INOCULANT DEVELOPMENT

Reshid Abafita Abawari (Ph.D)

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Screening of Phosphate solubilizing Microorganisms

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Screening of Phosphate solubilizing Microorganisms

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MANUAL FOR PHOSPHATE SOLUBILIZING INOCULANT DEVELOPMENT: Screening of Phosphate solubilizing Microorganisms

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PREFACE

The field of phosphate solubilizing microorganisms and their role in organic agriculture has been undergoing rapid change in recent years. Because of greater consciousness of the effect of chemical fertilizers on the environment has increased the call in some accommodations for the use of more environmentally friendly and non-hazardous to human health is unquestionable. This manual has been designed with the objectives to help the researchers and laboratory technicians to provide them with the basic knowledge and skills how to isolate and screen the target P-solubilizes and produce inoculants. These include a summary of aseptic technique and preserving microbial cultures, formulations and application methods of inoculants in the soil amendments. The various steps of selection, identification, performance, and bio-formulation and inoculum preparation are covered broadly and each section is grouped to reflect these steps.

ACKNOWLEDGEMENT

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Reshid Abafita Abawari (Ph.D)

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1. Introduction

Next to Nitrogen, Phosphorus (P) is a non-renewable macronutrient, the most important nutrient required by the plants. It is one of the major important elements that play a significant role in biological growth and development such as photosynthesis, respiration, energy storage and transfer in the living plant cells but for plants is also a limiting one due to its low availability of soluble forms in soil. However, most soil phosphorus, approximately 95–99%, is present in the form of insoluble phosphates and hence cannot be utilized by plants. Unlike for nitrogen there is no large atmospheric source that can be made biologically available for P availability. Most natural ecosystems in tropical and subtropical areas are predominantly acidic, rich in iron and extremely P deficient due to their strong fixation of P as insoluble phosphates of iron and aluminum resulting P deficiency in the soil. To ease P deficiency in these regions, chemical fertilizers containing soluble forms of P are widely used on large scale to keep soil fertility in crop cultivation. However, only about 25% of the phosphorus applied to the soil is available for the crops. This in turn leads to a need for excessive and repeated application of soluble P fertilizers, which in addition to the economic restriction can pose a serious threat to groundwater. In lines with these, the cost of chemical fertilizers and their associated risks on the environmental safety calls for the search of alternative means of plant nutrient management practices, such as phosphate solubilizing inoculants. To achieve maximum benefits in terms of fertilizer savings and better growth, the P-solubilization based inoculation technology should be utilized along with appropriate levels of fertilization.

The soil solution remains to be the main source of phosphorus supply to plants. The biggest reserves of P in soil are in mineral forms such as Rock Phosphate (apatite), hydroxyapatite and oxyapatite and in organic forms such as inositol phosphate, phosphomonoesters, phosphodiesters and phosphotriesters, which cannot be directly assimilated by plants, because a large proportion of P is present in insoluble forms and is consequently not available for plant nutrition. Low levels of P reflect the high fixation of phosphate with other soluble components, such as iron and aluminum in acid soils (pH<5) and calcium in alkaline soils (pH>7). The phosphorus content of agricultural

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soil solutions are typically in the range of 0.01–3.0 mg per liters representing a small portion of plant needs. The rest must be obtained from the solid phase through intervention of biotic and abiotic processes where the phosphate solubilizing activity of the microbes has a role to play. Soil microbes help in phosphorus (P) release to the plants that absorb only the soluble phosphorus forms like monobasic ($H_2PO_4^-$) and dibasic ($H_2PO_4^{2-}$) forms. They make P available through mechanisms of solubilization to convert inorganic and mineralization to convert organic into bioavailable form facilitating uptake by plant roots. Solubilization of inorganic P from an insoluble chemical form is usually mediated by the ability of the bacteria and fungi to acidify growth medium, to release organic anions such as citrate, oxalate, gluconate and succinate to increase free P in the medium.

One of the main problems to be solved in the next decades is to minimize dependence on phosphate fertilizers. To meet this challenge, phosphate solubilizing microbes (PSMs) must play a bigger role. It is known and well-documented that PSMs are able to solubilize rock phosphate. Phosphate-solubilizing bacteria (PSB) and fungi can not only play key role in liberating P from pools of inorganic phosphate but also can prevent the liberated P from being re-fixed. Exploitation of these potentials may be a promising technique for plant phosphate nutrition. Thus, manual development for selection, identification, performance, and bio-formulation should be carried out to meet this need. Therefore, this manual has been designed with the objectives to help the researchers to provide them with the basic knowledge and skills how to isolate and screen the target P-solubilizes and produce inoculants. These include a summary of aseptic technique and preserving microbial cultures, formulations and application methods of inoculants.

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2. Sample collection, isolation and screening of phosphate-solubilizing Bacteria and Fungi

2.1. Collection of Vermicomposts samples

Matured Vermicomposts will be collected and air dried under shade for the isolation of phyto-beneficial microbe (bacteria and fungi). The air dried samples of vermicomposts will be collected into polythene bags, sieved through a 4mm mesh and stored at 4°C for further analysis.

2.2. Collection of rhizosphere soil samples

Soil samples will be collected from a depth of 0-15cm from the rhizosphere adhering to roots of plants. The samples will be randomly collected from fields within 1 to 2 km interval between the samples. During the soil sample collection, surface will be cleaned by removing the litter and top soil layer. All samples will be placed in polythene bags, brought to the laboratory in iceboxes, and will be stored at 4^{o}C in refrigerator for further analysis.

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3. ISOLATION OF MICROBES

3.1. ASEPTIC TECHNIQUE

1. Disinfect the tables with antibacterial cleaner.
2. Wear gloves and lab aprons.
3. When labeling Petri plates, always write on the bottom of the plate.
4. When using incinerators, give them ample time to warm up before sterilizing loops or needles. DO NOT leave loops or needles unattended in the incinerators. They will melt! You will burn your fingers!
5. When inoculating cultures, always sterilize your loop or needle before going into a culture and after transferring it. Sterilize the loop even if you are going back into the same culture again.
6. Make sure you let your loop cool first – you don't want to kill the bacteria.
7. After removing the lid of a test tube, briefly flame the mouth of the tube before inserting your inoculating loop, and flame again before replacing the cap.
8. Don't put anything down on the table – loops, needles, pipettes, test tube lids, etc. Once they touch the table they are no longer sterile.
9. Loops/needles can be placed temporarily in slots on the sides of incinerators.
10. Test tube lids can be held with pinky finger.
11. Pipettes should remain in canister until just before use.
12. Don't leave media open to the air –bacteria and fungi in the air can contaminate the medium.
13. Don't over-inoculate! It doesn't take much inoculum to start a culture. Simply touch the loop or needle to the bacterial growth and obtain a small amount on the loop. Don't "scrape" the culture, and don't dig into the agar.
14. When streaking onto an agar plate or slant, make sure the loop doesn't break the surface of the agar. A gentle gliding motion is all that is necessary to distribute the bacteria and fungi on the plate.

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15. When finished, disinfect the tables again with antibacterial cleaner. Dispose of gloves in the biohazard trash, and place aprons back in your designated drawers. Wash your hands!

3.2. VIABLE MICROBIAL PLATE COUNTS

Primary isolation and identification of fungal and bacterial P-solubilizes will be done on Pikovskaya's agar (PVK), containing per liter:0.5g yeast extract, 10g dextrose, 5g $Ca_3(PO4)2$, 0.5g (NH4)2SO4, 0.2g KCl, 0.1g, $MgSO4.7H_2O$,0.0001g, $MnSO4.H_2O$,0.0001g, $FeSO4.7H_2O$ and 15g agar, pH 7.2) supplemented with tri-calcium phosphate (5 g/L) as an insoluble inorganic phosphate source by plate count method. 10 ml/L Rose Bengal will be added for control of bacterial growth at concentration 1 /1 5000 to PVK for fungal growth. A 10g of soil and vermicomposts will be separately suspended in 90ml sterile distilled water in Erlenmeyer flask and mixed thoroughly for 30 minutes using a mechanical shaker at 110 rpm. Then 1mL of aliquot from each will be transferred with sterile pipettes for ten-fold serial dilution. Serial dilutions are a step-wise set of dilutions which sequentially dilute the bacterial and fungal cultures. One or more of the dilutions are then plated on the agar plates to determine the number of colonies present in the original culture. Only plates containing between 30 and 300 colonies are counted to ensure statistically significant data for bacterial culture. To estimate the number of bacterial and fungal in the original culture, the number of colonies on the plate is multiplied by the total dilution plated. For example, suppose 0.1 ml of a 10^{-6} dilution was plated, and 123 colonies were counted following incubation. The total dilution plated would be 10^{-7} (since only 0.1 ml was plated), and the number of bacteria/ml of the original culture would be: (123) x $1/10^{-7}$ = 1.23 x 10^9 CFU/ml. Note that the results are expressed as "colony forming units (CFU)" per ml. A subsequent serial dilution will be prepared **from 10^{-1}-10^{-7}** (Figure 1).

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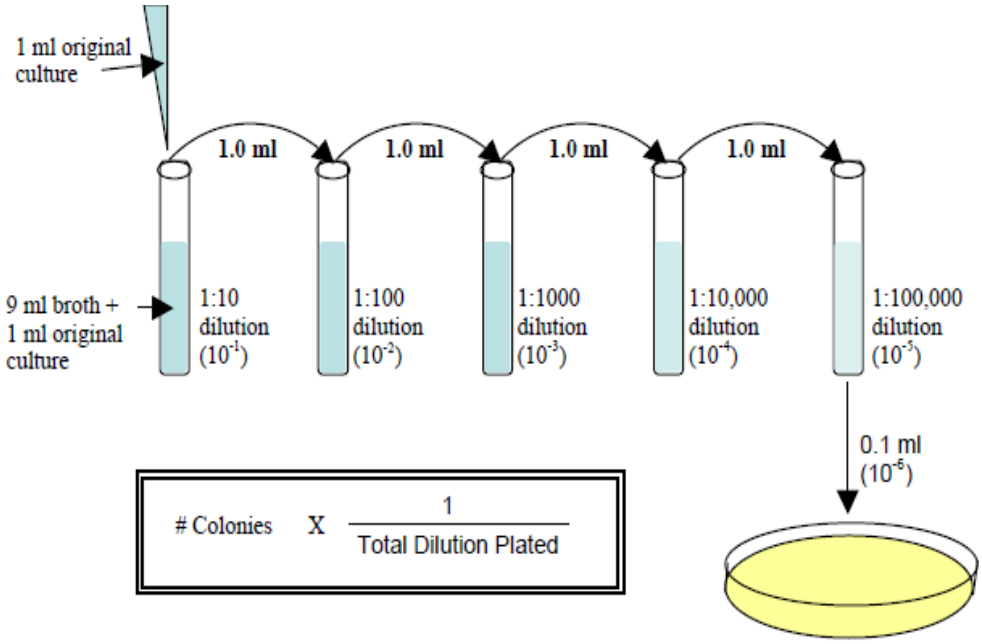

Figure 1 Serial dilution steps

From appropriate serial dilution, 0.2ml of aliquot will be transferred to sterile Petri-plate containing pre-solidified PVK medium. The inoculated plates will be incubated for 7–14 days at 30^0C for bacterial isolation and incubated for 6 days at25±2°C for fungal isolation. From the total colonies, only those colonies which showed clear zones will be re-streaked onto PVK medium to obtain pure cultures of phosphate solubilizing colonies following the given protocol (Figure 2).

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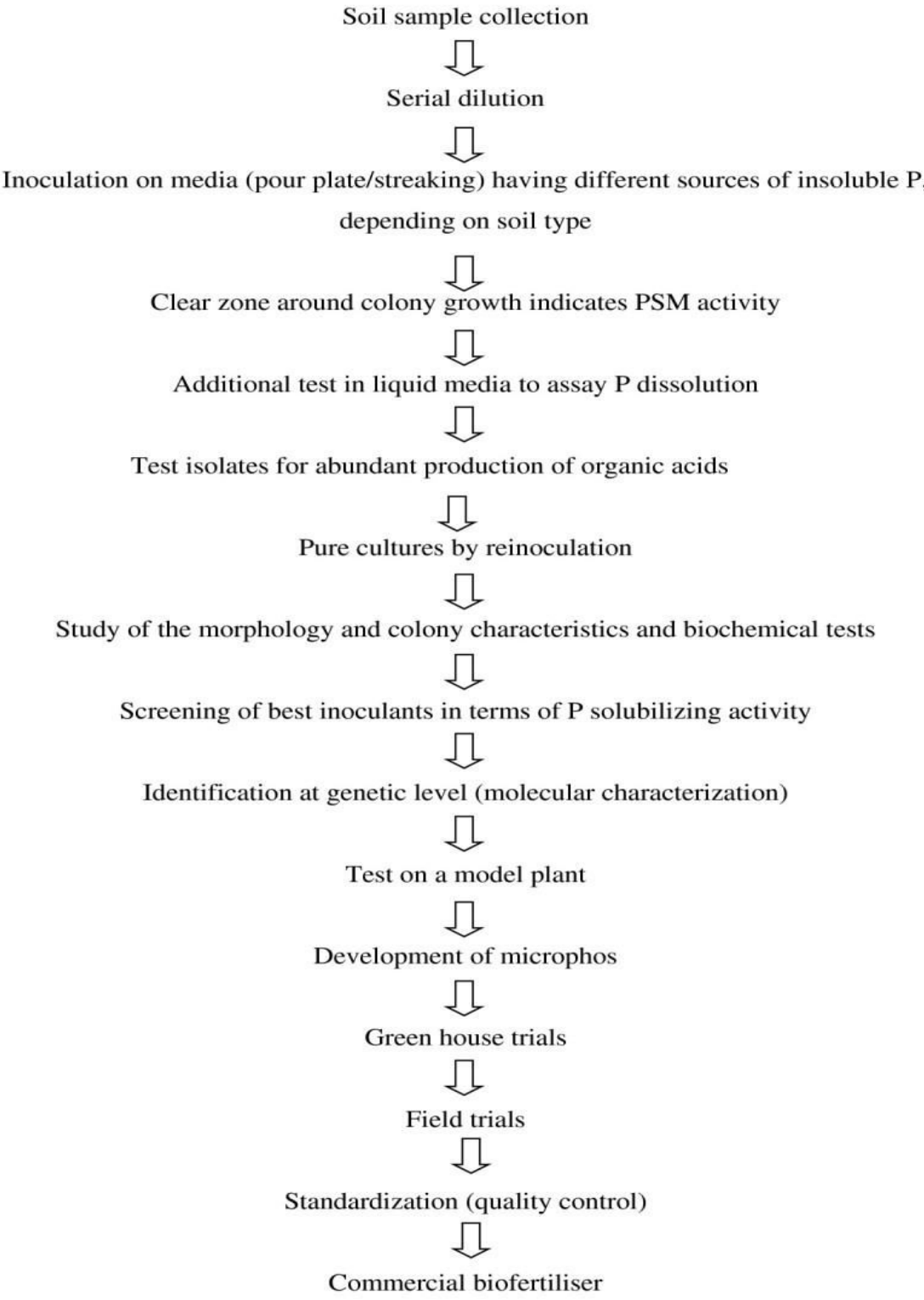

Figure 2. Protocol for isolation and effective inoculants development of PSM based bio fertilizer.

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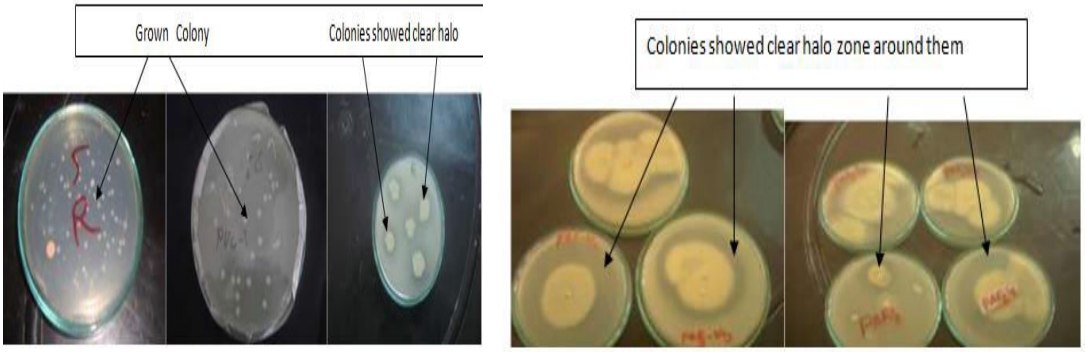
The pure colonies that showed clear zones around them will be maintained in PVK slants at 4^0C for subsequent analysis (Figure 3).



A B

Figure 3. Colony growth and halo zone appearance on Pikovskaya's agar medium (A-Bacteria, B-Fungi)

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4. Characterizing the Isolates

The selected PSB and fungi isolates will be studied for their morphological, physiological and biochemical tests based on the methods defined in Bergey's Manual of Determinative Bacteriology. Bacterial colonies from purified culture will be grown on PVK solid medium by streak plate method and incubated at 30^{o}C until colonies appeared. Gram staining reaction of isolates will be observed under light microscope. Morphological characters of the fungal isolates will be observed by growing them on PVK agar and stained with lectophenol cotton blue stain for observation of morphological characteristics of the hyphae, spores, and conidiophores under light microscope.

4.1. Gram staining of bacterial isolates

For gram staining the following reagents will be prepared; Crystal violet solution (Crystal violet- 10g, Ethyl alcohol-100ml, Ammonium oxalate-4g, Distilled water-400ml); Iodine solution(a) (Iodine-1g, Potassium iodide-2g, Ethyl alcohol-25 ml, Distilled water-100 ml); Iodinated alcohol

(Iodine solution (b)-5ml, Ethyl alcohol-95 ml) Counterstain (2.5% safranin in ethyl alcohol-10 ml, Distilled water-100 ml). Gram stain smear will be prepared with a loop full of a selected bacterium and spread over a slide in a drop of water and allowed to dry in air. The slide is dried in the vicinity of the flame and allowed to cool and then stained with crystal violet solution as follows: for 1 min followed by rinsing with water and removal of excess water, the slide is then flooded with iodine solution followed by decolorized with iodinated alcohol for one minute, for 5 min the slide is washed in water, drained and counterstained with safranin for 30 seconds. Finally the slide is washed in water, drained and air dried and observed under oil immersion. The pink colonies will show the gram negative bacteria and the purple colonies will show the gram positive bacteria (Figure 4).

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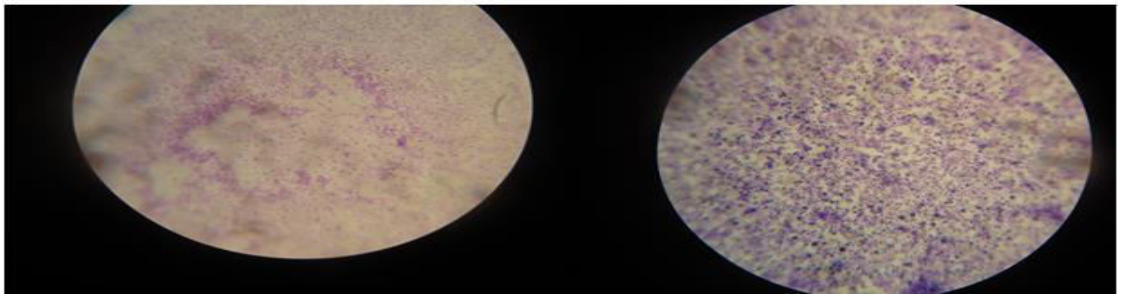

A B

Figure 4. Gram staining identification (A) Gram-negative (Pseudomonuas spp.) (B)-gram-positive (Bacillus spp.)

4.2. Morphological characterization of the fungi

Morphological characters of the fungi will be observed by growing it on Potato Dextrose Agar (PDA) (PA) and stained with lectophenol cotton blue stain (Figure.5). The isolate from each sample will be maintained on PVK slants for further analysis (Figure 5).

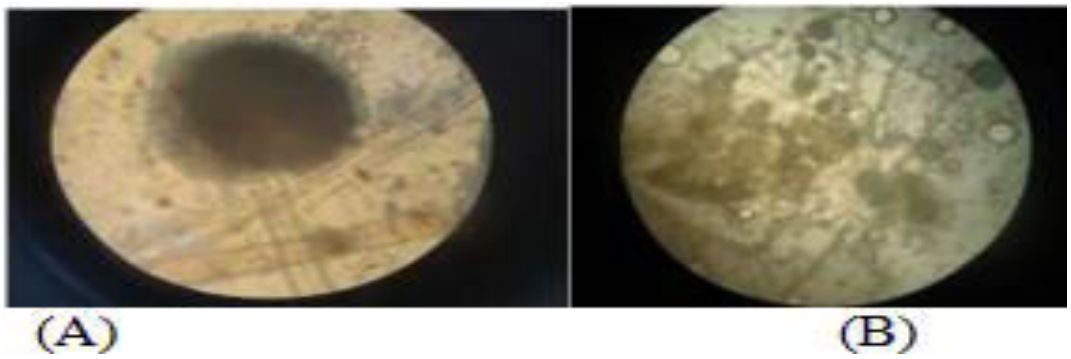

Figure 5. **Morphological characterization of the fungi** *(A)*Conidia*(Aspergillusspp)(B)* Conidia*(Penicilium sp)*

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5. In vitro Phosphate Solubilization tendency of isolates

5.1. The P-solubilization index (SI) of phosphate solubilizing bacterial and fungal

During qualitative analysis the bacterial and fungal characters are usually tested in an agar plate medium with precipitated tri-calcium phosphate, National Botanical Research Institute phosphate growth (NBRIP) medium, $FePO_4$, and $AlPO_4$ or rock phosphate (hydroxyapatite) which is clarified by the acid released from the bacterial and fungal colony and the efficiency of the solubilizers is measured by measuring the **P-Solubilization Index (SI)**. The P-solubilization index (SI) of phosphate solubilizing bacterial and fungal isolates will be checked on the PVK medium supplemented with tri-calcium phosphate (5g/L) as insoluble phosphates source. A pinpoint inoculation of bacterial and fungal isolates will be placed on the center of plates under aseptic conditions. The growth and solubilization of insoluble phosphates in the PVK medium by forming the halo zones will be determined by solubilization diameter after incubation at 30^0C for 7 days for bacteria and at 25±2°C for 6 days for fungi. Bacterial and fungal colonies surrounded by a halo, indicating phosphate removal, will be visually observed and measured (Figure 6).

(A) (B)

Figure 6. Colonies of Bacteria (A) and fungi (B) showing clearly visible large halo zones around their colonies

The solubilization index will be measured according to the formula:

PSI= *Colony diameter + halozone diameter*

Colony diameter

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5.2. Solubilization of inorganic phosphate in liquid medium

Phosphate solubilization tendency of bacteria and fungi can also be quantified and measured in a liquid culture medium. Pikovskaya's broth medium (125 ml) supplemented with 0.5% TCP (equivalent to 5000 mg L^{-1}) will be prepared. The medium in 125 ml amount will be dispensed into a 250 ml capacity Erlenmeyer flask. Three replicate flasks will be used for each bacterial and fungal isolate. Sterile un-inoculated medium will be served as control. The pH of the medium will be adjusted to 7.0 before autoclaving and each flask will be inoculated with 0.1 ml of 24h old active culture suspensions of each PS bacterial isolates with a cell density of 10^8cuf/ml. Similarly, the liquid medium in the flasks will be inoculated with fungal isolate using 8mm mycelia disks taken from 7 days old cultures.

The flasks will be kept on a rotary shaker (125 rpm) for 6 days until the day of sampling for bacteria and kept for 15 days until the day of sampling for fungi. To collect solublized inorganic P by bacteria, insoluble materials in each culture broth will be removed by centrifuging at 4,000 rpm for 20 minutes and filtered through Whatman filter paper No.1. To quantify solubilized P in each fungal culture, insoluble materials will be removed by centrifuging at 5,000 rpm for 25 minutes and filtered through Whatman filter paper No.42. From each culture, 0.625 ml of the filtrate will be transferred to a volumetric flask of 100 ml capacity and 13 ml of mixed reagent added. The volume will be top upped to 100 ml with distilled water. Soluble phosphorus will be determined following quantitative spectrophotometric analysis and the pH of the cultures will be measured accordingly using pH meter. The absorbance of blank and cultures of the inoculated treatment filtrates will be read at 882 nm wave length using. A calibration curve for standard, plotting absorbance against respective P concentrations will be prepared and the P concentration of treatment filtrates will be read from calibration curve.

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6. Phytobeneficial traits of bacteria isolated from vermicomposted soil and plant rhizosphere.

To get the maximum benefit of inoculation under different ecological zones, there is a need to develop the inoculants using a microbial strain with multifunctional attributes. Hence in order to identify isolates with such multifunctional attributes among isolated rhizobacteria and vermicomposts. We will screen all the isolates for Phosphate solubilization, indole acetic acid (IAA), hydrogen cyanide production (HCN), ammonia (NH_3) production, Siderophore production and nitrogen fixation. Because the mechanisms by which bacteria may influence plant growth at various stages of the host plant life cycle includes such phytobeneficial traits.

6.1. Siderophore production

Bacterial isolates will be assayed for siderophores production on the Chrome azurol S agar medium (Sigma, Ltd.). Chrome azurol S agar plates will be prepared and divided into equal sectors and spot inoculated with test organism (10ml of 10^6 CFU/ml) and will be incubated for 48h at 36±2°C. Development of yellow-orange halo around the growth will be considered as positive for siderophore production.

6.2. Production of Indole acetic acid (IAA)

Indole acetic acid (IAA) is generally considered to be the most crucial native auxin. Soil microorganisms are capable of synthesizing and catabolizing indole acetic acid.

To estimate the production of IAA, the potent bacterial cultures will be inoculated into nutrient broth with L-tryptophan (5 μg/mL) and incubated at 28±2^{0}C for 5 days. After incubation, cultures will be centrifuged at 3000 rpm for 30 min. A 2mL of the supernatant will be mixed with 2 drops of orthophosphoric acid and 4 mL of Salkowski's reagent (50 mL of 35% perchloric acid + 1 mL 0.5$FeCl_3$) and incubated in the dark for 25 minutes. Development of pink colour indicates IAA production (Figure 7).

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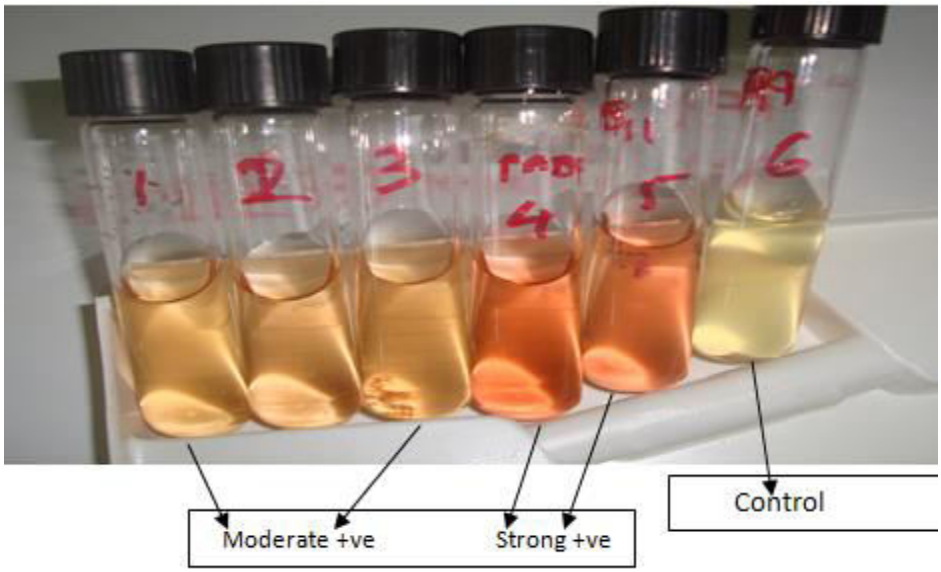

Figure.7: IAA production shown by bacterial isolates

6.3. Production of ammonia (NH3)

Bacterial isolates will be tested for the production of ammonia in peptone water. PSB will be grown for 48 h in nutrient broth (NB) medium at 36±2°C. Freshly grown cultures (100 µL of 24 h grown) will be inoculated into 10mL peptone water in each tube. Nessler's reagent (0.5 ml) will be added in each tube. Development of brown to yellow color will be taken as a positive test for ammonia production (Figure 8).

Figure 8: NH_3 production in bacteria

6.4. Hydrogen cyanide (HCN) production

To determine cyanide production Qualitatively PSB isolates will be sub cultured onto nutrient agar (NA) medium supplemented with glycine ($4.4gL^{-1}$). Whatman filter paper No.1 soaked in 2% sodium carbonate in 0.5% picric acid solution will be placed at the

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top of the plate fixed to the underside of the Petri-dish lids and sealed with parafilm before incubation at 28^0C for 48h. Changes in color from yellow to reddish brown will be taken as an indication of strongly cyanogenic potential of the bacteria (Figure 9).

Figure 9: HCN production in bacteria

6.5. Nitrogen Fixing Activity

Biological nitrogen fixation (BNF) is the potential biological process that maintains the soil nitrogen status under normal conditions. The diazotrophic character will be evaluated by the capability of the isolate to grow in the nitrogen free selective medium. In order to screen for nitrogen fixing ability among the isolated PSB, the pure cultures will be inoculated onto Jensen‘s medium containing g/L: Sucrose 20.0, Dipotassium phosphate 1.0, Magnesium sulphate 0.5, Sodium chloride 0.5, Ferrous sulphate 0.1, Sodium molybdate 0.005, Calcium carbonates 2.0, Agar 15.0.The inoculated Petri plates will be incubated at 37^0C for 5 days. Un-inoculated Petri plate served as control.

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7. Invitro test for eco-physiological properties

In addition to evaluating the bacterial strains for their phytobeneficial traits, the bacterial strains will be tested for their capability to adapt to different ecophysiological traits such as heavy metals, salinity and pH.

7.1. Evaluation for heavy metal tolerance

It is prime importance maintaining good soil quality for sustainable agriculture. To do so, soil biology is a significant component of soil quality and bacteria play vital roles in soil fertility and nutrient cycling. These bacteria promote uptake of heavy metals by promoting plant growth through the synthesis of different compounds such as siderophores or due to stimulation of certain other metabolic pathways such as nitrogen fixation along with the uptake of N, P, S, Mg, and Cu. When Various potentially toxic heavy metals, but needed in a trace amount for both animal and plants, are present in an elevated concentrations they are known to affect soil microbial populations and their associated activities. Therefore, evaluation of the impact of different concentration of heavy metal upon bacteria would be useful for clear understanding of the interaction of heavy metals on biogeochemical cycles in soil environment.

Tolerance to heavy metals will be measured using Pikovskayas medium supplemented with different concentrations of various soluble heavy metal salts. Freshly prepared agar plates will be amended with various soluble heavy metal salts namely Hg, Cu, Zn and Mn, at various concentrations ranging from 100 to 400µg/ml will be inoculated with overnight grown cultures in Pikovskayas medium. Heavy metal tolerance will be determined by the appearance of bacterial growth after incubating the plates at 37°C for 48 h. Isolates will be considered resistant when growth will be observed (“+’’) and (“–“) for absence of growth.

7.2. Evaluation for salinity tolerance

To determine the effect of increasing concentration of salts (NaCl) on the growth of phosphate solubilizing bacteria the isolate will be grown on Pikovskaya’s agar medium supplemented with different concentrations of NaCl (3%, 4%, 5%, 6% and 7% (w/v). These concentrations will be used to screen the P-solubilizers efficacy of different

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isolates and it will be incubated at 28^{o}C for 5 days. The results will be recorded qualitatively as ("+'') for presence and ("–") for absence of growth indicating the salt tolerance and sensitivity level of the microbes.

7.3. Screening for acid tolerance

PSB isolates will be tested for pH tolerance whether they can grow in a range of pH 4.0 to 10.0 at an increment of one unit pH, and hence all the active strains of PSB will be inoculated separately into test tubes containing 10ml of nutrient broth (NB) at varying pH levels adjusted with sterile 0.1N HCl and 1N NaOH. Bacteria will be incubated for 8 days at 25^{0}C. The optical density of the bacteria and pH value of the medium will be estimated after 2days intervals using a spectrophotometer (at 595 nm) and a pH meter, respectively. Each treatment will be replicated three times and data will be expressed as the mean value. The results will be recorded qualitatively as ("+'') for presence and ("–") for absence of growth.

7.4. Evaluation of the Fungal isolates tolerance to salinity

Three days old homogenized cultures fungal isolates will be inoculated into 50 ml of PVK broth containing 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 M NaCl and incubated at 28±2^{0}C for seven days. After incubation the cultures will be filtered through pre-weighed Whatman No. 1 filter papers and dried to constant weight at 60^{0}C in a hot air oven. The dry mycelial weight will be then recorded for each culture.

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8. PRESERVING MICROBIAL CULTURES

The following points highlight the top methods of preserving microbial culture. The methods are:

1. Agar Slant Cultures

2. Agar Slant Culture Covered with Oil (Paraffin Method)

3. Saline Suspension

4. Preservation at Very Low Temperature

5. Preservation by Drying in Vacuum

6. Preservation by Freeze Drying (Lyophilization)

8.1. Agar Slant Cultures

All microbiology laboratories preserve micro-organisms on agar slant. The slants are incubated for 24hr or more and are then stored in a refrigerator. These cultures are periodically transferred to fresh media. Time intervals at which the transfers are made vary with the origin and condition of growth.

8.2. Agar Slant Culture Covered with Oil (Paraffin Method)

The agar slants are inoculated and incubated until good growth appears. They are then covered with sterile mineral oil to a depth of 1cm above the tip of slant surface. Transfers are made by removing a loop full of the growth, touching the loop to the glass surface to drain off excess oil, inoculating a fresh medium and then preserving the initial stock culture. This is a simple and most economical method of preserving bacteria and fungi where they remain viable for several years at room temperature. The layer of paraffin prevents dehydration of the medium and by ensuring an aerobic condition, the microorganism remain in dormant state.

8.3. Saline Suspension

Sodium chloride in high concentration is frequently an inhibitor of bacterial growth. Bacteria are suspended in 1% salt solution (sublethal concentration in screw cap tubes to prevent evaporation). The tubes are stored at room temperature. Whenever needed the transfer is made on agar slant.

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8.4. Preservation at Very Low Temperature

The organisms are suspended in nutrient broth containing 15% glycerol. The suspension is frozen and stored at -15°C to -30°C. The availability of liquid nitrogen (temp -196°C) provides another main preserving stock culture. In this procedure culture are frozen with a protective agent (glycerol or dimethane sulphoxide) in sealed ampoules. The frozen cultures are kept in liquid nitrogen refrigerator.

8.5. Preservation by Drying in Vacuum

The organisms are dried over calcium chloride in vacuum and are stored in the refrigerator.

8.6. Preservation by Freeze Drying (Lyophilization)

In this process the microbial suspension is placed in small vials. A thin film is frozen over the inside surface of the vial by rotating it in mixture of dry ice (solid carbon dioxide) and alcohol, or acetone at a temperature of−78^{o}C .The vials are immediately connected to a high vacuum line. This dries the organism while still frozen. Finally, the ampules are sealed off in a vacuum with small flame. This culture can be stored for several years at 40°C. This method is also employed for preservation of toxins, sera, enzymes and other biological material. To revive microbial cultures it is merely necessary to break open the vial aseptically, add a suitable stale medium, and after incubation make further transfers. The process permits the maintenance of longer number of culture without variation in characteristics of the culture and greatly reduces the danger of contamination.

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9. Formulations and application methods of inoculants

Microbial formulation is a carrier-based preparation to provide microbes with better survival for longer duration. The formulation contains the organisms, which are useful in wide agriculture usages such as plant growth promoter, nutrient availability, and to improve soil and plant health and which has a shelf life of two years with an initial CFU count of 10^{10} and at the end of one year not less than 10^{8} at a wide temperature range of 5°C-40^{0}C. Microbial formulations for plant growth comprise beneficial bacteria, beneficial fungi, and carrier materials which extends the effective life time of the microbial inoculant. The microbial inoculant is effective for increasing plant productivity in legumes, non-legumes and vegetable crops. However, there are a number of disadvantages of the application process, such as the availability of enough inoculant quantity for total seed surface, contact with chemicals, bacterial movement, the material used as carrier (nature, particle size, and presence of contaminating microorganisms or viability of the bacteria), and the technology used for drying and preservation of bacteria (addition of nutrients and preservatives) in the substrates.

The seed treatment is a means to apply inoculants which are effective and economic. For small quantity of seeds (up to 5 kg quantity) the coating can be done in a plastic bag having size (21" x 10") or big size can be used. The bag should be filled with 2kg or more of seeds, closed in such a way to trap the airs as much as possible, squeezed for 2 minutes or more until all the seed are uniformly wetted and then the bag should be opened, inflated again and shacked gently. Stop shaking after each seeds gets a uniform layer of culture coating. The bag is opened and the seed is dried under the shade for 20-30 minutes.

For large amount of seeds coating can be done in a bucket and inoculant can be mixed directly with hand. Seed Treatment with *Rhizobium*, *Azotobacter, Azospirillum*, along with PSM can be done.

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10. Multiplication of bacterial and fungal isolates and inoculum preparation

10.1. Spore suspension for inoculation (Fungal inoculums)

For fungal inoculum preparation, phosphate solubilizing fungal (PSF) isolates will be mass cultured aseptically in 90mm diameter Petri plates each containing 15mL of autoclaved PVK. The plates will be incubated at 28 ±2°C for 10 days. On the tenth day, spore suspensions from the fungal isolates will be prepared by flooding the surface of the agar plates with 10mL sterile distilled water and the culture surfaces will be gently scraped using a sterile glass rod to dislodge the spores. The spore suspension will be transferred separately to 500mL flask containing 400mL sterile distilled water. Flasks will be shaken for 2 minutes to ensure that the spores will be properly mixed. The cultures will be filtered through Whatman No.42 filter paper into sterile glass bottle. The spore suspension of 25 ml (10^6 spore mL^{-1}) of fungal culture will be used per 50g of the sterilized carrier materials (VC) and immediately stored at 4ºC until use. The spore count will be determined using Haemocytometer with a formula mentioned below, so that the final counts will be determined as 1×10^6spores/mL:

Total cells per mL=Total counted x dialution factor x10^4 cells per mL

Number of squares

The mixed and inoculated carrier material will be evaluated for plant growth promotion as inoculants.

10.2. Multiplication of bacterial strain and inoculum preparation.

Inoculum of phosphate solubilizing bacteria will be prepared in Pikovskaya's broth medium. The viable count in the inoculum will be kept as 1x10^8CFU/ml before mixing with carrier materials. The media will be incubated at 28±2^{o}C under shaking at 100 rpm for three days. Vermicomposts (VC) (as carrier material) will be used for the mass multiplication of the isolates. The powdered dry Vermicomposts (VC), will be neutralized to pH 7 with $CaCO_3$ and Ca (OH)2 , and distributed in batches of 50g each in polyethylene bags and autoclaved for 30 minutes at 121ºC on three successive days, sieved and maintained proper water content in the carrier materials and inoculated with broth cultures of phosphate solubilizing spp (20mL per 50 g of VC) and will be

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incubated at 28±2^{o}C for three to five days and immediately stored at 4ºC until use (as much one week of storage at 4ºC). The mixed and inoculated carrier materials will be evaluated for plant growth promotion including phosphate solubilization at various concentrations as inoculants under greenhouse and field condition.

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11. In vivo screening the isolates for P-solubilization efficacy

11.1. Screening the isolate for P-solubilization efficacy in pots under greenhouse condition

Most literature reviews have shown that most of the experiments are carried out at laboratory and few papers have presented results in the pots to test efficiency level of the P-solubilizing isolates. Phosphate solubilization tendency of bacteria and fungi activity does not always guarantee the efficiency of inoculants in the field and hence in vivo experiments should be done under greenhouse and field condition to see whether the bacterial and fungal inoculum can enhance P availability and behaving as true biofertilizers to improve plant growth. So, since we know that results in laboratory conditions do not always reflect those of the in vivo, therefore, consistent in vivo results are a prerequisite for a massive adoption of bio-fertilizers based phosphate solubilizing microbes (PSMs).

Seeds of test crops will be surface sterilized by immersing in 3.25% (v/v) sodium hypochlorite for 1 min, followed by 70% (v/v) ethanol for 1 min and rinsing five times in sterile distilled water. The grains of each treatment, in polyethylene bag, will be moistened by adding 10ml of sugar solution (1spoon table sugar per 10ml water) and after mixing, the VC inoculant will be added and thoroughly mixed with the grains until uniformly surface coated. The inoculant will be added to grains at a rate of 15g/100g grains. A greenhouse experiment with Randomized complete Block Design (RCBD) in factorial form with three replications of each treatment and one control will be conducted. The phosphate solubilizing bacterial and fungal inoculums either separately or as consortium (1:1) will be tested for plant growth promoting activity.

Three seeds will be planted per pots. Seedlings will be thinned when they attained two pairs of true leaves and one uniformly growing Seedling will be left. The seedlings will be grown in a greenhouse until the emergence of six true leaves at a temperature of 28–32^{0}C and 85% relative humidity. After completion of the trial the plant will be uprooted, washed thoroughly with water and several parameters such as shoot and root length, leaf numbers, leaf area, stem girth, chlorophyll content of the leaves, fresh and dry weight of the whole plant will be measured using standard procedure.

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11.2. Field evaluation of phosphate solubilizing fungal and bacterial inoculums for increasing yield and yield parameters.

The use of soil inoculants has promise for use in agricultural systems for improving nutrient status, reducing plant diseases and pests, and improving yields. However, management practices such as rotating crops, growing cover crops and adding organic fertilizers and soil amendments provide similar benefits. All of these practices affect the numbers and diversity of the microorganisms in the soil. The complexity of the soil and agricultural production systems makes it difficult to predict whether soil inoculants will perform as expected. Without proper soil conditions, laboratory-raised inoculants often have a difficult time competing with native microorganism populations.

Therefore, to see these effects the phosphate solubilizing bacterial and fungal inoculums separately or as consortium (1:1) will be tested for plant growth promoting activity. A field experiment with randomized complete block design (RCBD) in factorial form with three replications of each treatment and one control will be conducted. Three seeds of *test crop* will be surface sterilized by immersing in 3.25% (v/v) sodium hypochlorite for 1 min, followed by 70% (v/v) ethanol for 1min and rinsing five times in sterile distilled water. The grains of each treatment, in polyethylene bag, will be moistened by adding 10ml of sugar solution (1spoon table sugar per 10ml water) and after mixing, the VC inoculant will be added and thoroughly mixed with the grains until uniformly surface coated. The inoculant will be added to grains at a rate of 15g/100g grains. Before establishment of the experiment, composite soil sample will be analyzed for selected physical and chemical properties. Soil texture, Soil pH, Soil organic carbon and available phosphorus in the soil will be determined based on the standard methods. Nutrient uptake (N, P and K) from growth media will be recorded at the end of the trial.

The field trials will be maintained as per the existing package of practice all the necessary plant pest and disease care practices will be followed strictly. Seedlings will be thinned when they attained two pairs of true leaves and one uniformly growing seedling will be left. Data will be obtained on germination percent, shoot and root length, stem girth, leaf number per plant, leaf area, fresh weight, dry weight and grain yield.

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