



ISOLATION, CHARACTERIZATION, PRODUCTION OF BIOFERTILIZER & ITS EFFECT ON  
VEGETABLE PLANTS WITH AND WITHOUT CARRIER MATERIALS

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*Solanum melongena*).

ABSTRACT

Biofertilizers are defined as preparations containing living cells or latent cells of efficient strains of microorganisms that help crop plants' uptake of nutrients by their interactions in the rhizosphere when applied through seed or to soil. They accelerate certain microbial processes in the soil which augment the extent of availability of nutrients in a form easily assimilated by plants. Very often microorganisms are not as efficient in natural surroundings as one would expect them to be and therefore artificially multiplied cultures of efficient selected microorganisms play a vital role in accelerating the microbial processes in soil. Use of biofertilizers is one of the important component of integrated nutrient management, as they are cost effective and renewable source of plant nutrients to supplement the chemical fertilizers for sustainable agriculture. In the present study biofertilizer with carrier materials was more effective than the without carrier materials.

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INTRODUCTION

Biofertilizers are defined as preparations containing living cells or latent cells of efficient strains of microorganisms that help crop plants' uptake of nutrients by their interactions in the rhizosphere when applied through seed or soil. They accelerate certain microbial processes in the soil which augment the extent of availability of nutrients in a form easily assimilated by plants. Very often microorganisms are not as efficient in natural surroundings as one would expect them to be and therefore artificially multiplied cultures of efficient selected microorganisms play a vital role in accelerating the microbial processes in soil. Use of biofertilizer is one of the important components of integrated nutrient management, as they are cost effective and renewable source of plant nutrients to supplement the chemical fertilizers for sustainable agriculture. Several microorganisms and their association with crop plants are being exploited in the production of biofertilizers. They can be grouped in different ways based on their nature and function. Plant growth promoting rhizobacteria (PGPR) have been studied for long. It has been suggested in the last few years that endophytic N<sub>2</sub>-fixing bacteria may be more

important than rhizospheric bacteria in promoting plant growth because they escape competition with rhizosphere microorganisms and achieve close contact with the plant tissues. The well known genera of PGPR are *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, and *Pseudomonas*, but some of these genera include endophytic species as well. The best-characterized endophytic bacteria include *Azoarcus* spp, *Gluconacetobacter diazotrophicus*, and *Herbaspirillum seropedicae*. Novel *Burkholderia* species, for instance, *B. unamae* and *B. tropica* have the potential for promoting plant growth and are found in rhizospheric and endophytic association with different agricultural crops. Bacterial mechanisms of plant growth promotion include biological nitrogen fixation (BNF), synthesis of phytohormones, environmental stress relief, synergism with other bacteria-plant interactions, inhibition of plant ethylene synthesis, as well as increasing availability of nutrients like phosphorus, iron and minor elements, and growth enhancement by volatile compounds. However, the expression of such bacterial activities under laboratory conditions does not guarantee in association with a host plant. This is especially true of nitrogen fixation as abundantly expressed in culture media by many bacterial species. The mechanisms of plant growth promotion have been analyzed in different organisms,

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especially in *Azospirillum* spp. and in few other PGPR. In this review, *Azospirillum* as a model for studying mechanisms of plant growth promotion will only be covered briefly but some other models and new mechanisms will be presented in more depth. Many definitions and interpretations of the term biofertilizer exist. In this chapter, a biofertilizer is a product that contains living microorganisms, which exert direct or indirect beneficial effects on plant growth and crop yield through different mechanisms.

The term biofertilizer as used here could include products containing bacteria to control plant pathogens, but these are frequently referred to as biopesticides. Cyanobacteria (or cyanophyceae) are non-motile, planktonic, occasionally forming blooms and belong to the kingdom of eubacteria and to the division of cyanophyta. They are gram negative and are common in some extreme environments. Cyanobacteria are a large and morphologically diverse group which can thrive in all kinds of waters with some species thriving in freshwater while others thrive in brackish water or the marine environment. Chemical fertilizers are needed to get good crop yields, but their use can be harmful for the environment and their cost cannot seem to make economic agricultural products. Thus, attempts have been undertaken to substitute chemical fertilizers with biofertilizers, such as cyanobacteria (blue green algae), which are capable of fixing atmospheric N. In addition, the use of cyanobacteria as biofertilizer can improve plant growth and crop yield since they add organic matter to soil, thus improving soil structure. The positive effect on crop yield was due to their release of various biologically active substances such as gibberellin, auxin, cytokinins, vitamins, amino acids, polypeptides, antibacterial and antifungal substances and polymers, especially exopolysaccharides. Cyanobacteria can also be used for bioremediation since they can take up heavy metals. However, cyanobacteria (some species of the genera *Microcystis*, *Nostoc*, *Oscillatoria*, and *Anabaena*) might be harmful as they can synthesize toxic secondary metabolites such as microcystins which can be accumulated in plant tissues and be carried through the food chain.

## MATERIALS AND METHODS

### Collection of soil

The waste soil should be collected from garden area and which isolation of microorganism is carried out. Waste soil sample of 4-5 days old is taken directly from that portion of filled from where possibility of finding of *Bacillus* sp. is maximum one gm of soil sample is taken and serially diluted using saline to prepare dilution of  $10^{-1}$  to  $10^{-1}$  respectively in different sterile test tubes. 1ml of sample from each test tube is taken with the help of micropipette and spread over the freshly prepared nutrient agar plates inside laminar air flow. Then petri plates are kept in incubator at  $37^{\circ}\text{C}$  for 24 hours. Microorganism are found throughout the environment in the air and water; on the surface of any objects such as cloth, walls, furniture, in soil and dust and on in our own bodies (skin, mucous membrane). Soil and water are rich sources of industrial organism, a gram of soil contains between  $10^6$  to  $10^9$  microorganism including bacteria filamentous fungi, yeast, protozoa, algae and microscopic milt cellular form. Since each microbial strain or species has certain

nutritional and environment requirements providing those requirements for all organism in one medium is impossible enumeration technique for bacteria using non-selective media are estimated to detect, optimistically. Only 0.1% of the population in a given soil or water sample. It is possible to favour the development of broad group of microorganism by dilution on selective media that favour the growth of one group of microorganism over competing types.

### Serial dilution method

The method was first developed by Joseph Lister and later perfected by Robert Koch. There are two variations of the dilution plating technique- the spread plate and the pour plate methods. In the first method, a measured quantity of a serially diluted sample of the original culture is spread evenly on the surface of the solidified growth medium. On the incubation at an optimum temperature, each viable bacterial cell forms a discrete colony on the surface of the medium. 1gm of soil sample is mixed to 10 ml sterile distilled water, shaker vigorously, it is stock solution. From the stock solution 1ml solution is transfer to 9 ml sterile distilled water aseptically, shaken vigorously, this is solution of  $10^{-1}$  dilution. This step is repeated 9 more times to make the dilution till  $10^{-10}$ . 0.1 ml of each solution is transferred aseptically to each plate containing nutrient media (nutrient agar = bacteria; potato dextrose = fungi) and spread with spreader on the surface of the medium. The plates are incubated (bacteria 48 hour;  $37^{\circ}\text{C}$ ; fungi 5-7 days in room temperature).

### Gram Staining Method

Transfer a loopful of the liquid culture to the surface of a clean glass slide, and spread over a small area. Two to four cultures may be stained on the same slide, which can be divided into 2-4 sections with vertical red wax pencil lines. To stain material from a culture growing on solid media, place a loopful of tap water on a slide; using a sterile cool loop transfer a small sample of the colony to the drop, and emulsify. Allow the film to air dry. Fix the dried film by passing it briefly through the Bunsen flame two or three times without exposing the dried film directly to the flame. The slide should not be so hot as to be uncomfortable to the touch.

1. Flood the slide with crystal violet solution for up to one minute. Wash off briefly with tap water (not over 5 seconds). Drain.
2. Flood slide with Gram's Iodine solution, and allow to act (as a mordant) for about one minute. Wash off with tap water. Drain.
3. 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. (Smears that are excessively thick may require longer decolorization. This is the most sensitive and variable step of the procedure, and requires experience to know just how much to decolorize). Drain the slide.
4. Flood slide with safranin solution and allow to counterstain for 30 seconds. Wash off with tap water. Drain and blot dry with bibulous paper. Do not rub.

All slides of bacteria must be examined under the oil immersion lens.

### Method of biofertilizer production

The production of carrier based biofertilizer involves five stages-

- 1) Isolation and characterization of Biofertilizer.
- 2) Culturing of microorganisms- starter culture and inoculum preparation.
- 3) Processing of carrier material.
- 4) Mixing the carrier and broth culture.
- 5) Packing.
- 6) Proper storage.

#### 1) Isolation of Biofertilizer

The strains of different microorganisms are isolated from the agricultural fields of West Bengal, of India by serial dilution method on Congo red yeast extract mannitol media, Pikovskaya's medium and N-free Mannitol agar medium, Dextrose Agar medium, Triple Sugar Iron Agar Media, Centrimide agar media. All the samples were pure cultured by agar plating method and characterized by colony morphology, Gram's staining and biochemical tests.

##### i) Isolation of *Rhizobium sp.*

The *Rhizobium* isolates were obtained from root nodules of Bean, Gram. Nodules located on the roots were spherical (2-4 mm in diameter) and pink. Root nodules were sterilized in 95% (v/v) ethanol for 10 s and then washed 7 times with sterile distilled water. Individual nodules were crushed with sterile glass rods and streaked onto yeast extract mannitol (YEM) agar containing 0.0025% (w/v) Congo red. After incubation for 2-3 days at 30 °C, single colonies were selected and restreaked on YEM agar for purity.

##### ii) Isolation and identification *Azotobacter sp.*

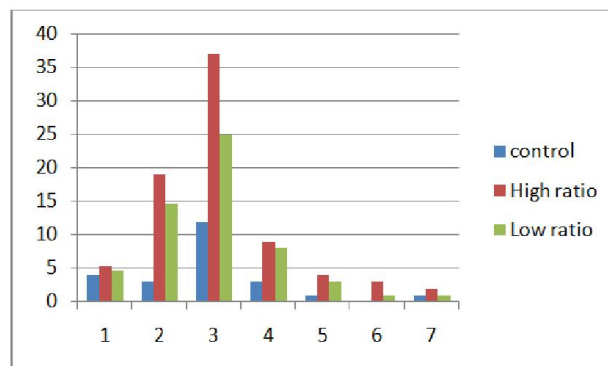
Samples of 1g of rhizosphere (roots and soil) were shaken with 10 ml of culture broth N-free Mannitol solutions for *Azotobacter sp.* An aliquot of 1 ml from each broth was added to a selective medium to purify the bacteria. Identification of grown isolated colonies was based on morphologic, biochemical and culturing characteristics.

##### iii) Isolation and Identification of Phosphate Solubilizing Bacteria

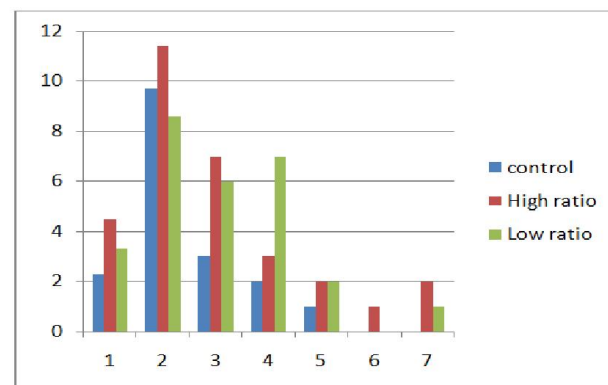
The soil samples were collected from rhizospheric region of Maize and Tomato plants in rural parts of West Bengal. Microorganisms mainly the bacterial strains were isolated by serial dilution of soil samples and an aliquot of 100 µl from decimal dilutions were considered. The samples were spread onto the Pikovskaya's agar medium, and incubated for 24 hours at room temperature for the detection of phosphate solubilising bacteria. The prominent colonies showing a clear zone on the Pikovskaya's agar were selected and purified by repeated culturing on nutrient agar medium. The bacteria were

identified and screened by their colony characteristics, Staining techniques, microscopic observations and biochemical characteristics and molecular characterization.

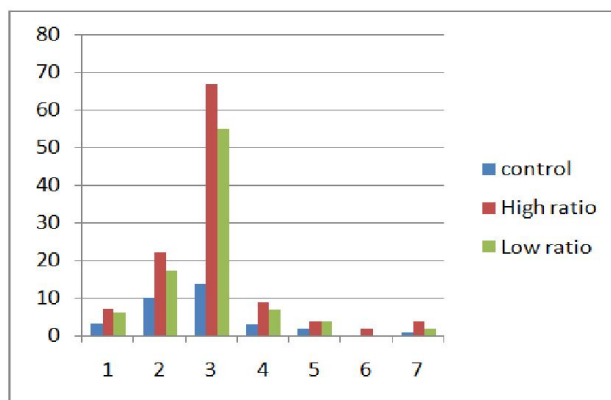
### Graph Analysis



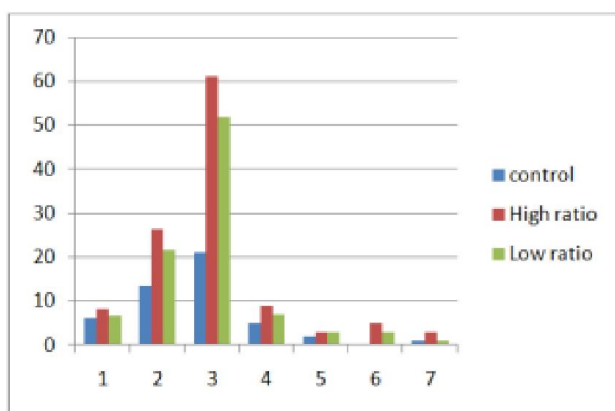
Graph 1. Analysis of Growth Parameter with *Bacillus sp.* + *Capsicum annuum*



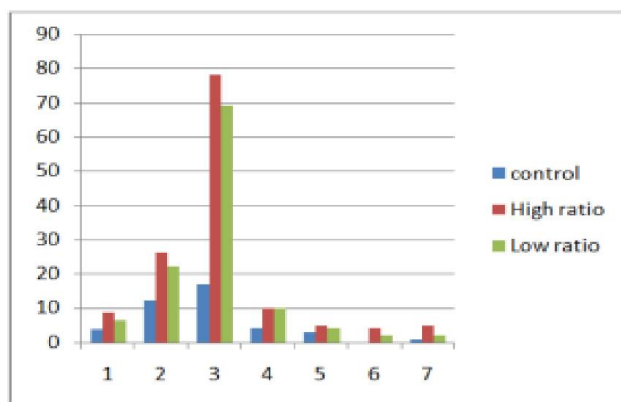
Graph 2. Analysis of Growth Parameter with *Yeast sp.* + *Solanum melongena*



Graph 3. Analysis of Growth Parameter with *Pseudomonas sp.* + *Capsicum annuum*



Graph 4. Analysis of Growth Parameter with *Azotobacter sp.* + *Solanum betaceum*



Graph 5. Analysis of Growth Parameter with *Rhizobium sp.* + *Solanum betaceum*

## 2. Important steps of biofertilizer production

Biofertilizers are carrier based preparations containing efficient strain of nitrogen fixing or phosphate solubilizing microorganisms. Biofertilizers are formulated usually as carrier based inoculants. The organic carrier materials are more effective for the preparation of bacterial inoculants. The solid inoculants carry more number of bacterial cells and support the survival of cells for longer periods of time.

## 3. Culturing of microorganism

Although many bacteria can be used beneficially as a biofertilizer the technique for production of *Rhizobium*, *Phosphobacteria* and *Azotobacter* are discussed here. The growth mediums used for mass culturing of different bacterial biofertilizers are as follows:

*Rhizobium sp.* Grown on Yeast extract mannitol broth Components Quantity (g L<sup>-1</sup>) Mannitol 10.0; K<sub>2</sub>HPO<sub>4</sub> 0.5 Mg SO<sub>4</sub> 7H<sub>2</sub>O 0.2; NaCl 0.1; Yeast extract 0.5; Agar 20.0; Distilled water 1 L. Add 10 mL of Congo red stock solution (dissolve 250 mg of Congo red in 100mL water) to 1 liter after adjusting the pH to 6.8 and before adding agar. *Rhizobium*

forms white, translucent, glistening, elevated and comparatively small colonies on this medium. Moreover, rhizobium colonies do not take up the colour of congo red dye added in the medium. Those colonies which readily take up the congo red stain are not rhizobia but presumably *Agrobacterium*, a soil bacterium closely related to rhizobium.

## Trace element solution

Sodium molybdate 200 mg; Manganoussulphate 235 mg; Boric acid 280 mg; Copper sulphate 8 mg; Zinc sulphate 24 mg; Distilled water 200 mL.

## *Azotobacter sp.*

Grown on N-free Mannitol agar medium-Components Quantity (g L<sup>-1</sup>); Mannitol 10.0; CaCO<sub>3</sub> 5.0; K<sub>2</sub>HPO<sub>4</sub> 0.5; MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2; NaCl 0.2; Ferric chloride Trace; MnSO<sub>4</sub>·4H<sub>2</sub>O Trace; N-free washed Agar 15.0; pH 7.0; Distilled Water 1 L.

## *Yeast sp.*

When a yeast cell is inactivated, a natural digestion process called "autolysis" starts. During this process the yeast's own enzymes break down proteins and other parts of the cell. This causes the release of peptides, amino acids, vitamins and other yeast cell components which, once the insoluble components have been removed, is called "Yeast Extract". YEPD or yeast extract peptone dextrose, also often abbreviated as YPD, is a complete medium for yeast growth. It contains yeast extract, peptone, double-distilled water, and glucose or dextrose. It can be used as solid medium by including agar. The yeast extract will typically contain all the amino acids necessary for growth. By being a complete medium, YEPD cannot be used as a selection medium to test for auxotrophs. Instead, YEPD is used as a growth medium to grow yeast cultures. The agar version of YEPD typically consists of 3% (mass/volume) yeast extract, 1% peptone, 1% glucose/dextrose, 2% agar, with the rest being distilled water. The broth version of YEPD typically contains 1% yeast extract, 2% peptone, 2% glucose/dextrose, and the rest is distilled water.

## Phosphobacteria (*Bacillus sp.* and *Pseudomonas sp.*)

Pikovskaya's Broth Glucose-10.0g; Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>-5.0g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-0.5g; KCl-0.2g; MgSO<sub>4</sub>·7H<sub>2</sub>O -0.1g; MnSO<sub>4</sub>·Trace; FeSO<sub>4</sub>·Trace; Yeast Extract-0.5 g; Distilled Water-1000 ml. Microorganisms mainly the bacterial strains were isolated by serial dilution of soil samples and an aliquot of 100µl from decimal dilutions were considered. The samples were spread onto the Pikovskaya's agar medium and incubated for 24 hours at room temperature for the detection of phosphate solubilizing bacteria. The prominent colonies showing a clear zone on the Pikovskaya's agar were selected and purified by repeated culturing on nutrient agar medium. The bacteria were identified and screened by their colony characteristics, Staining techniques, microscopic observations and biochemical characteristics.

## Methodology of Biofertilizer Production

The above broths are prepared in separate flasks and inoculum from respective mother culture is transferred to flasks. The culture is grown under shaking conditions at  $30 \pm 2^\circ\text{C}$  as submerged culture. The culture is incubated until maximum cell population of  $10^{10}$  to  $10^{11}$  cfu mL<sup>-1</sup> is produced. Under optimum conditions this population level could be attained within 4 to 5 days for Rhizobium; 5 to 7 days for Phosphobacteria; and 6-7 days for Azotobacter. The culture obtained in the flask is called starter culture. For large scale production of inoculant, inoculum from starter culture is transferred to large flasks and grown until required level of cell count is reached.

### Inoculum preparation

Prepare appropriate media for specific to the bacterial inoculant in 250 mL, 500 mL, 3 litre and 5 litre conical flasks and sterilize. The media in 250 mL flask is inoculated with efficient bacterial strain under aseptic condition. Keep the flask under room temperature in rotary shaker (200 rpm) for 5- 7 days. Observe the flask for growth of the culture and estimate the population, which serves as the starter culture. Using the starter culture (at log phase) inoculate the larger flasks (500 mL, 3 litre and 5 litre) containing the media, after obtaining growth in each flask. The above media is prepared in large quantities in fermenter, sterilized well, cooled and kept it ready. The media in the fermenter is inoculated with the log phase culture grown in 5 litre flask. Usually 1 -2 % inoculum is sufficient, however inoculation is done up to 5% depending on the growth of the culture in the larger flasks. The cells are grown in fermenter by providing aeration (passing sterile air through compressor and sterilizing agents like glass wool, cotton wool, acid etc.) and given continuous stirring. The broth is checked for the population of inoculated organism and contamination if any at the growth period. The cells are harvested with the population load of  $10^9$  cells per mL after incubation period. There should not be any fungal or any other bacterial contamination at  $10^6$  dilution level. It is not advisable to store the broth after fermentation for periods longer than 24 hours. Even at  $4^\circ\text{C}$  number of viable cells begins to decrease.

### Processing of carrier material

The use of ideal carrier material is necessary for the production of good quality biofertilizer. Peat soil, lignite, vermiculite, charcoal, press mud, farmyard manure and soil mixture can be used as carrier materials. The neutralized peat soil/lignite are found to be better carrier materials for biofertilizer production. The following points are to be considered in the selection of ideal carrier material. Cheaper in cost; Should be locally available; High organic matter content; No toxic chemicals; Water holding capacity of more than 50% ; Easy to process, friability and vulnerability.

### Preparation of carrier material

The carrier material (peat or lignite) is powdered to a fine powder so as to pass through 212 micron IS sieve. The pH of the carrier material is neutralized with the help of calcium carbonate (1:10 ratio), since the peat soil / lignite are acidic in

nature (pH of 4 - 5). The neutralized carrier material is sterilized in an autoclave to eliminate the contaminants. Mixing the carrier with different ratio and the broth culture and packing Inoculants packets are prepared with different ratio with carrier materials by mixing the broth culture obtained from fermenter with sterile carrier material as described below with high and low ratio of biofertilizer production.

**Table 1. Production of different (High & low) ratio of biofertilizer with carrier materials**

Ratio	Coal	Soil	Sand
1	50%	50%	50%
2	50%	50%	-
3	50%	-	50%
4	70%	30%	30%
5	70%	30%	-
6	70%	-	30%

### Preparation of Inoculants packet

The neutralized and sterilized carrier material is spread in a clean, dry, sterile metallic or plastic tray. The bacterial culture drawn from the fermenter is added to the sterilized carrier and mixed well by manual (after wearing sterile gloves) or by mechanical mixer. The culture suspension is to be added to a level of 40 - 50% water holding capacity depending upon the population. The inoculants packet of 200 g quantities in polythene bags, sealed with electric sealer and allowed for curing for 2 -3 days at room temperature (curing can be done by spreading the inoculants on a clean floor/polythene sheet or by keeping in open shallow tubs/ trays with polythene covering for 2 -3 days at room temperature before packaging).

### Specification of the polythene bags

The polythene bags should be of low density grade. The thickness of the bag should be around 50 - 75 micron. Each packet should be marked with the name of the manufacturer, name of the product, strain number, the crop(s) to which recommended, method of inoculation, date of manufacture, batch number, date of expiry, price, full address of the manufacturer and storage instructions etc.

### Storage of biofertilizer packet

The packet should be stored in a cool place away from the heat or direct sunlight. The packets may be stored at room temperature or in cold storage conditions in lots in plastic crates or polythene/gunny bags. The population of inoculant in the carrier inoculant packet may be determined at 15 days interval. There should be more than  $10^9$  cells per gram of inoculant at the time of preparation and  $10^7$  cells per gram on dry weight basis before expiry date.

### Fumigation of Soil

Each Pot is dugged, filled with Red sand loamy soil (1:1) and fumigated with 1% formaldehyde (100 ml in 1L of distill water), sprayed on the soil , covered with polythene cover and every alternate day tested for pH and spores colonization. On 5<sup>th</sup> day refumigation with 1% formaldehyde is repeated and covered

with polytene cover and every two days once tested for pH and AM spores. This is maintained for 15 days. Fumigation of the soil reduces population.

### Pot Experiment

The efficiency of the biofertilizers are tested by running a pot culture experiment and monitoring the growth of different vegetable plants. The different microorganisms are used in combination and ratio to check if it enhances the growth of the plants. The experiment was arranged in two treatments (control i.e. without addition of biofertilizer and test i.e. application of microorganism sp). Each treatment consisted of plants and the whole treatment is replicated many times. 0.04 gms of microorganisms (dry granules) are revived by placing in sterile distilled water and then mixed with 5 gm of biofertilizer under aseptic conditions. Seeds of vegetable plants are mixed in the prepared slurry and allowed to stand still for 30 min. Test and control seeds were directly sown into their respective pots. Each pot contained about 200 gm of soil. The phenotypic parameters of the plants are measured after the 90th day from sowing. Soil used for the whole experiment was taken from a single source (black farm soil provided by BCKV, West Bengal). The plants are kept in a poly house with humidity ranging from 70 – 80%. The temperature of the polyhouse is not controlled and varied with local conditions (max: 32°C, min: 15°C).

## RESULTS AND DISCUSSION

The culture was also subjected to various sugar fermentation tests and was found to be able to reduce glucose, mannose, xylose, galactose, raffinose, mannitol and trehalose.

These results are in consensus with all the previous reported tests done during the isolation of *Rhizobium* sp, *Yeasts* sp, *Bacillus* sp, *Pseudomonas* sp, *Azotobacter* sp. The sequence obtained from NCCS was found to of these microorganism sp after it was run through the NCBI database using BLAST. Maximum similarity was obtained at 99% with 95% query coverage (e value = 0).

From Table: 4; the *Bacillus* sp. biofertilizer was used on different vegetable plants and calculate no of flower; number of leaves ; number of branches; length of internodes; no of fruit; growth parameters. The high ratio of *Bacillus* sp biofertilizer effected on *Capsicum annuum*. In high ratio the growth of the plant is 19.1 cm and the other growth parameters more than control and low ration. The result of low ratio is good than control. The root (5.4 cm); shoot (19.1 cm); number of leaves (37); number of branches (9); length of internodes (4); number of fruit (3); number of flower (2). In the result the overall growth is good and the rate is too low than the other biofertilizer. The low ratio biofertilizer gave satisfactory result on *Capsicum annuum*. There was One fruit production of low ratio of *Bacillus* sp. biofertilizer and in high ratio there was maximum number of Fruits.

From the Table 5, the effect of *Yeasts* sp on *Solanum melongena* was observed and the high ration of the biofertilizer gave good results. The root (4.5 cm); shoot (11.5cm); number of leaves (7); number of branches (3); length of internodes (2); number of fruit (1); number of flower (2). In the result the overall growth is not good and the rate is too low than the other biofertilizer. The low ratio biofertilizer gave satisfactory result on *Solanum melongena*. The control plant gave normal result as plant growth rate. There was no fruit production of low ratio of *Yeast* sp. biofertilizer and in high ratio there was only one. Number of Fruit.

**Table 2. Staining technique for Morphological identification**

S.No	Observation	+/-	Microorganism
1.	Purple color, rod shaped, chain forming, dispersed, long chain forming, thick rod shaped, branched fine short forming structure.	Gram positive	Bacillus sp.
2.	Oval shaped, purple colour, round and oval, spike like structure,	Gram positive	Yeast sp.
3.	Fine and rod shape , pink coloured structure.	Gram negative	Pseudomonas sp.
4.	Fine and rod shape , pink coloured, shaded fine structure.	Gram negative	Rhizobium sp.
5.	Fine and very short rod shape , pink coloured, dispersed shaded fine structure.	Gram negative	Azotobacter sp.

**Table 3. Biochemical Test of the different microorganism**

Serial no	Test	Bacillus	Yeast	Pseudomonas	Rhizobium	Azotobacter
1.	Urease	positive	negative	negative	positive	positive
2.	Nitrate reduction	positive	negative	negative	positive	positive
3.	Starch hydrolysis	positive	positive	positive	positive	Positive
4.	Gelatin hydrolysis	negative	positive	positive	negative	Negative
5.	Casein hydrolysis	positive	negative	positive	negative	Negative
6.	Methyl Red test	positive	positive	positive	positive	Negative
7.	Vogues Proskuer	negative	negative	negative	negative	Negative
8.	Indole	negative	negative	positive	negative	Negative
9.	Citrate utilization	negative	positive	positive	positive	Positive
10.	Hydrogen sulphide production	negative	positive	negative	negative	Negative

**Table 4. Growth Parameter with *Bacillus* sp. + *Capsicum annuum* (90 days after inoculation)**

Trails	Growth Parameters		Number of Leaves	Number of Branches	Length of Internodes	Number of Fruit	Number of Flower
	Root (cm)	Shoot (cm)					
Control	4	3	12	3	1	0	1
High ratio	5.4	19.1	37	9	4	3	2
Low ratio	4.6	14.6	25	8	3	1	1

**Table 5. Growth Pamameter with Yeast sp. + Solanummelongena(90 days after inoculation)**

Trails	Growth Parameters		Number of Leaves	Number of Branches	Length of Internodes	Number of Fruit	Number of Flower
	Root (cm)	Shoot (cm)					
control	2.3	9.7	3	2	1	0	0
High ratio	4.5	11.4	7	3	2	1	2
Low ratio	3.3	8.6	6	7	2	0	1

**Table 6. Growth Pamameter with Pseudomonas sp. + Capsicum annum(90 days after inoculation)**

Trails	Growth Parameters		Number of Leaves	Number of Branches	Length of Internodes	Number of Fruit	Number of Flower
	Root (cm)	Shoot (cm)					
control	3.3	10.2	14	3	2	0	1
High ratio	7.5	22.1	67	9	4	2	4
Low ratio	6.2	17.5	55	7	4	0	2

**Table 7. Growth Pamameter with Azotobactor sp. + Solanumbetaceum( 90 days after inoculation)**

Trails	Growth Parameters		Number of Leaves	Number of Branches	Length of Internodes	Number of Fruit	Number of Flower
	Root (cm)	Shoot (cm)					
control	6.2	13.5	21	5	2	0	1
High ratio	8.1	26.3	61	9	3	5	3
Low ratio	6.7	21.5	52	7	3	3	1

**Table 8. Growth Pamameter with Rhizobium sp. +Solanumbetaceum(90 days after inoculation)**

Trails	Growth Parameters		Number of Leaves	Number of Branches	Length of Internodes	Number of Fruit	Number of Flower
	Root (cm)	Shoot (cm)					
control	3.7	12.1	17	4	3	0	1
High ratio	8.8	26.2	78	10	5	4	5
Low ratio	6.4	22.2	69	10	4	2	2

From Table : 6 the effect of *Pseudomonas sp.* on *Capsicum annum* was observed and the high ration of the biofertilizer gave good results. The root (7.5 cm); shoot (22.1cm); number of leaves (67); number of branches(9); length of internodes(4); number of fruit(2); number of flower(4). In the result the overall growth is very good and the rate is too high than the other biofertilizer. The low ratio biofertilizer gave satisfactory result on *Capsicum annum*. The control plant gave normal result as plant growth rate. The growth of the plant is too good than the low ratio of biofertilizer. The maximum number of flowers were observed using of *Pseudomonas sp.* Biofertilizer

From Table 6 the effect of *Pseudomonas sp.* on *Capsicum annum* was observed and the high ration of the biofertilizer gave good results. The root (7.5 cm); shoot (22.1cm); number of leaves (67); number of branches(9); Length of Internodes(4); no. of fruit (2); no of flower(4). In the result the overall growth is very good and the rate is too high than the other biofertilizer. The low ratio biofertilizer gave satisfactory result on *Capsicum annum*. The control plant gave normal result as plant growth rate. The growth of the plant is too good than the low ratio of biofertilizer.

From Table: 8; the effect of *Rhizobium sp.* on *Solanumbetaceum* was observed and the high ration of the biofertilizer gave good results. The root (8.8cm); shoot (26.2cm); number of leaves (78); number of branches (10); Length of Internodes (5); number of fruit (4); number of flower (5). In the result the overall growth is very good and the

rate is too high than the other biofertilizer. The low ratio biofertilizer gave satisfactory result on *Solanumbetaceum*. The control plant gave normal result as plant growth rate. The growth of the plant height, number of leaves and the number of fruit are too good than the low ratio of biofertilizer. The maximum number of fruits were produced using of ratio of *Rhizobium sp.* Biofertilizer.

**High ratio Yeast sp. biofertilizer in Solanummelongena**



Low ratio of *Yeast sp.* Biofertilizer in *Solanum melongena*



High ratio of *Pseudomonas sp.* Biofertilizer in *Capsicum annuum*



High ratio of *Azotobacter sp.* Biofertilizer in *Solanum betaceum*



Low ratio of *Pseudomonas sp.* Biofertilizer in *Capsicum annuum*



Low ratio of *Azotobacter sp.* Biofertilizer in *Solanum betaceum*



High ratio of *Bacillus sp.* Biofertilizer in *Capsicum annuum*





Low ratio of *Bacillus sp.* Biofertilizer in *Capsicum annuum*



High ratio of *Rhizobium sp.* Biofertilizer in *Solanum betaceum*



Low ratio of *Rhizobium sp.* Biofertilizer in *Solanum betaceum*

## Conclusion

In this work, the novel biofertilizers were developed using species of *Rhizobium sp.*, *Pseudomonas sp.*, *Yeast sp.*, *Bacillus sp.* and *Azotobacter sp.* which were isolated from the different soil of Agriculture are of West Bengal. These were characterized with the help of morphological and biochemical tests. The isolated strains of different microorganisms were used for the large production inoculum of *Rhizobium*, *Pseudomonas*, *Yeast*, *Bacillus* and *Azotobacter sp.* Using these strain, a biofertilizers were successfully produced using activated charcoal as the carrier and its effect were studied on *Capsicum annuum*, *Solanum betaceum*, *Solanum melongena* in combination with of *Rhizobium*, *Pseudomonas*, *Yeast*, *Bacillus* and *Azotobacter sp.* The overall increases in the growth of the plant treated with the formulation were observed after 90 days. Increase in root (cm); shoot (cm); number of leaves; number of branches; Length of internodes; no. of fruit; no of flower on *Capsicum annuum*, *Solanum betaceum*, *Solanum melongena* plants. These results indicate a significant increment in the productivity of plants treated with the newly developed biofertilizers. This novel biofertilizers can be produced at a higher commercial level and its effect checked on various high yielding agricultural plants. It is necessary to continue researching in this field as it has the potential to be highly profitable for farmers as well as provide a way to a more sustainable future.

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