

# Screening of Halophilic Microorganisms (Oceanobacillus Oncorhynchi and Pseudomonas Stutzeri) for the Effect of Plant Growth Promotion and Its Formulation as a Biofertilizer

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## Abstract

A quest for Plant Growth Promoting bacteria are increasing expeditiously as efforts are made to exploit them commercially as inoculum preparations i.e., biofertilizers. In this work, a comparative study is carried out to assess the ability of the halobacterial strains in plant growth promotion and in addition tested for the ability of the isolate whether it could solubilize phosphate. These halobacterial strains were isolated and identified as *Oceanobacillus oncorhynchi* and *Pseudomonas stutzeri* by 16s rRNA sequence. The effectiveness of the isolate in plants seed germination and growth were analyzed in crops like Chickpea, Cowpea, Green gram and Pearl millet etc in which *P.stutzeri* showed a significant increase in plant root and shoot

length than *O.oncorhynchi*. Thus, the ability to perform multifarious plant growth promoting activities together suggested uniqueness of *P. stutzeri* and its potential use in developing a cost-effective eco-friendly multifunctional biofertilizer and biocontrol agent in agriculture. Further the optimal growth conditions such as pH, NaCl concentrations and temperature were studied for the effective isolation of the isolate.

## Keywords

Plant Growth Promotingbacteria(PGPR), Biofertilizer, *Oceanobacillus oncorhynchi*, *Pseudomonas stutzeri*, Seed germination test

## I. INTRODUCTION

The term plant growth promoting bacteria (PGPB) was first brought in use by Kloepper and Schroth,1978 [11]. PGPB can directly or indirectly enhance plant growth. Direct mechanisms include the production of plant hormones such as indole acetic acid (IAA), gibberellins and cytokinins [13] [7] along with asymbiotic N<sub>2</sub> fixation [10] and solubilization of phosphates [15] [2]. On the other hand, indirect mechanisms are the production of iron chelators, siderophores, as well as cyanides [9] [1], since they act as antagonists of plant pathogens. The most important and well-studied genera of PGP halophilic bacteria are *Bacillus* and *Pseudomonas*. Thus the beneficial effects of PGPB have been exploited for many mechanisms employed such as stimulation of root branching, increase in the shoot and root biomass, increase nitrogen content, improves survival

of seedling, tolerance to heavy metal toxicity etc, . Halophilic PGPB has intense potential in saline environments when compared to other mesophilic commercial biofertilizers. Therefore, commercial application of plant growth promoting halotolerant bacteria in salt-affected soil is being developed for survival and performance in the field. Biofertilizers are preparations containing latent cells of efficient strains of nitrogen-fixing, phosphate solubilizing or cellulolytic microorganisms which are used for enhancement of plant growth by enabling the available nutrients in a form that can be assimilated by the plant. Hence the present study was conducted in an attempt to isolate, characterize and identify halotolerant bacteria from saline habitats, optimize their growth characters and evaluate their ability of plant growth promoting activity under saline stress condition. Based on the investigation of halophilic isolates for their ability to promote plant growth, a

novel bio-fertilizer was formulated using isolated halophilic PGPB.

## II. MATERIALS AND METHODS

### A) *Enrichment And Isolation Of Microorganisms*

The soil sample from saltpans situated in Thoothukudi District, Tamil Nadu, India was collected in sterile plastic containers. 10g of the collected soil sample was inoculated in 100ml of Halophilic broth medium and incubated at room temperature for 5 – 7 days in the shaker for enrichment. The enriched soil samples were inoculated on halophilic agar plates and incubated at room temperature for 3 – 5 days for development of isolated colonies. The isolated bacterial strains were screened for production of plant growth promoting factors.

## III. MORPHOLOGICAL STUDIES

### A) *Gram staining:*

Gram staining was performed by using air-dried slides which were fixed and simultaneously desalted in 2% acetic acid for 5 minutes, dried before staining by standard procedures [8]. A thin smear of the bacterial colony was prepared on a clean slide. The slide was fixed by passing through the flame. The smear was covered with crystal violet for 30 sec, then rinsed with water and drained. Next, the smear was covered with iodine for 30 sec, then with rinsed water. Decolorized with ethanol 95% and washed with water, then it was counterstained about 30 sec with safranin. Blot slide was dried and examined under oil immersion (1,000x). Colonial appearances were examined after incubated for 3-7 days.

## IV. BIOCHEMICAL TEST

### A) *Indole production*

Peptone broth was prepared and autoclaved. Test tubes were prepared to contain peptone broth. Test organisms were inoculated into the test tubes and incubated at 45°C for 48 hrs. After incubation, Kovac's reagent was added and tubes were observed for the change in color. Appearance of cherry red color indicates a positive result.

### B) *Methyl red test*

The MR-VP broth was prepared and autoclaved. Test tubes were prepared to contain MR-VP broth. Test organisms were inoculated into the tubes and incubated at 45°C for 48 hrs. After incubation methyl red indicator was added and test

tubes were observed for change in color. The appearance of red color in the tubes indicates a positive result.

### C) *Voges-Proskauer test*

MR-VP broth was prepared and autoclaved. Test tubes were prepared to contain MR-VP broth. Test organisms were inoculated into the tubes and incubated at 45°C for 48 hrs. After incubation, Barritt's reagent was added and test tubes were observed for change in color. The appearance of rose pink or red color indicates a positive result.

### D) *Citrate utilization*

Simmon's Citrate Agar was prepared and autoclaved. Slants were prepared in the sterile test tubes. Test organisms were streaked onto the slants and incubated at 37°C for 48 hrs. After incubation, the change in color of the slants was observed. Change in color from green to deep blue indicates a positive result.

## V. STARCH HYDROLYSIS

The isolated bacteria (GD3007 & DM0207) were tested for amylase production by starch hydrolysis method [12]. The 24hrs old cultures of isolated halophilic bacterial forms were streaked into the sterile starch agar medium and incubated at 37°C for 5 days. After incubation, the plates were flooded with iodine solution (iodine – 0.2%, KI – 0.4%, water 100 ml), the presence of clear zone was recorded and the selected bacterium species was noted for future investigations.

## VI. SIDEROPHORE PRODUCTION

Siderophore production was tested on Chrome Azurol (CAS) agar. The CAS agar plates were prepared and spot inoculated with pure cultures. Then plates were incubated at 37°C for 72 h. Development of yellow-orange halo around the growth confirms siderophore production.

## VII. ASSESSING THE ABILITY TO REDUCE NITRATE

Nitrate broth was prepared and autoclaved. Test tubes were prepared contain to nitrate broth. Test organisms were inoculated in the test tubes and incubated at 45°C for 48 hrs. After incubation alpha-naphthylamine and sulphanilic acid was added and the change in color of tubes was observed. Results were verified by adding zinc powder. The positive

result is indicated by the appearance of red color in the test tubes.

### VIII. PRODUCTION OF AMMONIA

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 7 days at 37°C. Nessler's reagent (0.5 ml) was added to each tube. Development of brown to yellow color was a positive test for ammonia production [3].

### IX. DETERMINING THE ABILITY TO SOLUBILIZE PHOSPHATE

Modified Pikovskaya agar plates were prepared and test isolates were streaked on plates, then the plates were then incubated at 37°C and observed for 2-7 days. The strains forming a zone of clearance were maintained by streaking on nutrient agar slants and stored at 4°C.

### X. CELL WALL DEGRADING ENZYME PRODUCTION

Protease activity (casein degradation) was determined from the clear zone in skimmed milk agar. Colonies were screened for cellulase activity by plating on CMC agar [5]. The agar plates were prepared and spot inoculated with test organism and incubated at 30°C. Development of halo zone around the colony was considered as positive for cell wall degrading enzyme production.

### XI. PHYSIOLOGICAL CHARACTERIZATION

#### A) NaCl Variation Assay

The isolates were screened for their salt tolerance level by growing them on to Minimal (MM63) broth tubes with concentrations of salt (NaCl) ranging from 0.5%, 1%, 2%, 3%, 5%, 10% & 20%, and subsequently measuring their optical density. pH was maintained at 7.0 and temperature at 37°C.

#### B) pH Variation Assay

Minimal (MM63) broth was prepared at different pH range (1, 3, 5, 7, 10 & 14) to detect the tolerance level of the strains and subsequently measuring their optical density at each pH. Salt concentration was maintained at 15% and temperature at 37°C.

#### C) Temperature Variation Assay

All the isolates were tested for the temperature tolerance by subjecting them to the different temperatures (30°C, 37°C, 40°C, 45°C, 50°C, 55°C & 60°C) in nutrient broth and subsequently measuring their optical density. Salt concentration was maintained at 15% and pH at 7.0.

### XII. SEED GERMINATION TEST

Chickpea, Cowpea, Green gram and Pearl millet seeds were collected from local agricultural centers. Ten seeds were surface sterilized with hydrogen peroxide solution for 1 – 2 minutes and then the treated seeds were soaked in culture (108 CFU/ml) of the selected isolate for 30 min. The seeds were placed in sterile Petri dishes that had filter paper moistened with 10 – 50mM concentration of NaCl. Germination of seeds was observed daily and shoot length and root length was recorded every 5th, 10th and 15th day [14].

### XIII. MASS CULTIVATION OF THE HALOPHILIC ISOLATES

#### A) Starter Culture

The isolates were inoculated into 100 ml of Minimal (MM63) medium and placed in a magnetic shaker for incubation of 4-7 days.

#### B) Mass Production

For the mass production of halophilic isolates, the starter culture is transformed to the 1000 ml Minimal medium and continuously agitated for 7 days. When the viable cell count reaches  $10^8 - 10^9$ /ml, the inoculums can now be used as inoculants along with carrier material. Carriers are an inert material which is used for easy handling, packaging, storing and transporting along with the viable cell cultures. Here the carrier used is garden soil.

### XIV. FORMULATION OF CARRIER-BASED INOCULUM

1 Kg of sieved garden soil was weighed, autoclaved and air dried. The pH was neutralized by adding Calcium carbonate. 200 ml of inoculums of the halophilic isolates was mixed with 1 Kg of the sterilized soil. The carrier-based inoculums were packed in sterile polyethene bags and stored at room temperature.

### XV. QUALITY CONTROL

As per BIS specification for a Phosphate Solubilising Bacterial inoculant, the viability of the

cells, pH & moisture percentage of the prepared formulation was determined. To check the cell viability, the sample was serially diluted, plated in Mineral (MM63) medium and incubated for 15 days. Initial and final viable colony on the medium was noted.

## XV. MOLECULAR IDENTIFICATION

Bacterial Genomic DNA was isolated by using the Insta Gene TM Matrix Genomic DNA isolation kit. The gene fragment of Sample 1 was amplified with 16s RNA F/16s RNA R and Sample 2 with Arch 16s-F/ Arch 16s-R.

## XVI. RESULTS AND DISCUSSION

Soil samples were collected and two bacterial strains were selected based on distinct morphology on halophilic agar (15% NaCl) plates. Colonies were selected based on color, shape, size, and abundance. These were screened for salt tolerance and growth in nutrient broth amended with various concentrations of NaCl. The two isolates designated as GD3007 & DM0207 were screened for the plant growth promoting factors. The above cultures were subcultured every 15 days and maintained at room temperature.

### A) Colony Morphology

The colony of the isolate GD3007 was creamy white, opaque, translucent and whereas DM0207 was reddish brown, flat, mucoid. Some of the colonies were very small, large, circular and irregular in shape. Smooth, mucoid and dry colonies were also recorded (Table 2). Furthermore, the results of the present study were supported by the results of Delgado-García et al., 2012 who has selected the isolates based on their colony morphology.

### B) Gram Staining

Both the halophilic isolates were subjected to Gram staining; the results of the isolate GD3007 was Gram-positive rod-shaped bacilli and DM0207 was Gram-negative rod-shaped bacilli. The study by Sawale et al., 2013 had revealed the presence of several gram-positive rod and cocci and varying size rod-shaped bacteria.

### C) Biochemical Tests

The results of the biochemical characteristics of the halophilic isolates were presented in Table I. In the same way, Saju et al., 2011 isolated and characterized the halophilic bacteria such as *Vibrio fischeri*, *Halobacillus salinus*,

*Halobacterium salinarum*, *Bacillus subtilis* and *Staphylococcus citreus* from salt pans of Kovalam in Kanyakumari district.

### D) Siderophore production

The isolate DM0207 was found to produce siderophore by the development of yellow-orange halo around the colony in CAS agar. According to Shinoda and Okomato, 1977 some strains of *Pseudomonas stutzeri* were found to produce siderophores during iron starvation.

### E) Nitrate reduction

Both the halophilic isolates GD3007 & DM0207 were found to reduce nitrate to nitrite which is confirmed by the color change in Nitrate broth (Figure 1). Experimental studies conducted by Curtis et al., 1982 shows that nitrate can be vigorously reduced to N<sub>2</sub> by *Pseudomonas stutzeri* and *P.denitrificans*.

### F) Ammonia production

Development of yellow-brown color after addition of Nessler's reagent indicates a positive test for ammonia production. Both the isolates GD3007 & DM0207 changed the color indicating all the isolates to be positive for ammonia production (Figure 2). The previous study by Tipre et al., 2013 shows that *Bacillus* sp. PUI<sup>T</sup> tested positive for ammonium production by showing growth in nitrogen-free NFb medium.

### G) Phosphate solubilization

Pikovaskaya's agar with NaCl, when inoculated with the halophilic isolate DM0207 showed the clear zone of hydrolysis around the bacterial growth, which indicated their ability to solubilize phosphates while the halophilic isolate GD3007 did not show any clear zone of hydrolysis around the bacterial growth, which indicated their inability to solubilize phosphates. *Pseudomonas stutzeri* is in accordance with previous studies showing that members of *Pseudomonas* genus display P solubilization [19].

### H) Screening for amylase, protease, and cellulase

Production of fungal cell wall degrading enzymes was analyzed because this is an important mechanism of fungal inhibition. The halophilic isolate DM0207 was found to produce cellulase, a fungal cell wall degrading enzyme. The isolates were also screened for amylase activity in Starch agar medium. After incubation, the two halophilic isolates GD3007 & DM0207 were found to have clear zone (Figure 3). Both the isolates had the ability to produce halo zones on skim milk agar

that showed protease activity (Figure 4). Halophilic and halotolerant bacteria produce several commercially important enzymes like amylase, protease and cellulase. Similarly, Kumar *et al.*, 2012 demonstrated screening and isolation of halophilic bacteria producing industrially important hydrolytic enzymes including amylase, and protease.

**I) Physical characterization**

Optimization conditions like pH, temperature, and NaCl were tested for the effective strain. The strain was found to resist up to 20% NaCl with optimal growth at 12%. It was able to grow at all the pH range with 7 being the optimal and room temperature being the effective and optimal growth temperature.

**J) Seed germination**

The PGP effect of halophilic isolates on seed germination was evaluated by pretreating Chickpea, Cowpea, Green gram, and Pearl millet seeds with the isolates. It was noted that halophilic isolate GD3007 increased seed germination by 10 to 20% over control and isolate DM0207 remarkably influenced the germination of seeds. The PGP isolate DM0207 significantly induced the height of seedlings than the isolate GD3007. (Figure 5). This result revealed that plant height increased in PGPR treated plants over un-inoculated control. The plant root length and shoot length was recorded at 3 different intervals 5th, 10th and 15th day. This result indicates the test isolates have the plant growth promoting activity. The lowest root length, shoot length and number of leaves were noted in uninoculated control (Table 4).

**K) Preparation of Biofertilizer**

A formulation was prepared using the halophilic isolate DM0207-Phosphobact (Figure 6), because of its denitrifying ability, phosphate solubilization and increased stimulation of root and shoot length than the halophilic isolate GD3007. The

BIS specification for biofertilizer was determined and the results were tabulated (Table II). The viability of the cells can be retained for 6 months by storing the packages at 4°C. Although we have developed biofertilizer using soil as a carrier; however field trials are yet to be conducted.

**L) Molecular Identification**

On the basis of phenotypic characteristics and the comparison of partial 16 S rRNA gene sequence, the isolates GD3007 & DM0207 were identified as *Oceanobacillus oncorhynchi* and *Pseudomonas stutzeri* respectively (Table III). Kumar *et al.*, 2012 had reported the morphological, biochemical and 16S rRNA analysis of halophilic bacteria *Oceanobacillus*, *Bacillus*, *Halomonas* and *Staphylococcus* genera isolated from salt pans.

**XVII. CONCLUSION**

The notion prevailing today is that PGP halophilic bacterial inoculants will complement the commercial use of chemical fertilizers already on the market. Commercial production of biofertilizer is relatively inexpensive and therefore it can be easily manufactured and modestly displace the chemical fertilizers. It is also easy to isolate novel biocontrol PGP bacterium that will increase root and shoot development. In conclusion, the halophilic isolate DM0207, which was identified as *Pseudomonas stutzeri* shows a wide range of properties as biofertilizer for moderately saline soils, a producer of hydrolytic enzymes and its plant growth promotion. It is thus an ideal candidate that can be exploited for agricultural and industrial application practices.

**XVIII. CONFLICTS OF INTEREST**

The author declares that there is no conflict of interest

**Table I Biochemical tests of the halophilic isolates**

**Table II Determination of Initial Viable Count**

TESTS	GD3007	DM0207
Indole	-	+
MR	+	+
VP	-	-
Citrate	+	+

DILUTION	INITIAL VIABLE COUNT	AFTER 25 DAYS	AFTER 30 DAYS	AFTER 35 DAYS
10 <sup>-1</sup>	TNTC	TNTC	TNTC	TNTC
10 <sup>-2</sup>	172	172	172	172
10 <sup>-3</sup>	136	136	136	136
10 <sup>-4</sup>	114	114	114	114
10 <sup>-5</sup>	108	108	108	108
10 <sup>-6</sup>	93	93	93	93
10 <sup>-7</sup>	67	67	67	67

Table III Identification of isolated halophilic strains based on 16S rRNA gene sequence and their accession numbers (BLAST similarity search results)

STRAIN ID	STRAIN NAME / GENUS	NUMBER OF NUCLEOTIDES OF 16S rRNA GENE	ACCESSION NUMBER OF 16S rRNA GENE	CLOSELY RELATED TAXA	SEQUENCE SIMILARITY (%) OF 16S rRNA GENE
GD3007	<i>Oceanobacillus</i>	1497	LT221188	<i>Oceanobacillus oncorhynchi</i>	92%
DM0207	<i>Pseudomonas</i>	1150	ABI26690	<i>Pseudomonas stutzeri</i>	90%

Table IV Comparison of measurements of root and shoot length in test plants and Control for different incubation days

TEST SEED	INCUBATION PERIOD (DAYS)	GD3007		DM0207		CONTROL	
		R.L	S.L	R.L	S.L	R.L	S.L
CHICK PEA	5	2.3 cm	5.2 cm	0.8 cm	7.4 cm	1.2 cm	1.3 cm
	10	2.5 cm	6.8 cm	3.4 cm	8.9 cm	1.9 cm	5.5 cm
	15	2.9 cm	10.3 cm	5.4 cm	14 cm	2.5 cm	9.6 cm
COW PEA	5	2.8 cm	4.5 cm	2.3	7 cm	1.5 cm	3.8 cm
	10	3.2 cm	9.6 cm	4 cm	10.6 cm	2.5 cm	6.5 cm
	15	3.8 cm	10 cm	6 cm	13.3 cm	2.7 cm	10 cm
GREEN GRAM	5	1.9 cm	3.6 cm	3.3 cm	2.8 cm	1 cm	0.8 cm
	10	2.8 cm	11.5 cm	3.6 cm	12.8 cm	3 cm	6.5 cm
	15	3 cm	11.8 cm	7 cm	13.4 cm	3.2 cm	9.6 cm
PEARL MILLET	5	2 cm	2.6 cm	2.5 cm	3.1 cm	1.8 cm	1.9 c,
	10	4 cm	3.2 cm	6 cm	4 cm	4 cm	2.3 cm
	15	5.5 cm	4.5 cm	8.5 cm	5.3 cm	5 cm	3.5 cm

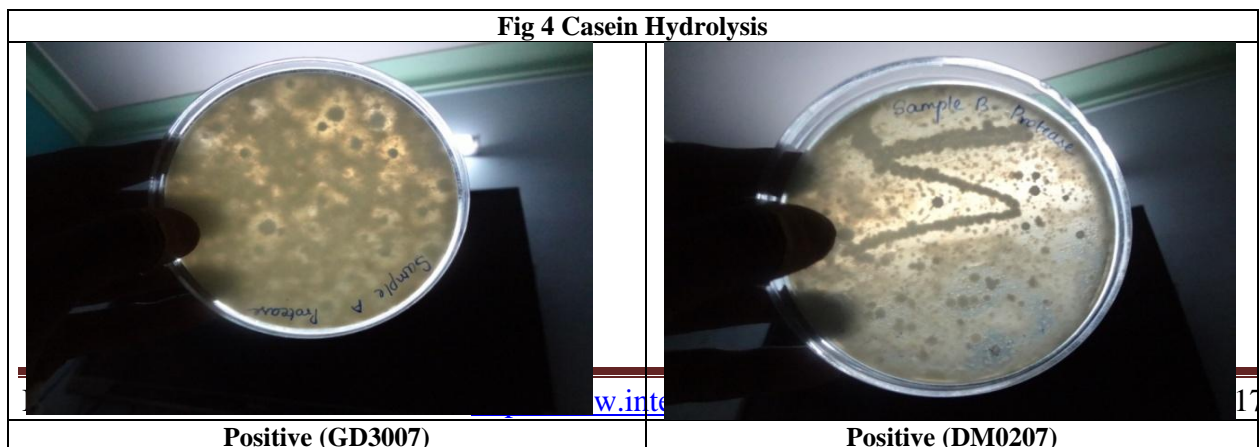
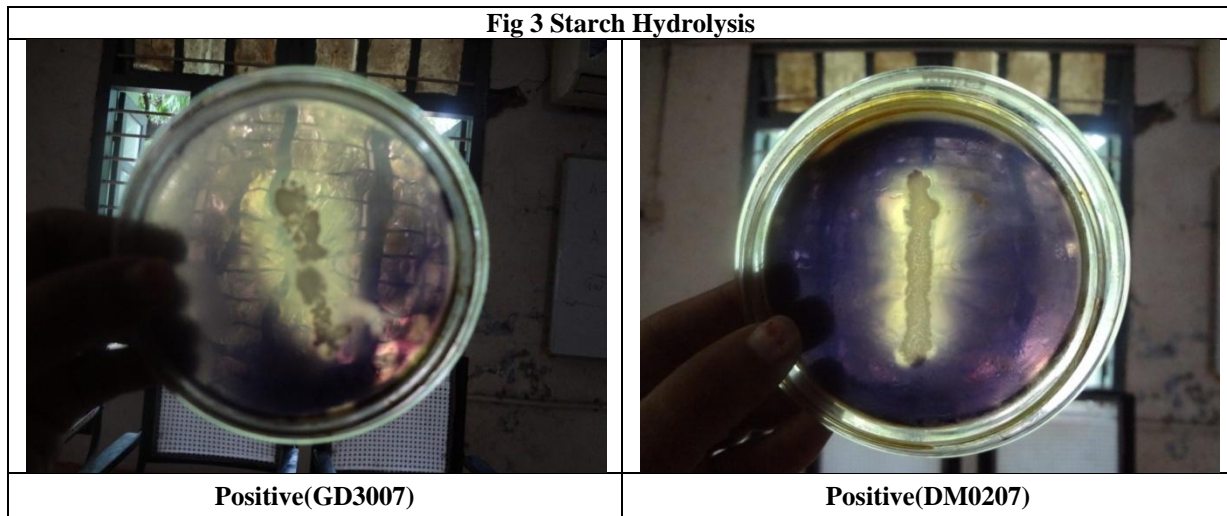
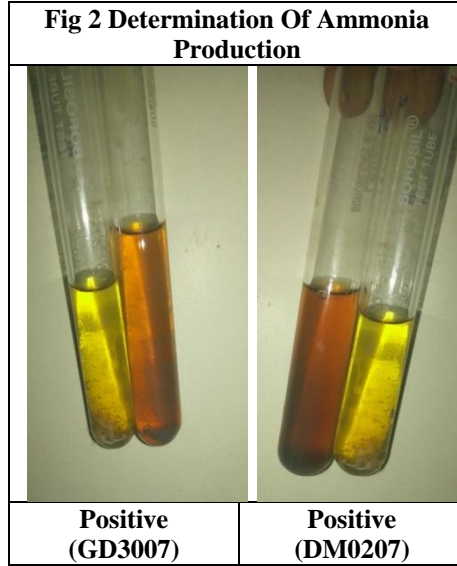
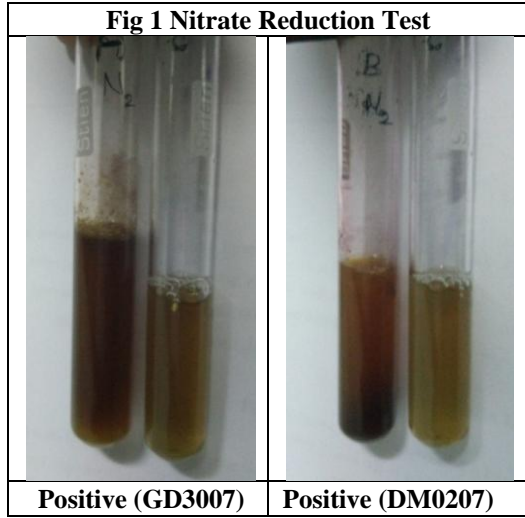














Fig 5 Seed Germination Test

INCUBATION DAYS	CHICK PEA	COW PEA	GREEN GRAM	PEARL MILLET
DAY 5				
DAY 10				
DAY 15				

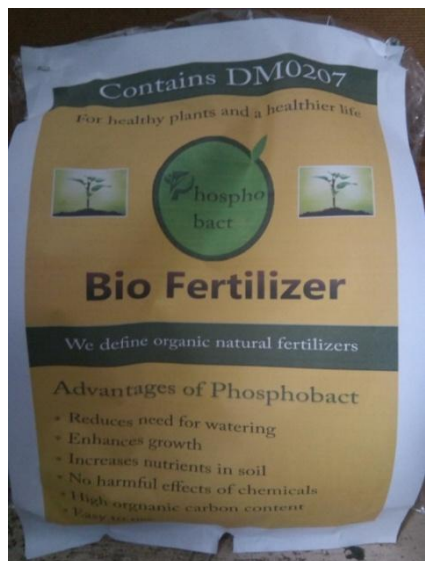
\* C- Control

\*GD-GD3007

\*DM-DM0207



**Fig 6 Phosphobact Biofertilizer**



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