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

G. Thirumal¹, R. Subhash Reddy², S. Triveni³ and M. H. Bhave⁴

¹,²,³Department of Agricultural Microbiology and Bioenergy
⁴Department of Statistics & Mathematics

College of Agriculture, Prof Jaya Shankar Telangana State Agricultural University, Rajendranagar, Hyderabad - 500 030, Telangana, India

*Corresponding Author E-mail: thirumalgummula18@gmail.com

Received: 26.06.2017 | Revised: 7.07.2017 | Accepted: 8.07.2017

**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 ± 2°C in an incubator shaker till they attained log phase with a cell load of $1 \times 10^9$ cfu ml⁻¹. For biofertilizer production Vermicompost, Vermiculate, Lignite, and Sodium Alginate carriers were used for bioinoculant preparation and stored at different temperatures i.e. 4°C and 28± 2°C. In first month log₁₀ values are 9.62, 9.82 (in Vermicompost), 9.73, 9.84 (Sodium Alginate), 9.50 9.75 (Lignite), 9.70, 9.82 (Vermiculate) showed at 4°C and 28± 2°C respectively, Even though bioinoculant populations were higher at first month gradually deceasing upto 8th month, at the end of the experiment log₁₀ values are 8.20, 6.50 (in Vermicompost), 8.90, 7.20 (Sodium Alginate), 7.80, 6.00 (Lignite), 8.50, 6.80 (Vermiculate) showed at 4°C and 28± 2°C respectively. Finally concluded that Sodium Alginate is best carrier materials among them when compared to specification of biofertilizers, carrier should be minimum $5 \times 10^7$ cfu g⁻¹ (7.6 log₁₀) 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.
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 realize the useful aspects of biofertilizers and implement its application to modern agricultural practices.

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. Inoculam, 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 organisms.

*Pseudomonas aeruginosa* has been shown to withstand biotic and abiotic stresses. *P. fluorescens* MSP-393 produces osmolytes and salt-stress induced proteins that overcome the negative effects of salt. *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+. Few strains of *Pseudomonas* conferred plant tolerance via 2,4-diacetylphloroglucinol (DAPG). Interestingly, systemic response was found to be induced against *P. syringae* in Arabidopsis thaliana by *P. fluorescens* DAPG.

*Pseudomonas spp.* was found to cause positive affect on the seedling growth and seed germination of *A. officinalis* L. under water stress. Heavy metals such as cadmium, lead, mercury from hospital and factory waste accumulate in the soil and enter plants through root. *Azospirillium spp*, *Phosphobacteria spp* and *Glucanacetobacter spp.* isolated from rhizosphere of rice field and mangroves were found to be more tolerant to heavy metal specially iron. *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).

**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 solublization like Phosphorus Solubilisation\textsuperscript{12} Zinc Solubilization\textsuperscript{13}, Potassium releasing\textsuperscript{14} Plant growth promoting substances such as IAA production, biocontrol activity such as HCN production\textsuperscript{15} and Siderophore production\textsuperscript{16} 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 by\textsuperscript{17}.

**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 bioformulations**
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 1×10⁹ 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 (1x10$^9$ 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 1x10$^9$ cfu ml$^{-1}$ Sodium alginate beaded inoculant was prepared as per the methods described$^{18}$. 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±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) upto 8 months.

Treatments

T 1: $S_1C_1O_1$ (Autoclaved Vermicompost with Pseudomonas spp)

T 2: $S_1C_2O_1$ (Autoclaved Sodium alginate with Pseudomonas spp)

T 3: $S_1C_3O_1$ (Autoclaved Lignite with Pseudomonas spp).

T 4: $S_1C_4O_1$ (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±2°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 ± 2°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 9.70, 7.80, vermiculite showed log$_{10}$ value 9.80, 6.00, at 4°C, 28 ± 2°C storage temperature respectively. When compared among carrier materials sodium alginate having highest population and lignite having least population at both temperatures.

From 1$^{st}$ month to 8$^{th}$ month Pseudomonas population gradually decreased (table no.1. and 2. ) But survival rate of Pseudomonas cells was more upto 8$^{th}$ 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 compared to 4°C.
As per specification of biofertilizers, carrier should be minimum 5x10^7 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 5x10^7 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 materias, 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 5x10^7 cfu g^-1 (log_{10} 7.6) viable count of powdered form of carrier based biofertilizer.

At 28 ± 2°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 5x10^7 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±2°C) and log_{10} 8.90 Refrigerator (4°C ).

| MONTH | VERMICOMPOST (28±2°C) | SODIUM ALGINATE (28±2°C) | LIGNITE (28±2°C) | VERMICULITE (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                 |

| MONTH | VERMICOMPOST (4°C) | SODIUM ALGINATE (4°C) | LIGNITE (4°C) | VERMICULITE (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              |
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).

REFERENCES
1. Bhardwaj, Mohammad, W. A., Ranjan Kumar, S. and Narendra, T., Biofertilizers function as key player in sustainable agriculture by improving soil fertility, planttolerance and crop productivity. Microbial Cell Factories 13: 66 (2014).
2. Khosro, M. and Yousef, S., Bacterial biofertilizers for sustainable crop production: a review. ARPN Journal of Agricultural and Biological Science. 7(5): (2012).
3. Pandey, P.K., Yadav, S.K., Singh, A., Sarma, B.K., Mishra, A., Singh, H.B., Cross-Species Alleviation of Biotic and Abiotic Stresses by the Endophyte Pseudomonas aeruginosa PW09. J Phytopathol. 160: 532–539 (2012).
4. Paul, D. and Nair, S., Stress adaptations in a plant growth promoting Rhizobacterium (PGPR) with increasing salinity in the coastal agricultural soils. *J Basic Microbiol.* **48**: 1–7 (2008).

5. Yao, L., Wu, Z., Zheng, Y., Kaleem, I., Li, C., Growth promotion and protection against salt stress by Pseudomonas putida Rs-198 on cotton. *European J Soil Biol.,* **46**: 49–54 (2010).

6. Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot-Bonnefoy, C., Reimmann, C., Notz, R., Defago, G., Haas, D., Keel, C., Autoinduction of 2,4 diacetylphloroglucinol biosynthesis in the biocontrol agent Pseudomonas fluorescens CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. *J Bacteriol.,* **182**: 1215–1225 (2000).

7. Weller, D.M., Mavrodi, D.V., van Pelt, J.A., Pieterse, C.M., van Loon, L.C., Bakker, P.A., Induced systemic resistance in Arabidopsis thaliana against Pseudomonas syringae pv. tomato by 2,4-diacetylphloroglucinol-producing *Pseudomonas* fluorescens. *Phytopathology.* **102**: 403–412 (2012).

8. Liddycoat, S.M., Greenberg, B.M., Wolyn, D.J., The effect of plant growth promoting rhizobacteria on asparagus seedlings and germinating seeds subjected to water stress under greenhouse conditions. *Can J Microbiol.,* **55**: 388–394 (2009).

9. Gill, S.S., Khan, N.A., Tuteja, N., Cadmium at high dose perturbs growth, photosynthesis and nitrogen metabolism while at low dose it up regulates sulfur assimilation and antioxidant machinery in garden cress (*Lepidium sativum* L.). *Plant Sci.,* **182**: 112–120 (2012).

10. Samuel, S. and Muthukkaruppan, S.M., Characterization of plant growth promoting rhizobacteria and fungi associated with rice, mango and effluent contaminated soil. *Curr Bot.** 2**: 22–25 (2011).

11. Baharlouei, K., Pazira, E., Solhi, M., Evaluation of Inoculation of plant Growth-Promoting Rhizobacteria on Cadmium. Singapore: *International Conference on Environmental Science and Technology IPCBEE. 6:* (2011)

12. Pikovskaya, R.I., Mobilization of phosphorus in soil connection with the vital activity of some microbial species. *Microbiologiya,* **17**: 362–370 (1948).

13. Saravanan, V.S., Subramoniam, S.R. and Raj, S.A. Assessing in Vitro solubilization potential of different zinc solubilizing bacterial (ZnSB) isolates. *Brazilian Journal of Microbiology.* **34**: 121-125. (2003).

14. Prajapati, K.B. and Modi, H.A., Isolation and characterization of potassium solubilizing bacteria from ceramic industry soil. *CIB .Tech Journal of Microbiology.* **1**:2-3 (2012).

15. Castric, K.F., and Castric, P.A., Method for rapid detection of cyanogenic bacteria. *Applied and Environmental Microbiology.* **45**: 700–702 (1983).

16. Schwyn, B. and Neilands, J.B., Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry.* **160**: 47-56 (1987).

17. Barthalomew, J.W and Mittewer, T. A simplified bacterial strain. *Stain Technology.* **25**:153 (1950).

18. Hegde, S. V. and Brahmaprakash, G. P., A dry granular inoculant of *Rhizobium* for soil application. *Plant and Soil.* **144** (2): 309–311 (1992).