

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

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ABSTRACT

In this study focus on shelf life of different carrier based bioinoculants for this Pseudomonas cultures collected from different resource labs, then cultures were studied morphologically and biochemically for purity confirmation. The screening test results revealed that, among all Pseudomonas isolates, (RGP-1) showed best plant growth promoting abilities in in-vitro conditions. The selected Pseudomonas (RGP-1) 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 $1 \times 10^9 \text{ cfu ml}^{-1}$. For biofertilizer production Vermicompost, Vermiculite, Lignite, and Sodium Alginate carriers were used for bioinoculant preparation and stored at different temperatures i.e. 4°C and $28 \pm 2^\circ\text{C}$. In first month \log_{10} values are 9.62, 9.82 (in Vermicompost), 9.73, 9.84 (Sodium Alginate), 9.50 9.75 (Lignite), 9.70, 9.82 (Vermiculite) showed at 4°C and $28 \pm 2^\circ\text{C}$ respectively, Even though bioinoculant populations were higher at first month gradually decreasing upto 8th month, at the end of the experiment \log_{10} values are 8.20, 6.50 (in Vermicompost), 8.90, 7.20 (Sodium Alginate), 7.80, 6.00 (Lignite), 8.50, 6.80 (Vermiculite) showed at 4°C and $28 \pm 2^\circ\text{C}$ respectively. Finally concluded that Sodium Alginate is best carrier materials among them when compared to specification of biofertilizers, carrier should be minimum $5 \times 10^7 \text{ cfu g}^{-1}$ ($7.6 \log_{10}$) viable count of powdered form of carrier based biofertilizer at both storage temperatures.

Key words: *Pseudomonas, carriers, Vermiculite Vermicompost, Sodium alginate, temperatures.*

INTRODUCTION

Environmental stresses are becoming a major problem and productivity is declining at an unprecedented rate. Our dependence on chemical fertilisers and pesticides has

encouraged the thriving of industries that are producing life-threatening chemicals and which are not only hazardous for human consumption but can also disturb the ecological balance.

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Biofertilizers can help solve the problem of feeding an increasing global population at a time when agriculture is facing various environmental stresses. It is important to realise the useful aspects of biofertilizers and implement its application to modern agricultural practices¹.

Commonly used organisms as biofertilizers are nitrogen (N) fixers, potassium (K) solubilizer and phosphorus (P) solubilizer, or with the combination of molds or fungi. These biological fertilizers would play key role in production and sustainable agriculture and also protect the environment as eco-friendly and cost effective inputs for the farmers. Inoculum, method of application and storage of the bioinoculant are all critical to the success of a biofertilizer. There are 6 major steps in making biofertilizer, and these includes choosing active microorganisms, isolation and selection of target microbes, selection of method and carrier material, selection of propagation method, prototype testing and large scale testing. First of all, active organisms must be decided. For example, we must decide to use whether organic acid bacteria or nitrogen fixer or the combination of some organisms².

Pseudomonas aeruginosa has been shown to withstand biotic and abiotic stresses³ *P. fluorescens* MSP-393 produces osmolytes and salt-stress induced proteins that overcome the negative effects of salt⁴. *P. putida* Rs-198 enhanced germination rate and several growth parameters viz., plant height, fresh weight and dry weight of cotton under condition of alkaline and high salt via increasing the rate of uptake of K⁺, Mg²⁺ and Ca²⁺, and by decreasing the absorption of Na⁺.⁵ Few strains of *Pseudomonas* conferred plant tolerance via 2,4-diacetylphloroglucinol (DAPG)⁶. Interestingly, systemic response was found to be induced against *P. syringae* in *Arabidopsis thaliana* by *P. fluorescens* DAPG⁷.

Pseudomonas spp. was found to cause positive affect on the seedling growth and seed germination of *A. officinalis* L. under water stress⁸. Heavy metals such as cadmium, lead, mercury from hospital and factory waste

accumulate in the soil and enter plants through root⁹. *Azospirillum spp.*, *Phosphobacteria spp.* and *Glucanacetobacter spp.* isolated from rhizosphere of rice field and mangroves were found to be more tolerant to heavy metal specially iron¹⁰. *P. putida* strain 11 (P.p.11), *P. putida* strain 4 (P.p.4) and *P. fluorescens* strain 169 (P. f.169) can protect canola and barley plants from the inhibitory effects of cadmium via IAA, siderophore and 1-aminocyclopropane-1-carboxylate deaminase (ACCD)¹¹.

MATERIAL 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 *Pseudomonas* 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 *Pseudomonas* 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 Indole 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 Solubilisation¹² Zinc Solubilization¹³, Potassium releasing¹⁴, Plant growth promoting substances such as IAA production, biocontrol activity such as HCN production¹⁵ and Siderophore production¹⁶ and antifungal activity with soil born plant pathogens all fifteen 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 by¹⁷.

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. Suspension spread on slide using the tip of inoculation needle to make a thin suspension 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.

Growing of culture.

King's medium B Base

Kings Medium B Base is recommended for non-selective isolation, cultivation and pigment production of *Pseudomonas* species

Composition

Ingredients	Gms / Litre
Proteose peptone	20.000
Dipotassium hydrogen phosphate	1.500
Magnesium sulphate. Heptahydrate	1.500
Agar	20.000
Final pH (at 25°C)	7.2±0.2

Directions

Suspend 42.23 grams of dehydrated medium in 1000 ml distilled water containing 15 ml of glycerol. Heat to boiling to dissolve the medium completely. Mix well. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Aseptically pour into sterile Petri plates.

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 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

a. Preparation of carrier based bio-formulations

The selected isolate was multiplied in large quantities in appropriate culture broth by incubating at 28±2°C in an incubator shaker till they attained log phase with a cell load of 1×10^9 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 121°C 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., *Pseudomonas* (RGP1) 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.

b. Preparation of Alginate based inoculant

Pseudomonas (RGP1) 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 described¹⁸. 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 CaCl_2 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 $28 \pm 2^\circ\text{C}$ 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 ($28 \pm 2^\circ\text{C}$) and refrigerator (4°C) upto 8 months.

Treatments

T 1: $\text{S}_1\text{C}_1\text{O}_1$ (Autoclaved Vermicompost with *Pseudomonas spp*)

T 2: $\text{S}_1\text{C}_2\text{O}_1$ (Autoclaved Sodium alginate with *Pseudomonas spp*)

T3: $\text{S}_1\text{C}_3\text{O}_1$ (Autoclaved Lignite with *Pseudomonas spp*).

T4: $\text{S}_1\text{C}_4\text{O}_1$ (Autoclaved Vermiculite with *Pseudomonas 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 ($28 \pm 2^\circ\text{C}$) and Refrigerator (4°C). 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

First month results revealed that vermicompost based bioinoculant showed \log_{10} value 9.62, 9.82, sodium alginate, showed \log_{10} value 9.73, 9.84, lignite, showed \log_{10} value 9.50, 9.75, vermiculite showed \log_{10} value 9.70, 9.82, at 4°C , $28 \pm 2^\circ\text{C}$ storage temperature respectively. When compared among carrier materials sodium alginate having highest population and lignite having least population at both temperatures.

Eight month results revealed that vermicompost based bioinoculant showed \log_{10} value 8.20, 6.50, sodium alginate, showed \log_{10} value 8.90, 7.20, lignite, showed \log_{10} value 7.80, 6.00, vermiculite showed \log_{10} value 8.50, 6.80, at 4°C , $28 \pm 2^\circ\text{C}$ storage temperature respectively.

From 1st month to 8th month *Pseudomonas* population gradually decreased (table no.1. and 2.) But survival rate of *Pseudomonas* cells was more upto 8th month at 4°C compared to $28 \pm 2^\circ\text{C}$. 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 $28 \pm 2^\circ\text{C}$ temperature compared to 4°C .

As per specification of biofertilizers, carrier should be minimum 5×10^7 cfu g^{-1} (\log_{10} 7.6) viable count of powdered form of carrier based biofertilizer. So that the above results revealed that at 4°C, lignite carrier based *Pseudomonas* bioinoculants supported and maintained optimum viable count \log_{10} 7.80 more than 5×10^7 cfu g^{-1} (\log_{10} 7.6) viable count of powdered form of carrier based biofertilizer upto 8th month. but showed the viable count least among different carrier materilas, whereas sodium alginate, vermiculite and vermicompost based carrier bioinoculants supported and maintained optimum viable count \log_{10} 8.90, 8.50, 8.20, respectively upto 8th month fig no.1. Which is more than the minimum 5×10^7 cfu g^{-1} (\log_{10} 7.6) viable count of powdered form of carrier based biofertilizer.

At $28 \pm 2^\circ\text{C}$ storage temperature vermicompost and lignite carrier based *Pseudomonas* inoculants supported and maintained optimum viable count \log_{10} 8.10 and \log_{10} 8.00 upto 6th month only, but sodium alginate and vermiculite based carrier bioinoculants supported and maintained optimum viable count \log_{10} 8.01 and \log_{10} 7.80. more than 5×10^7 cfu g^{-1} (\log_{10} 7.6) upto 7th month fig no.2. When comparing all carrier based bio inoculants shelf life sodium alginate *Pseudomonas* carrier based biofertilizer showed highest viable count upto 8th moths at both storage temperatures viz., \log_{10} 7.20 Room temperature ($28 \pm 2^\circ\text{C}$) and \log_{10} 8.90 Refrigerator (4°C).

Table 1: *Pseudomonas* Population in different carrier based bioinoculants at 4°C upto 8th months

MONTH	VERMI COMPOST	SODIUM ALGINAT	LIGNITE	VERMICULITE
	4 °C	4 °C	4 °C	4 °C
1	9.62	9.73	9.50	9.70
2	9.57	9.71	9.54	9.68
3	9.52	9.65	9.46	9.60
4	9.42	9.59	9.35	9.55
5	9.24	9.44	9.12	9.34
6	9.03	9.26	8.60	9.14
7	8.74	9.10	8.40	8.90
8	8.20	8.90	7.80	8.50

Table 1: *Pseudomonas* Population in different carrier based bioinoculants at 28±2°C upto 8th months

MONTH	VERMI COMPOST	SODIUM ALGINATE	LIGNITE	VERMICULITE
	(28±2°C)	(28±2°C)	(28±2°C)	(28±2°C)
1	9.82	9.84	9.75	9.82
2	9.72	9.81	9.65	9.74
3	9.45	9.60	9.36	9.56
4	9.20	9.42	9.10	9.30
5	8.55	8.92	8.36	8.70
6	8.10	8.57	8.00	8.31
7	7.41	8.01	7.20	7.80
8	6.50	7.20	6.00	6.80

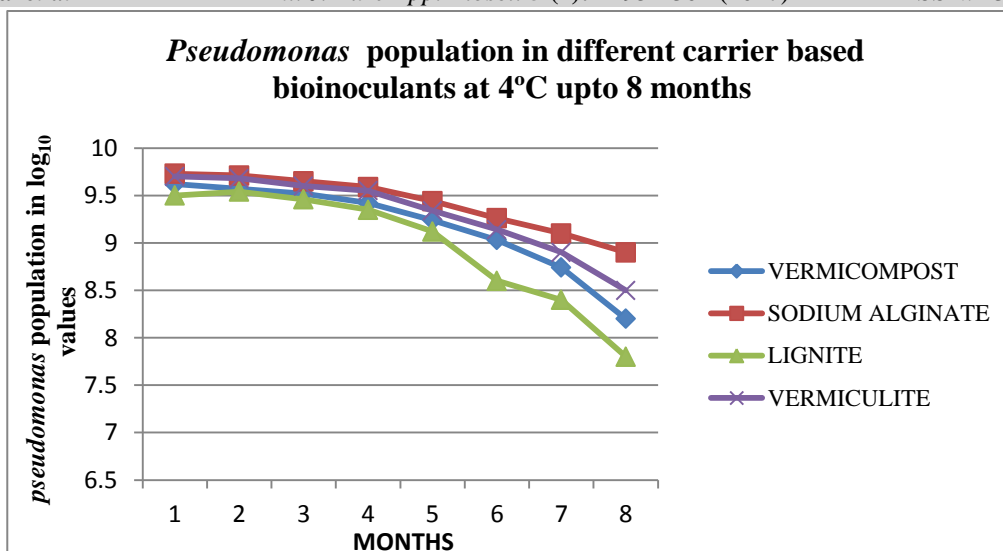


Fig. 1:

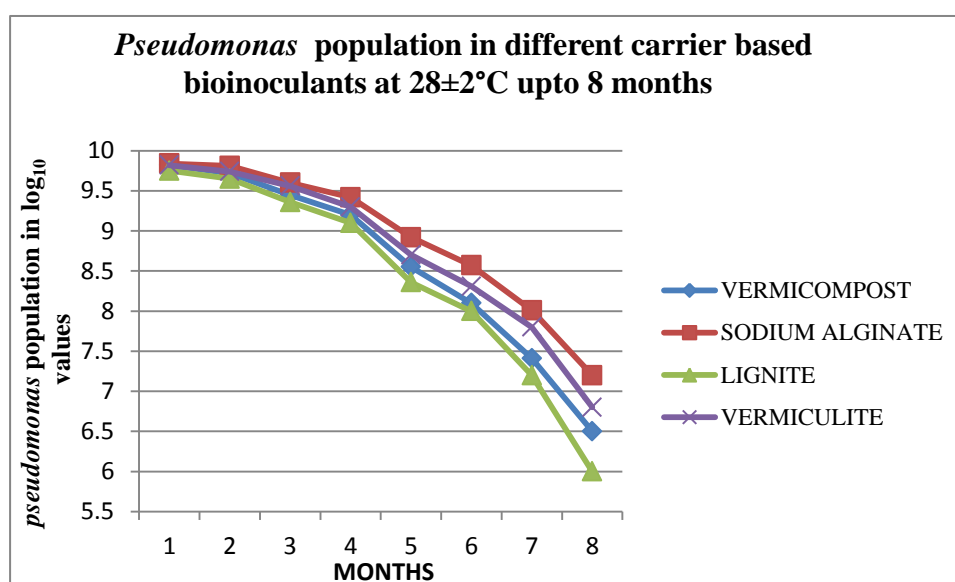


Fig. 2:

CONCLUSION

For the increasing the shelf life of *Pseudomonas* carrier based biofertilizers should be store at 4°C temperature then Room temperature (28±2°C), and also for best carrier based biofertilizer production sodium alginate and vermiculite carriers were showed highest population with long time storage capacity at both temperatures viz., Room temperature (28±2°C) and Refrigerator (4°C).

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