PS: 5330

PAKISTAN STANDARD

SPECIFICATION FOR BIO FERTILIZER

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PAKISTAN STANDARDS AND QUALITY CONTROL AUTHORITY,
STANDARDS DEVELOPMENT CENTRE,
PSQCA COMPLEX, PLOT NO. ST – 7/A, BLOCK NO. 3,
SCHEME – 36, GULISTAN-E-JAUHAR,
KARACHI.

0. FOREWORD

0.1 This Pakistan Standard was adopted by Pakistan Standards & Quality Control Authority on 26-11-2014 ,after the draft finalized by the Fertilizers and Allied Product Technical Committee had been approved by the National Standards Committee for Chemical Division.

0.2 Bio-fertilizers (BF) are becoming very popular as agricultural input to improve crop productivity. They are considered excellent supplements to mineral fertilizers for obtaining better yields. The advances made in this field during the recent years have played a critical role in popularizing bio-fertilizers among the agricultural community. Bio-fertilizers represents the formulations consisting of either beneficial microorganisms or their metabolites which affect plant growth positively through a variety of mechanisms, such as N_2-fixation, P-solubilization, Zn-solubilization, phytohormone production, siderophore production, chitinase activity and/or ACC-deaminase activity. Bio-fertilizers consisting of (P) solubilizing bacteria help in enhancing the bioavailability of insoluble fraction of P present in soil while those carrying N_2-fixers may improve N nutrition of plants. Similarly, bio-fertilizers prepared from Zn-solubilizers or siderophores producers could improve the Zn and Fe availability respectively in the root zone. Bio-fertilizers consisting of phytohormone producers alter the plant growth positively as these compounds play very critical role in plant growth and development. Sometimes bio-fertilizers are formulated from consortia of beneficial microorganisms to develop a multifaceted impact on plant growth which enhances the efficacy of such products. In a country like Pakistan where soils are alkaline in nature and poor in organic matter content, bio-fertilizers occupy special position in achieving food security as they are the cheapest, economical and environmental friendly products capable of improving crop productivity substantially. Since, biofertilizers are usually consist of living entity which survive on some kind of organic carrier, thus needs special criteria with respect to formulations, uses and shelf life. Thus standards should be approve by the PSQCA for avoiding any kind of mall practices.

0.3 Innovative bio-fertilizers can help in better fertilizer use efficacy particularly of P which gets fixed, precipitated or insolubilized when commercial and soluble chemical fertilizers are applied to soil. This will help resolve issue of P bioavailability to plants and improve farmers' benefit from applied P through p-solubilizing Bio-fertilizers. At this stage, standard for P, K, Zn solubilization is 20% and for nitrogen fixation 10 mg of nitrogen per g of carbon is to be published/notified.

0.4 The fertilizer committee responsible for the preparation of this draft felt that it is necessary to lay down the specifications on Bio-fertilizer to safeguard the interests of the farming community and to protect them from using wrong types or fake fertilizers of similar type as well as safe guard the interest of the manufactures.

0.5 While preparing this standard the Technical Committee have taken into consideration views of all the stake holders, including manufacturers, importers, testing authorities, technologists, experts, and consumers. In addition, the existing trade practices in this field in the country have also been reviewed. Furthermore, due weightage has been given to the standards prevailing in different countries of the world for similar products.

0.6 For the purpose of deciding whether a particular requirement of this standard is complied with, the final value, observed or calculated, expressing the result of a test or analysis shall be rounded off in accordance with PS: 103 – 1991 "Rules for Rounding Off Numerical Values". The number of places retained in the rounded off value should be same as those of specified value in the standard.

0.7 In order to keep abreast with the progress of trade and industry, the Pakistan Standards are revised periodically. Suggestions from the members are welcome and will be placed before the Technical Committees for consideration at the time of revision.

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0.8 This standard is intended mainly to cover the technical provisions relating to the supply of the material, and it does not cover all the necessary provision of a contract.

1. SCOPE

1.1 This standard provides requirement and methods for Bio fertilizers use as fertilizer.
The material is known as *Bio fertilizers*

2.0 REQUIREMENT:

2.1 The material shall consist essentially of Bio fertilizers and shall be in the form ofmoist powder. It shall be free from visible foreign matters and shall comply with the requirements specified in Tables given below and tested according to the methods prescribes in Appendices.

Table-1: REQUIREMENTS FOR PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

Sr. No.	Characteristics	Requirements	Method of tests
01	Base	Carrier based or liquid based (to be specified)	-
02	Physical condition	Dark brown moist Powder	Visual inspection
03	Moisture percent by weight, maximum in case of carrier based material	40%	Appendix – B
04	Contamination level	No contamination at 10^5 dilution	Appendix – C
05	Viable cells count	CFU minimum 10^7 cells/g of carrier material or 10^7 cells/mL of liquid material	Appendix – D
06	Particle size in case of carrier based material	Powder (material shall pass through 0.15 – 0.212 mm IS sieve)	Appendix – I
07	Shelf life	12 months from date of manufacturing	Appendix – C Appendix – D
08	Efficiency character	capable of promoting root growth	Appendix – J

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Table-2: REQUIREMENTS FOR RHIZOBIUM

Sr. No.	Characteristics	Requirements	Method of tests
01	Base	Carrier based or liquid based (to be	-

		specified)	
02	Physical condition	Dark brown moist Powder	Visual inspection
03	Moisture percent by weight, maximum in case of carrier based material	40%	Appendix – B
04	Contamination level	No contamination at 10^5 dilution	Appendix – C
05	Viable cells count	CFU minimum 10^7 cells/g of carrier material or 10^7 cells/mL of liquid material	Appendix – D
06	Particle size in case of carrier based material	Powder (material shall pass through 0.15 – 0.212 mm IS sieve)	Appendix – I
07	Shelf life	12 months from date of manufacturing	Appendix – C Appendix – D
08	Efficiency character	30% increase in dry weight of plant nodules	Appendix – J

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Table-3: REQUIREMENTS FOR PHOSPHORUS SOLUBILIZING BACTERIA (PSB)

Sr. No.	Characteristics	Requirements	Method of tests
01	Base	Carrier based or liquid based (to be specified)	-
02	Physical condition	Dark brown moist Powder	Visual inspection
03	Moisture percent by weight, maximum in case of carrier based material	40%	Appendix – B
04	Contamination level	No contamination at 10^5 dilution	Appendix – C
05	Viable cells count	CFU minimum 10^7 cells/g of carrier material or 10^7 cells/mL of liquid material	Appendix – D
06	Particle size in case of carrier based material	Powder (material shall pass through 0.15 – 0.212 mm IS sieve)	Appendix – I
07	Shelf life	12 months from date of manufacturing	Appendix – C Appendix – D
08	P-solubilization capacity	20%	Appendix – E

Table-4: REQUIREMENTS FOR NITROGEN FIXING BACTERIA

Sr. No.	Characteristics	Requirements	Method of tests
01	Base	Carrier based or liquid based (to be	-

		specified)	
02	Physical condition	Dark brown moist Powder	Visual inspection
03	Moisture percent by weight, maximum in case of carrier based material	40%	Appendix – B
04	Contamination level	No contamination at 10^5 dilution	Appendix – C
05	Viable cells count	CFU minimum 10^7 cells/g of carrier material or 10^7 cells/mL of liquid material	Appendix – D
06	Particle size in case of carrier based material	Powder (material shall pass through 0.15 – 0.212 mm IS sieve)	Appendix – I
07	Shelf life	12 months from date of manufacturing	Appendix – C Appendix – D
08	Nitrogen (N) fixation capacity	10 mg of nitrogen per g of carbon source	Appendix – F

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Table-5: REQUIREMENTS FOR ZINC SOLUBILIZATION BACTERIA (ZSB)

Sr. No.	Characteristics	Requirements	Method of tests
01	Base	Carrier based or liquid based (to be specified)	-
02	Physical condition	Dark brown moist Powder	Visual inspection
03	Moisture percent by weight, maximum in case of carrier based material	40%	Appendix – B
04	Contamination level	No contamination at 10^5 dilution	Appendix – C
05	Viable cells count	CFU minimum 10^7 cells/g of carrier material or 10^7 cells/mL of liquid material	Appendix – D
06	Particle size in case of carrier based material	Powder (material shall pass through 0.15 – 0.212 mm IS sieve)	Appendix – I
07	Shelf life	12 months from date of manufacturing	Appendix – C Appendix – D
08	Zn-solubilization capacity	20%	Appendix – H

TABLE-6: REQUIREMENTS FOR POTASH SOLUBILIZING BACTERIA

Sr. No.	Characteristics	Requirements	Method of tests
01	Base	Carrier based or liquid based (to be	-

		specified)	
02	Physical condition	Dark brown moist Powder	Visual inspection
03	Moisture percent by weight, maximum in case of carrier based material	40%	Appendix – B
04	Contamination level	No contamination at 10^5 dilution	Appendix – C
05	Viable cells count	CFU minimum 10^7 cells/g of carrier material or 10^7 cells/mL of liquid material	Appendix – D
06	Particle size in case of carrier based material	Powder (material shall pass through 0.15 – 0.212 mm IS sieve)	Appendix – I
07	Shelf life	12 months from date of manufacturing	Appendix – C Appendix – D
08	K- solubilization capacity	20%	Appendix – G

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3.0 PACKING AND MARKING:

3.1 **Packing**: -The material shall be packed and supplied in sound, strong, moisture proof packages or container as agreed to between producer/marketer/distributor and purchaser/vendor, such as:Aluminum foil with mono-film polyethylene liners.

3.2. **Marking-** The container / sack shall be securely closed and marked with the following information:

a. Name of the material, namely “Bio fertilizers”
b. Total microbial count with tolerance
c. Sterilization ofcarrier material (%) with tolerance
d. Types of bacteria present i.e. N- fixers, P, K and Zn solubilizers
e. Name and Address of the manufacturer/importer/distributor; trade mark if any
f. Net Weight in kg of the material in the container
g. Any information required by law enforcement agencies or by the buyer.

3.3 **Storage Requirements:**

a. Store in cool, clean, dry and well-ventilated area at room temperature.
b. Avoid contact with sunlight for more than 48 hours.
c. Store away from oxidizers, acids, and food and drinkable items.

4.0 SAMPLING:

4.1 Representative sample of the material shall be drawn as prescribed in Appendix – A.

APPENDIX – A

SAMPLING OF BIO FERTILIZERS

A-1 GENERAL REQUIREMENTS OF SAMPLING

Since, Bio fertilizers are sterilized products therefore special care is required in case of sampling.

A-1.1 Full sealed container of biofertilizers must be considered as one sample. Do not open or tear the container before reaching it to laboratory and in laboratory open it in Laminar flow cabinet.

A-1.2 The sampling instruments shall be clean, sterilized and dry when used.

A-1.3 Precautions shall be taken to protect the samples, the material being sampled, the sampling instruments and the containers for samples from adventitious contamination.

A-1.4 Full bag should be taken as individual sample.

A-1.5 Avoid putting the bag in direct sunlight. Samples shall be stored in a cool and dry place.

A-1.6 Do not store it with pesticides or fungicides or any other chemical fertilizer.

A-2 **SCALE OF SAMPLING:**

A-2.1 **Lot** – All the containers in a single consignment of the material drawn from a single batch of manufacture shall constitute a lot. If a consignment is declared to consist of different batches of manufacture, the batches shall be marked separately and the groups of containers in such batch shall constitute separate lots.

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A-2.2 The number of containers to be chosen from a lot shall depend on the size of the lot and shall be in accordance with column 1 and 2 of Table II.

TABLE – II
NUMBER OF CONTAINERS TO BE SELECTED FOR SAMPLING

Lot Size	No. of Containers to be selected
N	N
Up to 100	5
101 to 300	6
301 to 500	7
501 to 800	8
801 to 1300	9
1301 and above	10

A-2.3 These containers shall be chosen at random from the lot, and in order to ensure randomness of selection the following procedure may be adopted.

A-2.4 Arrange all the containers in the lot in a systematic manner and starting from any container, count them 1,2,3, ……… etc up to r and so on, r being equal to the integral part of N/n. Every r^{th} containers thus counted shall be withdrawn and all such containers shall constitute the sample

.

A-3 **TEST SAMPLE AND REFEREE SAMPLE:**

A-3.1 Draw, with an appropriate sampling instrument small, portions of the material from different parts of the containers selected, the total quantity taken out from each container being sufficient to conduct the tests for all characteristics given in table-1.

A-3.2 Mix thoroughly all portions of the material drawn from the same container to form an individual test sample. Equal quantities from all individual test samples so formed shall be mixed together to form a composite test sample.

A-3.3 All the individual test samples and the composite test sample shall be divided into three equal parts, thus forming three sets of test samples. These parts shall be immediately transferred to thoroughly dried bottles that shall then be sealed air tight with glass or appropriate stopper. One of these sets of test sample shall be sent to the purchaser and another to the vendor.

A-3.4 ***Referee Sample*** – The third set of test samples bearing the seals of the purchaser and the vendor, shall constitute the referee sample and shall be used in case of dispute between the purchaser and the vendor. It shall be kept at a place agreed to between the purchaser and the vendor.

A-4 **NUMBER OF TEST:**

A-4.1 Test for the determination of moisture % shall be conducted on each of the individual test samples.

A-4.2 Test for the determination of Contamination levelshall be conducted on each of the individual test samples.

A-4.3 Test for the determination of Microbial Population shall be conducted on each of the individual test samples.

A-4.4 Test for the determination of P solubilization shall be conducted on each of the individual samples only to differentiate Bio fertilizers from other chemical phosphatic fertilizers. This test shall be conducted only if required by any of the stakeholders as defined in 0.5 of foreword.

A-4.5 Test for the determination of N fixation shall be conducted on each of the individual samples only to differentiate Bio fertilizers from other chemical fertilizers. This test shall be conducted only if required by any of the stakeholders as defined in 0.5 of foreword.

A-4.5 Test for the determination of K solubilization shall be conducted on each of the individual samples only to differentiate Bio fertilizers from other chemical fertilizers. This test shall be conducted only if required by any of the stakeholders as defined in 0.5 of foreword.

A-4.6 Test for the determination of Zn solubilization shall be conducted on each of the individual samples only to differentiate Bio fertilizers from other chemical fertilizers. This test shall be conducted only if required by any of the stakeholders as defined in 0.5 of foreword.

A-4.4 Test for the remaining characteristics given in table-1, table-2, table-3, table-4, table-5, table-6 shall be conducted on the composite test sample.

A-5 **CRITERION FOR CONFORMITY:**

APPENDIX -B

DETERMINATION OF MOISTURE IN BIO FERTILIZERS (OVEN METHOD)

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B-1 APPARATUS:

- Weighing bottle- size 50 mm x 30 mm, fitted with ground glass stopper with a hole.
- Air oven, heated electrically with temperature control system.
- Balance

B-2 PROCEDURE

B-2.1 Preparation of Sample – Weigh 5 gram of the sample into a weighing bottle using an analytical balance.

B-2.2 Determination – Place the weighing bottle containing the sample in the air oven maintained at 100 ± 2^0C. After 4 hours take the sample bottle out, and cool in a desiccator for 15 – 20 minutes. Silica-gel is desirable as desiccating agent. Reweigh the sample using an analytical balance.

B-3 CALCULATION

$$\text{Moisture percentage} = \frac{(A - B) \quad X\ 100}{\text{Sample weight (g)}}$$

Where

- A = Weight in gram before heating
- B = Weight in gram after heating.

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APPENDIX – C

DETERMINATION OF CONTAMINATION LEVEL

C-1 PRINICIPLE

To check the sterilization andnumber of bacteria in the active form per gram or milliliter of Bio-fertilizers

C-2 APPARATUS:

- Sterilized Test tubes
- Test tube stands
- Beakers
- Petri plates
- Conical flasks
- Aluminum foil
- Micropipette with sterilized tips
- Glass slides & cover slips
- Inoculum needle
- Glass droppers
- Wash bottle

C-3 EQUIPMENT:

- Laminar Air Flow Cabinet

- pH Meter
- Incubator
- Analytical Balance
- Autoclave
- Light Microscope with immersion oil

C-4 **SAMPLE PREPARATION**

C-4.1 **PROCEDURE**

Dissolve 1 g of sample in 100 ml of sterilized distilled water and mix it. Place it for 2 hours in incubator at 28±2^{0}C by covering it with aluminum foil.

C-4.2 **SERIAL DILUTION**

C-4.2.1**Principle:** Serial dilution is used to create highly diluted solutions and to reduce the concentration of cells in sample. This method involves removing a small amount of original solution and bringing that amount up to original volume using sterilized water.

C-4.2.2**Procedure:**

a. Clean and sterilized the laminar air flow cabinet with spirit or 70% alcohol.
b. Take 9 sterilized test tubes and labeled them as A, B, C, D, E, F, G, H, I. Arrange all test tubes in a stand in laminar flow cabinet.
c. Fill all test tubes with 9 ml sterilized distilled water.
d. Take 1 ml of sample from beaker and fill the test tube A and shake it well.
e. Then draw 1 ml of sample from test tube A with a pipette and transfer it to test tube B and mix thoroughly. Now, thesolution has a volume of 10 mL in test tube B.
f. Continue the same process to make the dilution upto 10^{-9}.
g. After each dilution, use a new tip for next dilution.
h. Take a loop of water from each dilution and streak it on the LB agar plates.
i. Incubate the plates for 24-48 hours at 28±2℃ in an incubator.

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C-5**PREPARATION OF LAURIA BERTANI (LB) MEDIA**

C-5.1 **CHEMICALS**

- Tryptone
- Yeast extract
- NaCl
- Agar

C-5.2 COMPOSITION OF MEDIA

Media is composed of (g/L) tryptone, 10; yeast extract, 5.0; NaCl, 5.0 and agar 15-20%. The pH should be adjusted to 7.5

C-5.3 PROCEDURE

Dissolve the chemicals in distilled water and autoclave at 121 °C for about 15–20 minutes.

C-6 GRAM STAINING

C-6.1 PRINCIPLE

The Gram stain procedure enables bacteria to retain color of the stains, based on the differences in the chemical and physical properties of the cell wall.**Gram positive bacteria** stain dark purple due to retaining the primary dye called Crystal Violet in the cell wall.**Gram negative bacteria** stain red or pink due to retaining the counter staining dye called Safranin.

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C-6.2 CHEMICALS

- Crystal violet
- Gram's Iodine
- Safranine
- Ethyl Alcohol (95%)

C-6.2.1 Standard Solution for Gram Staining (Ready solutions are available in the market):

I. Crystal Violet Solution:

Crystal violet	10 g
Ammonium oxalate	4 g
Ethanol	100 µl
Distilled water	400 ml

II. Iodine Solution

Iodine	1g
Potassium iodide	2g
Ethanol	25ml
Distilled water	100ml

III. Iodated Alcohol

Iodine solution II	5 ml
Ethanol	95 ml

IV. Counter Stain

2.5 % safranine methanol	10 ml
Distilled water	100 ml

C-6.3 PROCEDURE

a. Transfer a loopful of culture using a sterile cool loop on a clean glass slide, and spread over a small area. Allow the film to air dry and fix it by passing through the Bunsen flame without exposing the dried film directly to the flame. The slide should not be so hot as to be uncomfortable to the touch.
b. Flood the slide with crystal violet solution for up to one minute. Wash off briefly with tap water (not over 5 seconds). Drain.
c. Flood slide with Gram's Iodine solution, and stay it for about one minute. Wash off with tap water. Drain.
d. Remove excess water from slide and blot, so that alcohol used for decolorization is not diluted. Flood slide with 95% alcohol for 10 seconds and wash off with tap water. Drain the slide.
e. Flood slide with safranin solution and allow counterstaining for 30 seconds. Wash off with tap water and allow it to air dry.
f. All slides of bacteria must be examined under the oil immersion lens.

APPENDIX – D
DETERMINATION OF MICROBIAL POPULATION

D-1 PRINCIPLE

The most widely used method for determining bacterial numbers is the **viable/platecount method.** The standard plate count method consists of diluting a sample with sterile saline solution until the bacteria are dilute enough to count accurately.

D-2 APPARATUS/ EQUIPMENT

Same as given in Appendix-C (C-2, C-3)

D-3 PROCEDURE

a. Sterilized all glassware by autoclaving
b. Label the bottom of petri plates 1-9.
c. Label test tubes of saline 10^{-1} to 10^{-9}.
d. Using aseptic technique, the initial dilution is made by transferring 1mL of sample to a 9mL sterile saline blank.
e. The 10^{-1} dilution is then shaken by grasping the tube between the palms of both hands and rotating quickly to create a vortex. This serves to distribute the bacteria and break up any clumps.
f. Immediately after the 10^{-1} dilution has been shaken, uncap it and aseptically transfer 1mL to a second 9mL saline blank.
g. Repeat the process several times to produce a 10^{-9} dilution.
h. Aseptically transfer 1.0 mL to one petri plate. Do the same for all dilutions.
i. Carefully remove the cover from the petri plate and aseptically pour the LB agar into it. The agar and sample are immediately mixed gently moving the plate in a figure-eight motion or a circular motion while it rests on the tabletop. Repeat this process for the remaining eight plates.
j- After the pour plates have cooled and the agar has hardened, they are inverted and incubated at 28 ± 2^0C for 24 hours.

k- Calculate the number of bacteria (CFU) per milliliter or gram of sample by dividing the number of colonies by the dilution factor multiplied by the amount of specimen added to liquefied agar.

$$\textbf{Number of colonies (CFUs)} = \frac{\text{No. of bacteria/mL}}{\text{Dilution factor X amount plated}}$$

j. Record the final results for all dilutions.

APPENDIX – E
DETERMINATION OF PHOSPHATE SOLUBILIZATION

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E-1 PRINCIPLE

Phosphate solubilizing bacteria (PSB) used to convert insoluble rock P material into soluble forms available for plant growth. The principleconversion is carried out through acidification, chelation and exchange reactions and production of organic acids, acid phosphatases which play a major role in the mineralization of organic phosphorus in soil.

E-2 CHEMICALS

- Glucose
- $MgSO_4.7H_2O$
- $CaCl_2$, $Ca_3(PO_4)_2$
- $(NH_4)_2SO_4$
- NaCl,
- Yeast Extract
- $MnSO_4.H_2O$
- $FeSO_4.7H_2O$
- Agar

E-3 COMPOSITION OF PIKOVSKAYA MEDIA

Media is composed of (g/L) glucose, 10; $MgSO_4.7H_2O$, 0.1; $CaCl_2$, 0.2; $Ca_3(PO_4)_2$, 5.0; $(NH_4)_2SO_4$, 0.5; NaCl, 0.2; yeast extract, 0.5; $MnSO_4.H_2O$, 0.002; $FeSO_4.7H_2O$, 0.002; and agar 15-20%.

E-4 PROCEDURE

The initial pH of the medium was adjusted to 7. The plates were incubated at 37 °C for four days and the ability of isolates to solubilize the phosphate was assessed by observing halo zone around the growing colonies indicating phosphate solubilization.

E-5 QUANTITATIVE ESTIMATION OF PHOSPHATE SOLUBILIZATION

Bacterial isolates were inoculated in Pikovskaya's broth (100 mL) in 250 mL of Erlenmeyer flasks and incubated at 28 ± 2°C for 5 days in rotary shaker at 200 rpm. Triplicates were maintained for each treatment. After incubation the bacterial cultures were filtered through Whatman No.1 filter paper and were clarified by centrifugation at 10,000 rpm for 10 minutes. Uninoculated broth served as control. To determine phosphorus (P) in the cell free culture 1 mL of the supernatant was taken, 2.5 mL of Barton's reagent (see below) was added and volume was made up to 50 mL with distilled water. After 10 minutes, the intensity of yellow color was read on spectrophotometer at 430nm and the amount of P-solubilized was extrapolated from the standard curve.

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Barton's Reagent:

a. Dissolve 22.5g of ammonium heptamolybdate $[(NH_4)_6Mo_7O_{24}.H_2O]$ in 400 mL of distilled water.
b. Dissolve 1.25 g of Ammonium metavendate $[NH_4VO_3]$ in 300 mL hot distilled water.
c. Add (b) to (a) in one liter volumetric flask and let the mixture cool to room temperature.
d. Slowly add 250 mL concentrated nitric acid (HNO_3) to the mixture, cool the solution to room temperature and bring to 1L volume with distilled water.

Standard stock P solution:

Weigh 0.2195 g of potassium dihydrogen phosphate (KH_2PO_4) (dried in oven at 60ºC for 1 hr and cooled in a dessicator) and dissolved in 1L of distilled water. For preparation of working solution, 150 mL of this solution was taken and volume was made up to 250 mL with distilled water. Standard curve was prepared by taking 2, 3, 5, 8, 10, 15, 20, 25 and 30 mL of the working solution in each 50 mL volumetric flask and 2.5 mL of Barton's reagent was added to each flask and the volume was made up to 50 ml with distilled water. After 10 minutes, the intensity of yellow color developed was read at 430nm spectrophotometrically.

The following formula is used for calculations:

$$\% \text{ P Contents} = \frac{\text{ppm reading from calibration curve} \times \text{Dilution factor}}{10,000}$$

APPENDIX – F

DETERMINATION OF NITROGEN FIXATION

F-1 PRINCIPLE

Atmospheric nitrogen is fixed by Rhizobium (rhizobia) bacteria in the root nodules of legumes in the form of complex compounds of nitrogen. Some bacteria are free-living organisms and have the capacity to absorb atmospheric N_2 to synthesis organic nitrogenous compounds and make it available to plants.

F-2 CHEMICALS

- KH_2PO_4
- K_2HPO_4
- $MgSO_4$
- NaCl
- $CaCl_2$
- $FeCl_3$. $6H_20$
- $NaMoO_4.2H_20$
- Yeast extract
- L-malic acid
- Mannitol
- Agar

F-3 COMPOSITION OF NITROGENFREE MALATE-MANNITOL MEDIA

The media is composed of (g/L) KH_2PO_4, 0.4; K_2HPO_4, 0.1; $MgSO_4$, 0.097; NaCl, 0.1; $CaCl_2$, 0.0196; $FeCl_3.6H_20$, 0.002; $NaMoO_4.2H_20$, 0.002; yeast extract, 0.001; L-malic acid, 3.58; mannitol, 5.0 and agar 15-20%.

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F-4 PROCEDURE

The initial pH of medium was adjusted to 7.0 with KOH prior to the addition of the agar. Nitrogen free Malate media, containing bromothymol blue (BTB) as an indicator, was used for preliminary screening and incubated at 37ºC up to 24h. The blue colored zone producing isolates were marked as nitrogen fixers in the solid culture conditions. The coloring zone was calculated by deducting the colony diameter from the coloring zone diameter.

F-4 QUANTITATIVE TEST FOR NITROGEN

1. Take 2 g of sample.
2. Add 4 mL of concentrated H_2SO_4 and 0.1 g Salicylic acid (2.5 %) and kept it overnight for digestion. Add 0.2 g $Na_2S_2O_3$ and heat it upto 150 ºC for 1 hr.
3. Take 1 g of digestion mixture and kept it at 375 ºC for 2 hrs.
4. Fill ¼ of distillation tube with distilled water.
5. Take 5 mL of boric acid in flask and put it for distillation by adding alkali.
6. Take about 50 mL of distillate (green coloured) and titrate it against N/50 H_2SO_4 in the burette until the color become pink.
7. Take the reading and calculate total N according to the formula:

$$\text{N content} = 14 \times \frac{\text{(Millimeters of 0.1 N acid for sample- Millimeters of 0.1 N acid for blanks)}}{\text{Mass of sample taken}}$$

APPENDIX – G

DETERMINATION OF POTASH SOLUBILIZATION

G-1 PRINCIPLE

Potassium solubilizing bacteria (KSB) are able to solubilize rock K mineral powder, such as micas, illite and orthoclases, through production and excretion of organic acids. KSB increased K availability in soils and increased mineral content in the plant.

G-2 CHEMICALS

- Glucose
- $MgSO_4.7H_20$
- $FeCl_3$
- $CaCO_3$
- Potassium Mineral
- $Ca_3(PO_4)_2$
- Agar

G-3 COMPOSITION OF ALEKSANDROV'S MEDIA

The media is composed of (g/L) glucose, 5.0; $MgSO_4.7H_20$, 0.005; $FeCl_3$, 0.1; $CaCO_3$, 0.1; potassium, 2.0; $Ca_3(PO_4)_2$, 2.0 and agar 15-20%.

G-4 PROCEDURE

Bacterial strains were screened for their ability to solubilize potassium (K). 1 microlitres of 24 hours old bacterial culture were spot inoculated into the well. Then incubate at 28±2^{0}C for 15 days and clearing zone was measured.

G-5 QUANTITATIVE ESTIMATION OF K

The isolates showing zone of solubilization on Aleksandrov agar were futher examined for their ability to release K from broth medium (supplemented with 1 percent potassium mineral). 1 mL of overnight culture of each isolate was inoculated to 25 mL of Aleksandrov broth in triplicates. All the inoculated flasks were incubated for two weeks at 28±2^{0}C. The broth culture was centrifuged at 10,000 rpm for 10 minutes to separate the supernatant from the cell growth and insoluble potassium. The available K content in the supernatant was determined by flame photometry. 1 mL of the culture supernatant was taken and the volume was made to 50 mL with distilled water and mixed thoroughly. After that the solution was fed to flame photometer and K content was determined. The amount of potassium solubilized by isolates was calculated from the standard curve.

Preparation of standard curve:

Potassium chloride was dried at 60 ^{0}C and 1.908 g of it was dissolved in distilled water and made up volume to one liter. 10 mL of this was further dissolved to 100 mL with distilled water to obtain 2 ppm solution and used for preparation of standards 0, 2, 4, 6, 8, 10 ppm. These standards were fed to flame photometer to obtain K standard curve.

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APPENDIX – H

DETERMINATION OF ZINC SOLUBILIZATION

H-1 PRINCIPLE

Bacteria produce organic acids and these acids act as agent causing zinc solubilization

H-2 CHEMICALS

- Glucose
- $(NH_4)_2SO_4$
- KCl
- K_2HPO_4
- $MgSO_4$
- ZnO
- Agar

H-3 COMPOSITION OF MINERAL SALT MEDIA

The media is composed of (g/L) glucose, 10; $(NH_4)_2SO_4$, 1.0; KCl, 0.2; K_2HPO_4, 0.1; $MgSO_4$, 0.2; ZnO, 10 and agar 15-20%. The pH should be adjusted to 7.0

H-4 PROCEDURE

Bacterial strains were screened for their ability to solubilize zinc in mineral salt agar medium. 1 microlitres of 24 hours old bacterial culture were spot inoculated onto the medium then incubate at 28±2^{0}C for 15 days and clearing zone was measured.

H-5 QUANTITATIVE ASSAY FOR ZINC SOLUBILIZATION

All the strains were further evaluated for Zn solubilization efficiency. The cultures were grown in mineral salts broth (100 mL) amended with 0.1 % of insoluble zinc salts (Zn0). Flasks containing 150 mL of sterile broth were inoculated with 100 mL of 4 h old actively growing test bacterial cultures and incubated at 28±2^{0}C for 10 days with a shaking at 140 rpm. The sample was drawn and centrifuged at 10,000 rpm for 10 min to remove cell debris. Available Zn was assessed by atomic absorption spectrophotometry.

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APPENDIX – I

DETERMINATION OF PARTICLE SIZE

I-1 APPARATUS / EQUIPMENT

- Stainless steel sieves of the required mesh size with lid & bottom pan.
- Sieves Shaker
- Top loading balance
- Brush

I-2 METHOD

I-2.1 Arrange the individually tare sieves in descending order of mesh size from top to bottom.
I-2.2 Place receiving pan on the bottom of stack
I-2.3 Weigh about 200 to 300g of sample taken through sample divider.
I-2.4 Transfer sample onto the top sieve place lid on top of stack.
I-2.5 Place the sieve stack on shaker and tighten the belts evenly on both sides.
I-2.6 Set timer of vibrator to 5 minutes amplitude at 3.0 mm and start the vibrator.
I-2.7 After shaking stops switch-off the vibrator and remove the sieves one by one.
I-2.8 Weigh sieve + sample on top loading balance.
I-2.9 Calculate the weight of samples retained on each sieve.

I-3 CALCULATIONS

I-3.1 Calculate weigh percent on each sieve by following formula

$$W = \frac{\text{Weight (g) on sieve}}{\text{Total weight of sample}} \times 100$$

APPENDIX – J

DETERMINATION OF PGPR ACTIVITY

J-1 PRINCIPLE

Pant growth promoting rhizobacteria (PGPR) are soil bacteria that colonize the roots of plants following inoculation onto seed and enhance plant growth. The ineffectiveness of PGPR in the field has often attributed to their inability to colonize plant roots.

J-2 IMPORTANT STEPS

a. Take 4-5 kgs of good garden soil and sterilize it in an autoclave at 121 °C for about 30 minutes. After cooling fill the soil in 6 small pots.
b. Select the right combination of seed and bio fertilizers.
c. Treat half of the seeds with appropriate bio fertilizer using standard seed treatment method.
d. Pots should be sown with treated seeds while 3 pots are sown with untreated seeds to serve as control.
e. Keep the pots in protected open space and ensure daily watering.
f. Observe after 15-20 days for root length.

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J-3 OBSERVATIONS

J-3.1 FOR LEGUMES

In case of leguminous seeds treated with right type of Rhizobium bio fertilizers, the treated plants will show adequate nodulation on roots with higher plant weight compared to untreated control, which will show long roots as compared to control.

J-3.2 FOR NON LEGUMES

In case of non-legume, treat appropriate test host seeds (such as wheat, rice, maize etc.) with bio fertilizers, the treated plant will be healthier, having green color and higher plant weight compared to untreated control which will show lesser plant weight and pale plant color. The roots should be longer than the control.