ISOLATION OF MICROBES FOR THE PRODUCTION OF BIOFERTILIZER AND CHECKING IT'S EFFICIENCY ON PLANTS

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ABSTRACT:

Biofertilizers are biological preparations of efficient microorganisms that promote plant growth by improving nutrient acquisition.
They enhance soil productivity by fixing atmospheric nitrogen, solubilizing soil phosphorus, and stimulating plant growth.
Biofertilizer technology has integrated plant nutrient management for sustainable agriculture through BNF. The formulation of biofertilizers is a crucial multistep process that includes mixing of a suitable carrier with inoculant, providing optimal conditions during storage, packaging, and dispatch and ensuring survival and establishment after introduction into soils.
INDEX TERM: Biofertilizers, Nitrogen Fixing, Phosphate Solubilizing, Microbial

INTRODUCTION:

Biofertilizers are microbial inoculants or carrier based preparations containing living or latent cells of efficient strains of nitrogen fixing, phosphate is solublizing and cellulose decomposing microorganisms intended for seed or soil application

The need for the use of Biofertilizers has arisen primarily due to two reasons i.e. though chemical fertilizers increase soil fertility, crop productivity and production, but increased / intensive use of chemical fertilizers has caused serious concern of soil texture, soil fertility and other environmental problems, use of Biofertilizers is both economical as well as environment friendly and designed to improve soil fertility and plant growth by increasing the number and biological activity of beneficial microorganisms in the soil.

The objects behind the application of Biofertilizers /microbial inoculants to seed, soil or compost pit is to increase the number and biological / metabolic activity of useful microorganisms that accelerate certain microbial processes to augment the extent of availability of nutrients in the available forms which can be easily assimilated by plants.

Mechanisms of phosphate solubilization: The principal mechanism for mineral phosphate solubilization is the production of organic acids, and acid phosphatase play a major role in the mineralization of organic phosphorus in soil. It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms. Production of organic acids results in acidification of the microbial cell and its surroundings. The production of organic acids by phosphate solubilizing bacteria has been well documented. Gluconic acid seems to be the most frequent agent of mineral phosphate solubilization. Also, 2-ketogluconic acid s another organic acid identified in strains with phosphate

solubilizing ability. Strains of Bacillus were found to produce mixtures of lactic, isovaleric, isobutyric and acetic acids. Other organic acids, such as glycolic, oxalic, malonic, and succinic acid, have also been identified among phosphate solubilizers. Strains from the genera Pseudomonas, Bacillus and Rhizobium are among the most powerful phosphate solubilizers.

Nitrogen fixing bacteria: Biological nitrogen fixation (BNF) refers to a microbial mediated process based upon an enzymatic "Nitrogenase" conversion of atmospheric nitrogen (N2) into ammonium readily absorbable by roots. N2-fixing microorganisms collectively termed as "diazotrophs" are able to fix biologically N2 in association with plant roots.

Specifically, the symbiotic rhizobacteria induce structural and physiological modifications of bacterial cells and plant roots into specialized structures called nodules.

Other N2-fixing bacteria are free-living fixers that are highly diverse and globally widespread in cropland.

They represent key natural source of nitrogen (N) in natural and agricultural ecosystems lacking symbiotic N fixation (SNF).

In this paper, the importance of Azotobacter species was highlighted as both important free-living N2-fixing bacteria and potential bacterial biofertilizer with proven efficacy for plant nutrition and biological soil fertility.

MATERIALS & METHODS

1. SOIL SAMPLE COLLECTION

i. Soil was collected from hilly region (Bhramhagiri Nashik).
ii. Soil sample were collected and placed in sterile plastic bags, then taken to the laboratory.
iii. The debris from the soil sample were removed after collection (As in Figure no. 1).

Figure no 1 (Soil Sample Collection)

2. ISOLATION OF PHOSPHATE SOLUBILIZING BACTERIA FROM SOIL SAMPLES

- Soil bacteria were isolated by the Standard Serial Dilution and Spread Plate method.

i Requirement:-

a. Soil sample
b. Saline solution
c. Sterile test-tubes
d. Sterile Nutrient Agar plates
e. Micropipette
f. L-shape glass rod

ii Protocol:-

i. In which 1gm of each soil sample was weighed and mixed in 10 ml of Sterile Saline.
ii. Then the 1ml from first tube is added to the next tube containing 9 ml Sterile Saline, Continue the Dilution till the 9^{th} tube. (as in figure no.2)
iii. Out of the 6 dilutions, 100µl from the each dilution (10^{-2}, 10^{-4}, 10^{-6} 10^{-8}, 10^{-4}) were spreaded on sterilized Nutrient Agar Plates by sterile spreader under aseptic conditions.
iv. These plates were incubated in incubator at 37^0C for 24 hrs. for bacterial colony. (as in figure no. 3)

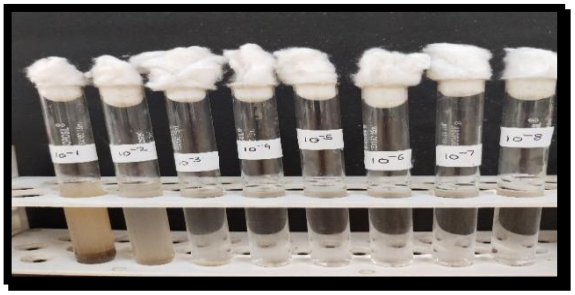


Figure No. 2 (Standard Serial Dilution)

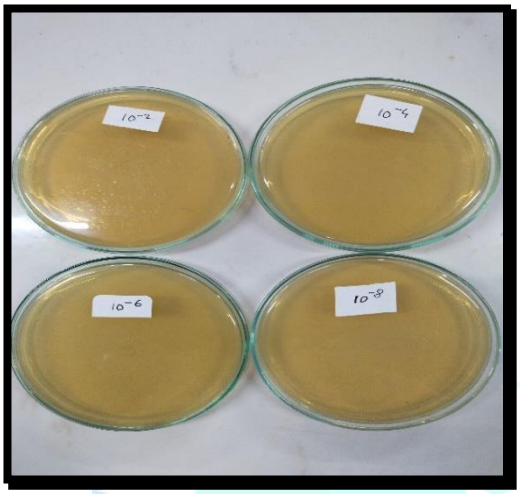


Figure No. 3 (Spread Plate Method)

3. PREPARATION OF PURE CULTURE AND SCREENING OF BACTERIAL STRAINS

iii Requirement:-

a. Inoculating loop (wire loop)
b. Sterile nutrient agar slants
c. Plates of serial dilutions
d. Pikovaskya Agar plates.

iv Protocol:-

i. The inoculating loop was sterilized by putting it in flame till red hot.
ii. After cooling it down, the well isolated colonies were picked from the spread plates and were streaked on the Nutrient Agar slants
iii. Slants were incubated for 24hrs at 37^0C. Then stored at 4^0C for subsequent studies. (As in figure no.4).
iv. After incubation this culture streaked on Pikovaskya Agar plates.
v. Incubate the plates at 37°C for overnight.
vi. Next day pure isolated Phosphate solubilizing bacteria confirmed.

Figure No. 4 (Pure Culture)

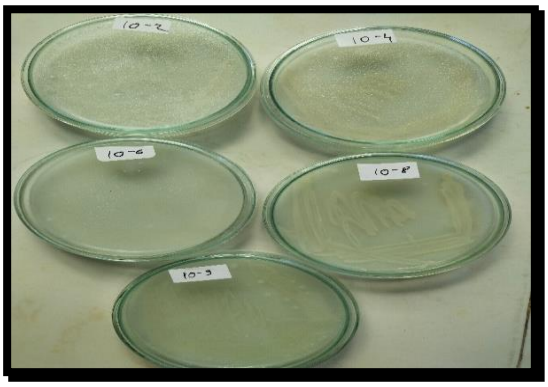


Figure No.5 (phosphate solubilizing bacteria)

3. ISOLATION OF NITROGEN FIXING BACTERIA FROM SOIL SAMPLE

- **Requirement:-**
- soil sample
- ASHBY's broth media.

Protocol:-

i. Add 1 gm of soil sample in the autoclaved ASHBY's broth media.
ii. Incubate the flask at 37°c for 7-8 days.
iii. After incubation streaked on autoclaved ASHBY's mannitol agar plates.
iv. Incubate the plates at 37°c for 24-48 hours.

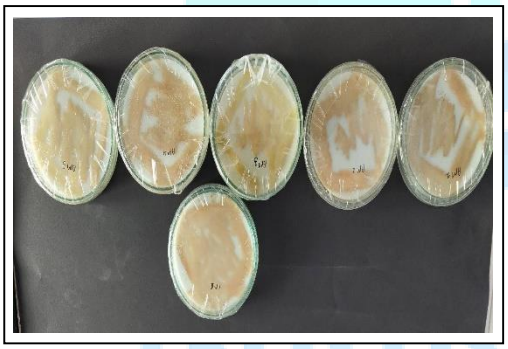


Figure No. 6(NF BACTERIA)

4. MORPHOLOGICAL CHARACTERIZATION OF BACTERIA

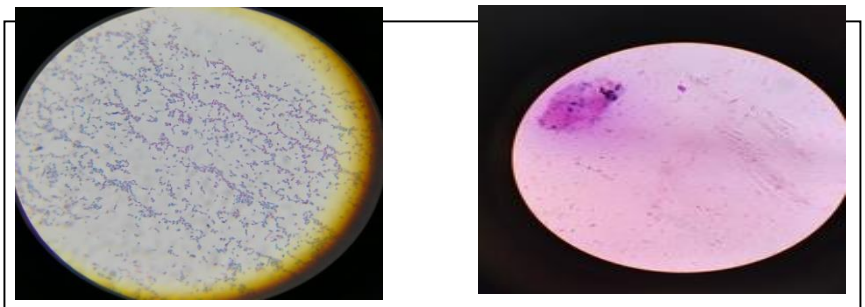
➢ Morphological characterization of bacteria was performed by Gram's Staining.

v Requirements:-

a. Bacterial suspension
b. Wire-loop
c. Slides
d. Crystal violet
e. Decolorizer (ethanol)
f. Gram's iodine
g. Distilled water
h. Tap water
i. Burners

vi Protocol:-

i. Bacterial suspension was made in sterile saline and was used.
ii. Under aseptic conditions, loop full suspension was picked and smear was made on a clean grease free slide. Then they were air-dried and heat-fixed.
iii. Stain the smear with Crystal Violet Stain for 1 minute & wash the slide in gentle and indirect stream of tap water for 2 sec.
iv. Flood the smear with Gram's Iodine and wait for 1 minute & wash the slide in gentle and indirect stream of tap water for 2 sec.
v. Add drop by drop Gram's Decolorizing agent to smear till slide runs clear.
vi. Flood slide with counter stain, Safranin and wait for 30 seconds & Wash the slide in gentle and indirect stream of tap water until no color appears and then blot dry with absorbent paper.
vii. Smear was covered with Oil Immersion and observed under microscope.(as fig.no.7)



(PS bacteria) (NF bacteria)

Figure No. 7 (Gram's staining)

5. BIOCHEMICAL CHARACTERIZATION OF BACTERIA

5.1. CATALASE TEST

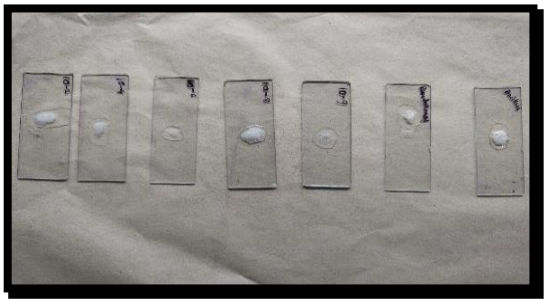
- **Requirement:-**
  a. Wire loop
  b. Ethanol
  c. Slides
  d. 3% H_2O_2 (Hydrogen Peroxide)
  e. Pure bacterial suspension

Protocol:-

i. Taken a loop full of bacterial suspension and make smear on an ethanol cleaned grease free slide.
ii. Then add 1 drop of 3% H_2O_2 (Hydrogen Peroxide) (do not mix or cover the slide with cover slip) and place slide in Petri plate and observe instantly. (figure no. 8)

Figure No. 8 (Catalase Test)

5.2. METHYL RED TEST



vii Requirement:-

a. MRVP Broth
   Buffered peptone…….7 g/L
   Glucose……5 g/L
   Dipotassium phosphate……5 g/L
   pH……6.9

b. Methyl Red Solution (0.02%)
   Methyl red…….0.1 g
   Ethyl Alcohol……300 ml
   Distilled Water……..makeup volume to 500 ml

c. Wire loop
d. Pure bacterial culture
e. Sterile test-tubes
f. dropper

viii Protocol:-

i. Take 2 ml MRVP Broth in tubes and inoculate it with pure bacterial culture.
ii. Incubate the tubes in incubator at 37^0C for 24 hrs.
iii. Add 2-3 drops of Methyl red Indicator to tubes and observe it immediately. (figure no. 9)

(PS bacteria) (NF bacteria)

Figure No. 9 (Methyl Red Test)

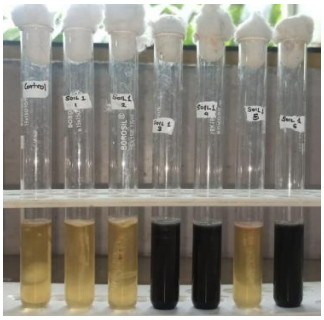
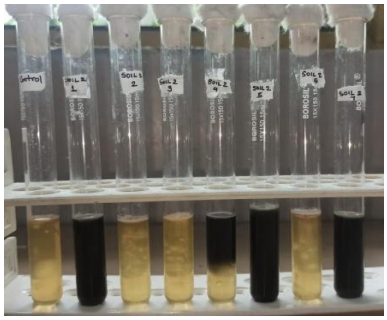
5.3. MOTILITY TEST

ix Requirement:-

a. SIM (Sulphur, Indole, Motility) Media
   Pancreatic Digest of Casein……20 g/L
   Peptic Digest of Animal Tissue…..6.1 g/L
   Agar……3.5 g/L
   $Fe(NH_4)_2(SO_4)_2.6H_2O$ …… …0.2 g/L
   $Na_2S_2O_3.H_2O$…… … …..0.2 g/L
   Ph……7.3

b. Wire-loop
c. Pure bacterial culture
d. Sterile test-tubes

- **Protocol:-**
  i. Take pure bacterial culture with wire loop and stab the colony in SIM Media to 1/3 inch in middle of tube and remove.
  ii. Incubate the tubes in incubator for 7 days at 35-37^{0}C.(figure.no.10)

(PS bacteria) (NF bacteria)

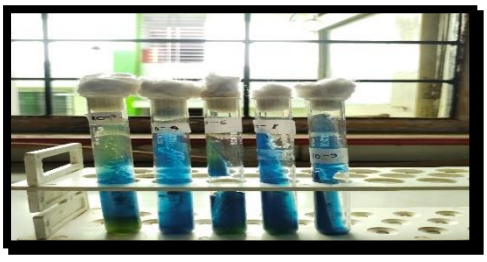
Figure No. 10 (Motility Test)

5.4. CITRATE UTILIZATION TEST

- **Requirement:-**
  a. Simmons's Citrate Agar
     $NaCl_2$.....5 g
     Sodium Citrate (dehydrate)…….2 g
     Ammonium dihydrogen phosphate…….1 g
     Dipotasium phosphate……..1 g
     Magnesium Sulphate (heptahydrate)…0.2 g
     Bromothymol Blue……...…..0.08 g
     Agar……..……15 g
     Deionized water…….....1000 ml
     Ph……6.9
  b. Pure bacterial culture
  c. Sterile test-tubes
  d. Wire-loop
- **Protocol:-**
  i. Make the Simmons's Citrate Agar slants.
  ii. Pickup pure bacterial culture with wire loop and streak from center.
  iii. Incubate the tubes in incubator for 4-7 days at 35-37^{0}C.(figure no.11)

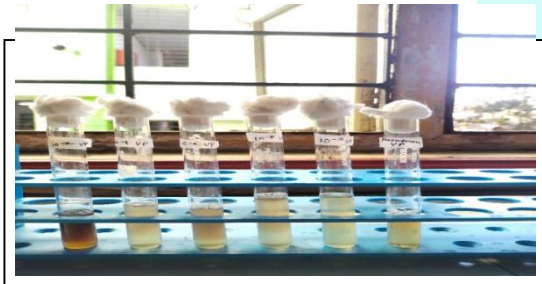
(PS bacteria) (NF bacteria)

Figure No. 11 (Citrate Utilization Test)

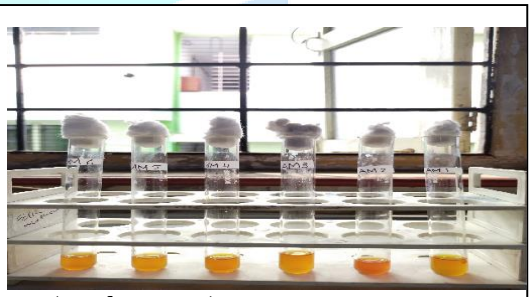



5.5. VOGES-PROSKAUER TEST

- **Requirement:-**
  a. MRVP Broth
     Buffered peptone……7 g/L
     Glucose……5 g/L
     Dipotassium phosphate……5 g/L
     pH……6.9
  b. Voges-Proskauer Reagent A
     α-napthol 5%.....50 g
     Absolute Ethanol……..1000 ml
  c. Voges-Proskauer Reagent B
     Potassium hydroxide……400 g
     Deionized Water……1000 ml
  d. Pure bacterial culture
  e. Sterile test-tubes
  f. Wire-loop
- **Protocol:-**
  i. Take 2 ml of MRVP Broth in test-tubes and inoculate tubes with pure bacterial culture. Incubate tubes in incubator at 37^0C for 24 hrs.
  ii. Add 6 drops of Voges-Proskauer Reagent A in test-tubes and mix well to aerate & Add 2 drops of Voges-Proskauer Reagent B in test-tubes and mix well to aerate.
  iii. Observe for pink-red color at surface within 30 minutes.
  iv. Shake the tubes vigorously during 30 min period. (figure no. 12)



(PS bacteria)



(NF bacteria)

Figure No. 12 (Voges-Proskauer Test)

➢ **All biochemical results in table no. 1, table no. 2**

Sr. no.	Gram's staining	Catalase Test	Indole Test	Methyl Red Test	Motility Test	Citrate Utilization Test	Voges-Proskauer Test
1	+ve	+ve	-ve	-ve	-ve	-ve	-ve
2	+ve	+ve	-ve	+ve	-ve	-ve	-ve
3	-ve	+ve	-ve	-ve	+ve	+ve	+ve
4	-ve	+ve	-ve	-ve	+ve	+ve	+ve
5	+ve	+ve	-ve	+ve	-ve	-ve	+ve
6	-ve	+ve	-ve	-ve	+ve	-ve	+ve
Control	------	------	-ve	-ve	-ve	-ve	-ve

Table No. 1 (Phosphate Solubilizing)

Sr. no.	Gram's staining	Catalase Test	Indole Test	Methyl Red Test	Motility Test	Citrate Utilization Test	Voges-Proskauer Test
1	-ve	+ve	-ve	+ve	+ve	-ve	+ve
2	-ve	+ve	-ve	+ve	+ve	+ve	-ve
3	-ve	+ve	+ve	-ve	-ve	-ve	-ve
4	+ve	+ve	-ve	+ve	+ve	+ve	+ve
5	+ve	+ve	+ve	+ve	+ve	+ve	+ve
6	+ve	+ve	+ve	-ve	-ve	-ve	+ve
7	+ve	+ve	+ve	-ve	+ve	-ve	+ve
Control	------	------	-ve	-ve	-ve	-ve	-ve

Table No. 2 (Nitrogen fixing)

6. PRODUCTION OF BIOFERTILIZERS USING MICROBIAL ISOLATES

- **Requirement:-**
  1. microbial isolates
  2. charcoal powder
  3. sterile saline
- **Protocol:-**
  i. Weigh 5gm charcoal powder.
  ii. Prepare 5 ml suspension of microbial isolates using sterile saline.

iii. Mix the suspension with charcoal powder and allow to dry it Packed the biofertilizers and stored it for the further experiment (Figure no 13)

Figure No. 13 (production of biofertilizer)

7. CHECKING EFFICIENCY OF BIOFERTILIZERS USING POT ASSAY

Experiments performed with pot-grown plants are probably the most common in plant research. This pot test is a quick and simple method to make comparable studies.

- Wheat (Triticum aestivum) seeds were selected to carry out pot trials. (As they contain less vigour and therefore would not give biased results).
- The soil was mined to a depth of 20 cm after removing surface litter and was passed through a sieve /screen in the field via a large mesh.
- All seeds were washed thoroughly and soaked in distilled water for 30 minutes.
- Seeds were sowed in pots containing soil.
- They were given biofertilizer treatments and allowed to grow for 3 week until seedlings developed
- At maturity, plants were harvested and different yield parameter and Growth attributes were measured of the given seedlings.

8. RESULT AND DISSCUSION

➢ SOIL ANALYSIS

Sr. no	Tested nutrient	Available content
1	Nitrogen	206.57 kg/ha
2	Potassium	468.83 kg/ha
3	Phosphorus	19.49 kg/ha
4	Ph	8.5
5	Electrical conductivity	0.34 mS/m
6	Organic carbon	0.78 %
7	Sodium	61.65 %
8	Magnesium	16.06 %
9	Calcium	33.85 %
10	Calcium carbonate	8.50 %

Table 3: soil analysis

- **POT ASSAY**
  - Effects of microorganisms on plant growth parameters such as Shoot length, Root length, fresh weight, Dry weight, of selected crop were studied influence of biofertilizers treatments.
  - The biofertilizers was produced using microorganisms by isolating bacillus and azotobacter was added in Triticum aestivum, and allowed to grow up to mature seedlings.

A. Triticum aestivum showed growth when treated with biofertilizer treatment.

- Triticum aestivum+ phosphate solubilizing biofertilizer

Wet weight(gm)	2.080
Dry weight(gm)	0.645

Sr. No.	Week 1					Week 2					Week 3				
Crop	Wheat					Wheat					Wheat				
Sample	Test				con-trol	Test				con-trol	Test				con-trol
Shoot Height	3.4	4	3.8	4.7	3	6.4	6.5	6.9	6.1	5	6.8	8.1	7.1	8.5	7
Rot Height	6	6.9	7	9.4	5	11	10.5	12	9	10	13	11.8	12.1	13.4	11

Pot trials and growth parameter of wheat + phosphate solubilizing biofertilizer

- Triticum aestivum+ nitrogen fixing biofertilizer

Wet weight	2.940
Dry weight	1.100

Sr. No.	Week 1					Week 2					Week 3				
Crop	Wheat					Wheat					Wheat				
Sample	Test				con-trol	Test				con-trol	Test				con-trol
Shoot Height	4	2.5	4.2	5	3	7.1	6.5	8	6.9	5	7.3	8.1	9.3	8.5	7
Rot Height	7	5	8.4	7.1	5	12	10.5	11	12.4	10	13	12.9	13.7	12.3	12

Pot trials and growth parameter of wheat + nitrogen fixing biofertilizer

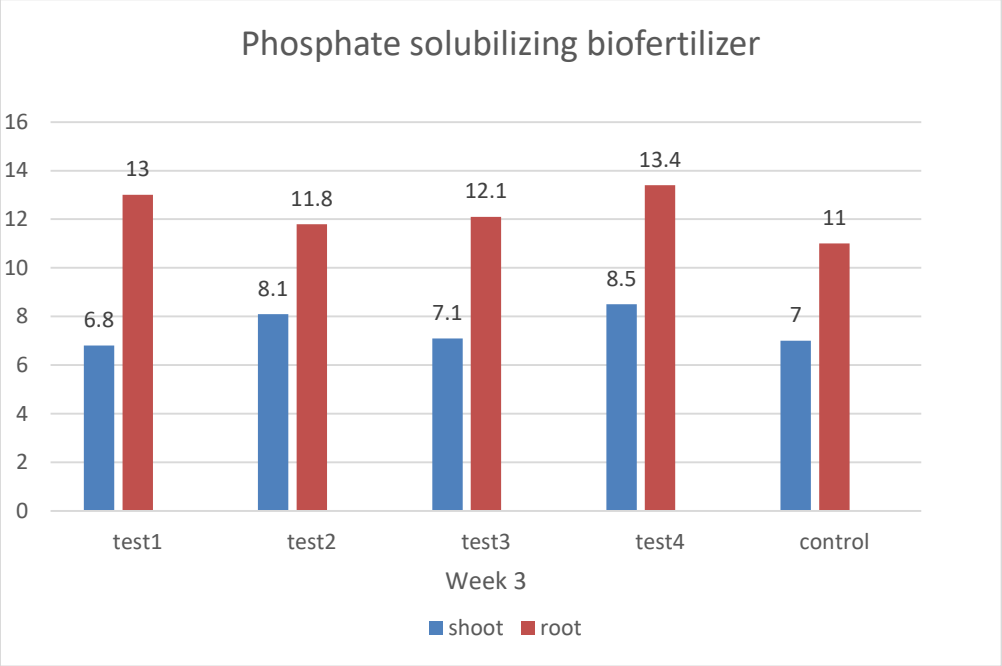


Fig.14: Growth parameter of phosphate solubilizing biofertilizer

Fig.15: Growth parameter of nitrogen fixing biofertilizer

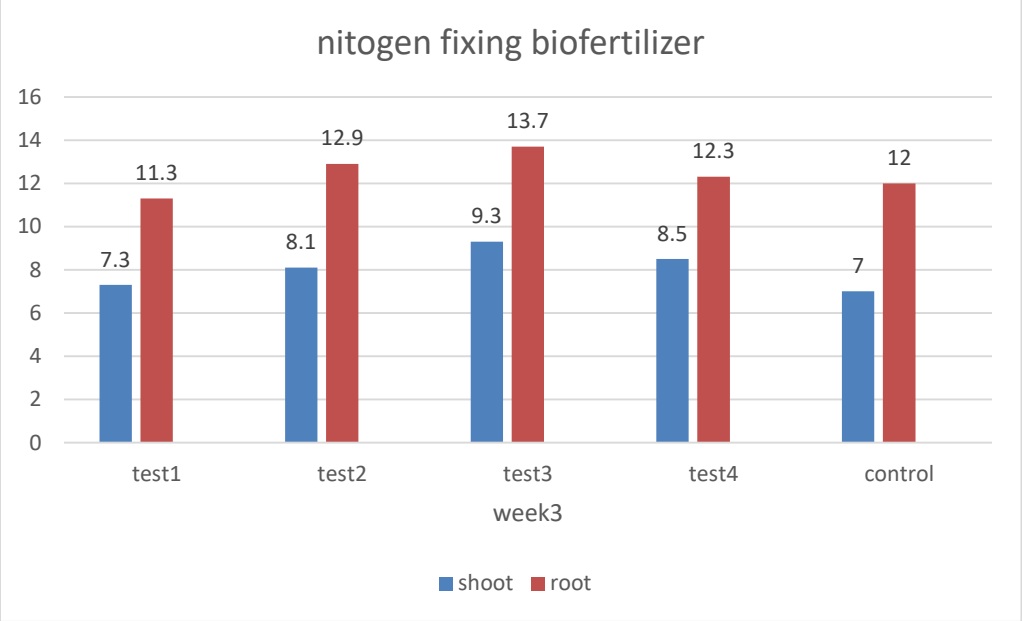


Fig.16: PSB POT ASSAY

Fig.17: NF ASSAY

Fig.18: Shoot & Root length of wheat

Phosphate solubilising bacteria are very important in solubilization of insoluble phosphate to soluble phosphate by release of organic acids. Thus the biofertilizers production through the selective and optimized media and mass production and then tested on plants.

Nitrogenous biofertilizer help in increasing crop productivity by way of increased BNF, increased availability or uptake of nutrients or increased absorption and stimulation of plant growth through hormonal action or antibiosis, or by decomposition of organic residues. Furthermore, nitrogenous biofertilizers as to replace part of the use of chemical N- fertilizers reduces amount and cost of chemical N-fertilizers and thus prevents the environment pollution from extensive application of these fertilizers.

CONCLUSION:

In conclusion, biofertilizers have emerged as a promising alternative to conventional chemical fertilizers in modern agriculture. These natural and environmentally friendly products harness the power of beneficial microorganisms to enhance soil fertility and plant nutrition. Biofertilizers contain living microorganisms such as bacteria, fungi, or algae that form symbiotic relationships with plants or directly release essential nutrients into the soil. They contribute to sustainable farming practices by reducing the reliance on synthetic fertilizers, minimizing environmental pollution, and promoting soil health.

Several benefits of biofertilizers include:

1. Nutrient enrichment: Biofertilizers can fix atmospheric nitrogen, solubilize phosphorus, and enhance the availability of other essential nutrients. This helps in improving soil fertility and nutrient uptake by plants.
2. Increased crop productivity: The application of biofertilizers has shown positive effects on crop yield, quality, and overall productivity. They can stimulate plant growth, increase root development, and enhance resistance to diseases and pests.

3. Environmental sustainability: Biofertilizers reduce the use of chemical fertilizers, which can have detrimental effects on soil health and water quality. They contribute to sustainable agricultural practices by minimizing pollution and preserving the natural balance of ecosystems.
4. Cost-effectiveness: Biofertilizers can be produced at a lower cost compared to synthetic fertilizers, making them economically viable for small-scale farmers. They also offer long-term benefits by improving soil structure and fertility over time.

However, it is important to note that the effectiveness of biofertilizers may vary depending on factors such as soil type, climate, crop species, and specific microbial strains used. Proper application methods and dosage should be considered for optimal results.

Overall, biofertilizers hold significant potential for promoting sustainable agriculture, reducing environmental impacts, and ensuring food security. Continued research and development in this field can further enhance their effectiveness and application in diverse farming systems.

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