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Evaluate the Shelf Life of Rhizobium Carrier Based Biofertilizer Stored at Different Temperatures at Different Intervals

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ABSTRACT

Keywords

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In the present study a total of ten Rhizobial bacterial isolates collected from different sources. These cultures were studied morphologically and biochemically for purity confirmation. The screening test results revealed that, among all Rhizobial isolates, GNR-1 showed best plant growth promoting abilities in *in-vitro* conditions. This promising culture was selected for main experiment to evaluate the shelf life of carrier based bio inoculants in different carrier materials stored at different temperatures in different intervals. The selected efficient PGP *Rhizobium* (GNR-1) and isolate was multiplied in large quantities in appropriate culture broth by incubating at $28 \pm 2^\circ\text{C}$ in an incubator shaker at 120 rpm till they attained log phase with a cell load of $1 \times 10^9 \text{ cfu ml}^{-1}$. For biofertilizer production Vermicompost, Vermiculite, Lignite, and Sodium Alginate carriers were used for bio inoculant preparation and stored at different temperatures *i.e.* 4°C and 28°C . Though the bacterial population decreased from 1st month to 8th month the survival rate of rhizobial cells was more upto 8th month at 4°C . Similarly with respect to evaluation of shelf life of bacterial cells in different carrier materials like sodium alginate vermicompost lignite and vermiculite, sodium alginate based inoculants maintained good population count upto 8th month.

Introduction

Biofertilizers are identified to have a supplementary role to chemical fertilizers to increase soil fertility and crop production in sustainable farming. Microbial inocula not only increased the assimilation of nutrients by plant (total N, P and K), but also improved soil properties, such as organic matter content and total N in soil (Wu *et al.*, 2005).

Biofertilizers with solid carrier material can be more advantageously used because they can increase the supply of phosphorus to plant, resistance to soil borne plant pathogens

and biological degradation of organic pollutants (Warren *et al.*, 2009). Several alternative carrier material, such as, soil, perlite, polyacrylamide gel, ground rock phosphate, alginate, coal, decomposed saw dust, compost, farmyard manure and volcanic pumice have been evaluated as inoculant carrier (Stephens and Rask, 2000).

No one can deny the importance of chemical fertilizers but high inputs of chemical fertilizers have not only caused environmental problems but also generated human health

damages. Major portion of applied fertilizers is not available to the plants because it becomes fixed onto the soil particles while remaining part enters into fresh water bodies and cause eutrophication which ultimately deteriorates the water quality (Barlog and Grzebisz, 2004).

Conventional agriculture plays a significant role in meeting the food demands of growing human population, which has also led to an increasing dependence on chemical fertilizers and pesticides (Santoso *et al.*, 2007). About 50% of applied nitrogen fertilizers enters into the environment from the agricultural lands in the form of nitrates, N_2 and NH_3 (Tilman, 1998) whereas 90% of phosphatic fertilizer gets precipitated and causes pollution. Furthermore, excessive use of chemical fertilizers has enhanced emission of greenhouse gases into the environment which damages ozone layer (Ma *et al.*, 2007) and causes global warming (Frink *et al.*, 1999).

John *et al.*, (2006) reported that the increased pathogen attack in aquatic ecosystem might be due to high accumulation of phosphorus and nitrogen from the agricultural lands. Several efforts have been made globally to overcome this major problem. Use of biofertilizer along with the complementary measures is the ultimate strategy for maintaining soil fertility and agricultural sustainability (Graham and Vance, 2000).

Materials and Methods

The present study was carried out at the Department of Agricultural Microbiology & Bioenergy, College of Agriculture, Rajendranagar, PJTSAU, and Hyderabad. Pure cultures of Plant Growth Promoting *Rhizobium* isolates collected from different laboratories. Attempts were made to assess the screening and characterization of isolates with multiple beneficial properties then the

efficient PGPR isolate were selected for preparation of carrier based biofertilizers.

Collection of rhizobial isolates from different sources

Promising bacterial isolates are collected from different laboratories and these isolates were tested for their purity and preservation in Dept. of Agricultural Microbiology & Bioenergy, College of Agriculture, PJTSAU Rajendranagar, Hyderabad.

Identification of bacterial isolates purity checking

Morphological and biochemical characterization

The isolated bacteria were studied for their morphological like gram reaction, pigmentation, cultural characteristics and biochemical characteristics like In dole production, methyl red, Voges-Praskaure's test, citrate utilization test, oxidase, catalase and sugar fermentation tests.

Screening for plant growth promoting properties

Screening will be carried out for different plant growth promoting properties such as mineral solubilization like Phosphorus Solubilisation (Pikovskaya, 1948), Zinc Solubilization (Saravanan *et al.*, 2003), Potassium releasing (Prajapati and Modi, 2012), Plant growth promoting substances such as IAA production (Gorden and Weber, 1951), biocontrol activity such as HCN production (Castric and Castric, 1983) and Siderophore production (Schwyn and Neilands, 1987) and antifungal activity with soil born plant pathogens all ten isolates were checked for their purity and then studied for the colony morphology and pigmentation. The cell shape and gram reaction were also

recorded as per the standard procedures given by Barthalomew and Mittewar (1950).

Gram's staining

A drop of sterile distilled water was placed in the center of glass slide. A loopful of inoculum from young culture was taken, mixed with water, and placed in the center of the slide. The suspension was spread out on slide using the tip of inoculation needle to make a thin suspension.

The smear was dried in air and fixed through mild heating by passing the slide 3 to 4 times over the flame. The smear was then flooded with Crystal violet solution for 1 min and washed gently with flow of tap water.

Then the slide was flooded with Iodine solution. After incubation at room temperature for 1 min, Iodine solution was drained out followed by washing with 95% decolorizer. After that, it was washed with water within 15 to 30 sec and blot carefully. The smear was incubated with Saffranin solution for 1 min. The slide was washed gently in flow of tap water and dried in air. The slide was examined under microscope at 100X power with oil immersion and data was recorded.

Cultural characterization

Morphological characteristics of the colony of each isolate were examined on specialized medium. Cultural characterization of isolates observed by different characteristics of colonies such as shape, size, elevation, surface, margin, colour, odour, pigmentation etc were recorded.

Yeast Extract Mannitol Agar with Congo red test (YEMAC)

The isolates were streaked on YEMAC media plates and incubated at $28 \pm 20^{\circ}\text{C}$ for 48-72h.

Rhizobial colonies do not absorb colour and remain white in colour.

Collection of carrier materials

Sodium alginate collected from Department of Agricultural Microbiology and Bioenergy College of Agriculture Rajendranagar, Hyderabad. Vermiculate collected from Navaratna Crop Science Pvt Ltd, Cherlapally, Hyderabad. Lignite collected from RKVY project in the Department of Agricultural Microbiology and Bioenergy College of Agriculture Rajendranagar, Hyderabad. Vermicompost collected from NIRD Rajendranagar, Hyderabad.

Physico-chemical properties of carriers

For the preparation of bioformulation, the collected different carriers such as lignite, vermicompost, sodium alginate and vermiculite were tested for their moisture content (Aeron *et al.*, (2011) and pH at initial and end of the experiment.

Autoclave sterilization

Lignite, Vermicompost, Vermiculate, and Sodium Alginate were sterilized in Tyndalization process in an Autoclave at 15lb psi (121°C) for 20 min three times on succeeding days.

Preparation of biofertilizers

Preparation of carrier based bio-formulations

The selected isolate was multiplied in large quantities in appropriate culture broth by incubating at $28 \pm 2^{\circ}\text{C}$ in an incubator shaker till they attained log phase with a cell load of $1 \times 10^9 \text{cfu ml}^{-1}$ and were used for inoculant preparation. The individual carrier materials were powdered and the pH was brought to neutral by adding CaCO_3 after sterilized at 15

psi 121⁰c for 1 hour in three successive days after and then mixed with the log phase culture (1×10^9 cfu ml⁻¹) of the selected plant growth promoting bacterial isolate viz., *Rhizobium* (GNR1) in separate quantities of sterile carrier in shallow trays. The optimum moisture content was adjusted to (30-40%) prior to preparation, followed by curing in shallow trays for 24 hours in aseptic rooms and then packed in high density opaque polythene bag (12g) at the rate of 100g bag⁻¹ and sealed. Individual inoculant was prepared by mixing with lignite (1:3v/w), vermicompost (1:2v/w), vermiculite (1:2v/w) volumes of each culture broth with sterile carrier materials. The populations of individual Plant Growth Promoting Rhizobacteria in the inoculant carriers were assessed at monthly intervals upto 8 months.

Preparation of alginate based inoculant

Rhizobium spp was grown in respective medium to get a population of 1×10^9 cfu ml⁻¹ Sodium alginate beaded inoculant was prepared as per the methods described by Hegde and Brahmaprakash (1992). Two gram of sodium alginate was added to 100 ml of culture broth of PGPR and mixed for 30 minutes in a magnetic stirrer.

The mixture was added drop wise through a 10 ml syringe into 100 ml sterile 0.1N CaCl₂ to obtain uniform Alginate beads. One gram of material contained 16 to 17 beads, each bead approximately weighing 60mg. The beads were washed twice in sterile distilled water and incubated for seven days in a psychrotherm (model environ shaker) incubator at 28±2°C to allow PGPR to multiply inside the beads. The beads were again washed in sterile distilled water and air dried in Laminar air flow chamber under aseptic condition. The Alginate beads were then stored in polythene bags at room temperature (28±2°C) and refrigerator (4°C) up to 8 months.

Treatments,

T 1: S₁C₁O₁ (Autoclaved Vermicompost with *Rhizobium spp*)

T 2: S₁C₂O₁ (Autoclaved Sodium alginate with *Rhizobium spp*)

T3: S₁C₃O₁ (Autoclaved Lignite with *Rhizobium spp*).

T4: S₁C₄O₁ (Autoclaved Vermiculite with *Rhizobium spp*),

Determination of viable bacterial population in the carrier based inoculants by serial dilution and plating technique. Influence of storage temperature on the survival of the inoculants as consortium in different carrier materials. The carrier based microbial inoculants prepared with different carrier material was kept in different temperature levels viz., Room temperature (28±2°C) and Refrigerator (4°). The surviving populations of PGPB at different temperatures were determined and population was enumerated by dilution plate technique at different intervals i.e., monthly upto 8 months.

Results and Discussion

At 4°C storage temperature, autoclave sterilized vermicompost, sodium alginate, lignite, vermiculite based rhizobial inoculants, rhizobial bacterial population decreased from 1st month to 8th month table 1.

At 28 ± 2°C storage temperature also autoclave sterilized all carrier based rhizobial inoculants, rhizobial bacterial population decreased from 1st month to 8th month table 2. But survival rate of rhizobial cells was more upto 8th month at 4°C compared to 28 ± 2°C. This results revealed that 4°C storage temperature is best suitable for storage of carrier based inoculants because of low level of moisture content in the carrier inoculants stored at 28 ± 2°C temperature.

Table.1 Rhizobium population in different carrier based bio inoculants at 4°C up to 8 months

| Month | Vermicompost | Sodium alginate | Lignite | Vermiculite |
|-------|--------------|-----------------|---------|-------------|
| | 4°C | 4°C | 4°C | 4°C |
| 1 | 9.58 | 9.64 | 9.6 | 9.61 |
| 2 | 9.56 | 9.62 | 9.52 | 9.56 |
| 3 | 9.48 | 9.58 | 9.44 | 9.5 |
| 4 | 9.26 | 9.52 | 9.2 | 9.4 |
| 5 | 8.96 | 9.4 | 8.85 | 9.26 |
| 6 | 8.73 | 9.3 | 8.55 | 8.89 |
| 7 | 8.4 | 9.1 | 8.18 | 8.6 |
| 8 | 7.42 | 8.8 | 7 | 7.73 |

Table.2 Rhizobium population in different carrier based bio inoculants at 28±2°C up to 8 months

| Month | Vermicompost | Sodium alginate | Lignite | Vermiculite |
|-------|--------------|-----------------|---------|-------------|
| | 28°C | 28°C | 28°C | 28°C |
| 1 | 9.85 | 9.92 | 9.8 | 9.9 |
| 2 | 9.77 | 9.84 | 9.74 | 9.8 |
| 3 | 9.66 | 9.75 | 9.63 | 9.69 |
| 4 | 9.34 | 9.54 | 9.27 | 9.33 |
| 5 | 8.6 | 9.18 | 8.45 | 8.78 |
| 6 | 8.2 | 8.7 | 7.8 | 8.42 |
| 7 | 7.6 | 8.16 | 7.4 | 8 |
| 8 | 6.8 | 7.35 | 6.5 | 7 |

Fig.1

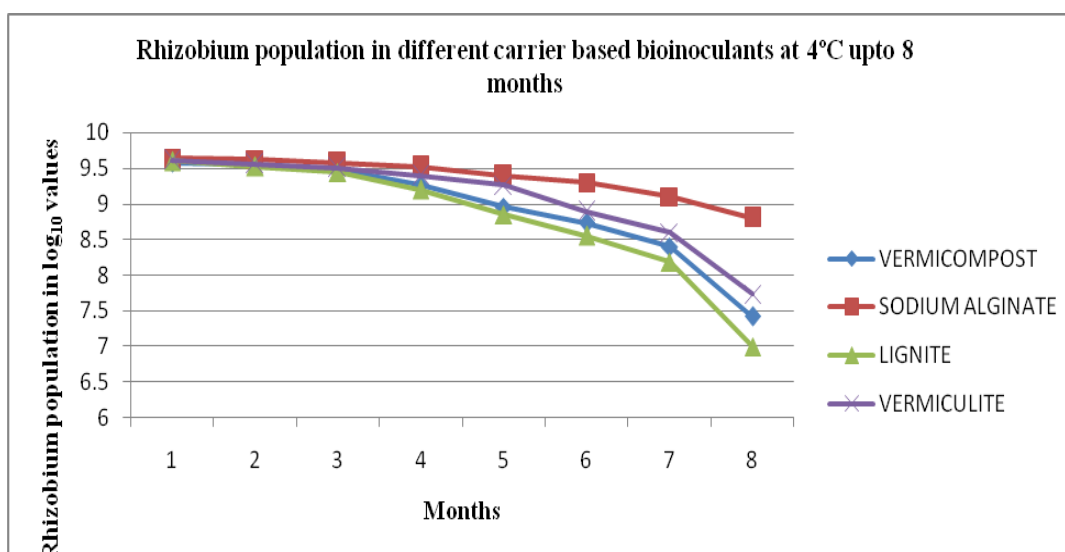
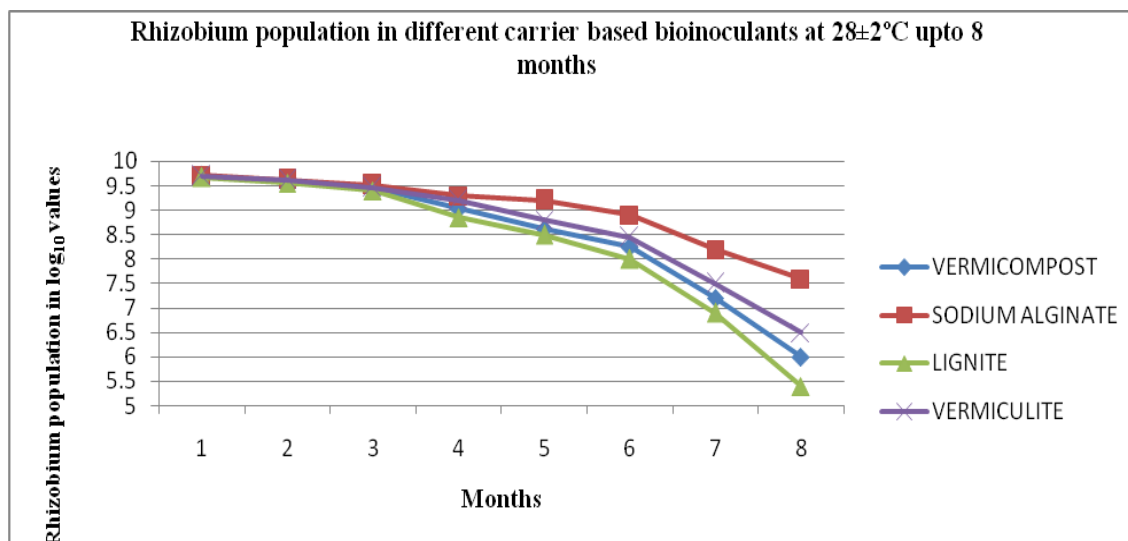


Fig.2



As per specification of biofertilizers, carrier should be minimum 5×10^7 cfu g^{-1} ($7.6 \log_{10}$) viable count of powdered form of carrier based biofertilizer. So that the above results revealed that at $4^\circ C$, autoclave sterilized vermicompost and lignite carrier based rhizobial bio inoculants supported and maintained optimum viable count (5×10^7 cfu g^{-1}) upto 7th month only, whereas sodium alginate and vermiculite based carrier bio inoculants supported and maintained optimum viable count (5×10^7 cfu g^{-1}) upto 8th month figure 1. At $28^\circ C$, autoclave sterilized vermicompost and lignite carrier based rhizobial inoculants supported and maintained optimum viable count (5×10^7 cfu g^{-1}) upto 6th month only, but sodium alginate and vermiculite based carrier bio inoculants supported and maintained optimum viable count (5×10^7 cfu g^{-1}) upto 7th month figure 2.

In conclusion, the production rhizobium carrier based biofertilizer sodium alginate is best in the view of shelf life of bio inoculants, for the longer storage purpose have to keeping in $4^\circ C$ or refrigerated to maintain viability.

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