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REGULAR ARTICLE

STUDIES ON SHELF LIFE OF *AZOSPIRILLUM LIPOFERUM*, *BACILLUS MEGATERIUM* AND *PSEUDOMONAS FLUORESCENS* IN VEMICOMPOST CARRIER

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SUMMARY

Inoculation of bioinoculants in crop cultivation plays very important role in sustainable farming. It is well known that the carrier-based bioinoculants are being very effective as carrier determines the shelf life of the inoculant. Hence the selection of better carrier is very essential for maintaining shelf life of the inoculant during storage and for better performance in the field use. In the present study, the effect of Vermicompost in maintaining the shelf life of bioinoculant such as *Azospirillum lipoferum*, Bacillus megaterium and *Pseudomonas fluorescens* was studied up to 12 months from the date of preparation of inoculant by comparing with lignite carrier. Comparatively, Vermicompost based bioinoculants showed longer shelf life than lignite based bioinoculants. Among Vermicompost based bioinoculants *B. megaterium* showed maximum population of 7.60 x 10 8 cfu/g of dry wt on 360th day followed by *Pseudomonas* 10 8 cfu/g of dry wt respectively.

Keywords: Bioinoculants, Vermicompost, *Azospirillum lipoferum*, *Bacillus megaterium*, *Pseudomonas fluorescens.*

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1. Introduction

In recent days, the use of laboratory grown microbial inoculants or microbial cultures i.e., Biofertilizers and Biocontrol agents has attracted the world towards sustainable agriculture farming. Biofertilizers are preparations containing agriculturally useful microorganisms, which help in mobilizing plant nutrients through their biological activity.

Inoculation of crop with beneficial microorganisms is gaining momentum in sustainable agriculture. In integrated plant nutrient system, one of the major components is the carrier based microbial inoculants, commonly referred to as "Biofertilizers". Biofertilizers generally are defined as preparation containing live or latent cells of efficient strains of N-fixing, P-solubilizing or cellulolytic microorganisms used for application to seed or soil[1].

Among the biofertilizer microorganisms, Rhizobium for legumes, Blue green algae for wet land rice; *Azotobacter* and *Azospirillum* for cereal crops can play a significant role in agriculture. The N fixing biofertilizers make an addition to N supplies by fixing atmospheric nitrogen for the soil- plant system. Several soil bacteria and fungi notably the species of Pseudomonas, Bacillus, Penicillin and *Aspergillus* secrete organic acids and lower the pH in their vaccinate to bring about solubilization of bound phosphates in soil and make it available to the plant root.

Pseudomonas sp. are receiving worldwide attention under the broad general category known as plant growth promoting rhizobacteria (PGPR) or plant health promoting rhizobacteria (PHPR)[2,3]. These bacteria, generally, improve the plant growth through direct effects on plants by producing plant growth promoting substances [4,5] by increasing the availability and uptake of mineral nutrients [4,6,7] and by suppressing the soil-borne pathogens or other deleterious rhozosphere microorganisms[2,4].

The biofertilizer microorganisms are mass multiplied under laboratory condition and mixed with a carrier for supply to the farmers. Carrier is a medium matrix on which the inoculated microorganisms grow to a reasonably higher population for an initial period and there after decline. The nature of the carrier often determines the subsequent performance of the inoculant. The introduction of artificial microbial culture in 1890's was in the form of agar-based culture. These early cultures were essentially the agar cultures of today but they changed to carrier-based culture to overcome short shelf life of agar and liquid cultures.

Presently, lignite powder is being used as carrier material by most of the bioinoculant producing units in India. Often it has also been found that its availability is also made difficult, as it is being used as fuel by thermal power stations, etc. Availability of quality lignite powder is also in doubt because of adulteration by agents and improper mesh size in the pulverizing unit. Several scientists have suggested compost as carrier material for biofertilizers. But the role of good compost in maintaining microbial population has not been studied much. The existing studies exhibit that the earthworm casts is ideal material for carrying microbial culture in the agriculture point of view. In the present study, Vermicompost was selected and studied its role in maintaining the shelf life of the widely used biofertilizer microorganisms such as *Azospirillum lipoferum* (Az 204), *Bacillus megaterium* (PB2) and *Pseudomonas fluorescens* (Pf1).

2. Materials and Methods

Mother culture of *Azospirillum lipoferum* (Az 204), *Bacillus megaterium* (PB2) and *Pseudomonas fluorescens* (Pf1) were obtained from Tamilnadu Agriculture University, Coimbatore, India and cultures were preserved in refrigerated condition.

Preparation of carrier material

Vermicompost

The vegetable waste from vegetable market was collected and large sized were chopped into small pieces and mixed with one Kg of cellulose degrading bacterial inoculant (Bacillus sp.-IMTECH-297) per ton of chopped waste. This mixture was made as heap on earth surface and watering was done for maintaining the moisture content at 50 –60 % up to 30 days. On 31st day this partially composted or Half-Decomposed Material (HDM) was collected and mixed with cow dung at 8: 2 ratio. This mixture was used as feed material for earthworms.

Coconut fiber waste was put as a primary layer in the pre constructed cement tub (10'x 3'x 1.5') under thatched shed. The HDM was filled in the tub over the layer of coconut fiber up to 6"

and watering was done to maintain moisture content at 50 – 60 %. Earthworms (*Esenia foetida*) were introduced into prepared vermibed. Moisture content of the vermibed was maintained up to 60% by spraying water daily till waste is fully digested by Earthworms. After complete digestion of filled material the second layer of HDM was prepared as done earlier and moisture was maintained. Similarly HDM was filled for the full height of the tub. After formation of earthworm casting of the complete material, watering to the bed was stopped for 3-5days. Due to lack of sufficient moisture the Earthworms settled at the bottom of the bed. The dried vermicompost was removed and screened to remove resistant residues and pulverized and sieved by using 100 mesh (IS).

Lignite powder having 100 mesh sized granules was obtained from lignite dealer and pH was adjusted to 7 by mixing with Calcium carbonate powder. The Physico-chemical properties of obtained vermicompost and lignite powder were studied by standard methods (Table-1). The moisture content of sieved vermicompost and pH saturated lignite powder was adjusted to 25% by using water and filled in autoclavable HM HDPE bags at 900gm per bag and autoclaved for 4 hours at 120°c and 15psi continuously. The sterilized packets were allowed to cool to room temperature.

Preparation of bacterial inoculants

Appropriate nutrients were dissolved one after another, in appropriate quantity of distilled water. The media for *A. lipoferum*, *B. megaterium* and *P. fluorescence*, were Nitrogen Free Bromothymoleblue medium [9], Nutrient medium and Kings B medium respectively with out agar. The liquid medium was poured into conical flask and 1/3rd of the volume of flask was left free. The mouth of the conical flask was closed with cotton plug. The cotton plug was covered with paper using a thread or rubber band. The medium was sterilized by autoclaving at 121° c for 15minutes. After sterilization the medium was kept at room temperature.

After the medium was cooled to the room temperature, it was inoculated with a loopful of bacterial culture from agar slant under aseptic conditions and kept in rotary shaker and allowed to grow for 5- 7 days for A. lipoferum, 1-2 days for *B. megaterium* and 5-7 days for *P. fluorescens*. The broth was harvested for inoculation with the sterilized carrier when cell count of about 109 cells/cc was achieved. The broth was injected into pre prepared sterilized and cooled vermicompost and lignite carrier material packets at 100ml per packet and inoculated packets were blended thoroughly manually to get uniform bacterial inoculant. The inoculated packets were stored in room temperature for the entire study period.

Enumeration of shelf life of bacterial culture in the carriers

The population test was carried out for both vermicompost and lignite based inoculants once in every 30 days after inoculation of culture for 12 months determined by serial dilution techniques [10].

One gram of sample was added to 100ml of sterile distilled water and shacked well for 10 – 15 minutes to obtain uniform suspension of inoculants. This will give a dilution of 1: 100 (10 2). 1ml of the supernatant was transferred from 102 dilution to 9 ml blank with a sterile 1.0 ml pipette which will give a dilution of 10 3. The above process repeated with the second 9.0ml blank, so that the second tube will have a dilution of 10 4. In the same way, dilutions up to 10 10 was prepared.

Enumeration of *Azospirillum lipoferum*

The population of *A. lipoferum* in both lignite and vermicompost carrier was enumerated by using semi solid Nitrogen Free Bromothymole blue medium by confirming the formation of a

thin pellicle 3-5mm below the surface of the medium through MPN technique [11]. Semisolid NFB medium was dispensed 5 ml quantities in test tubes and tubes were sterilized. The 8th, 9th and 10th dilution of sample were used for enumeration of population. One ml of each sample was transferred to semisolid NFB medium contained in test tubes with 5 replicates. The tubes were incubated at room temperature for 5-5 days. The development of sub surface pellicle and also change of the color of the medium from light yellow to blue was observed. The population of Azospirillum were calculated by using MPN table.

Enumeration of Bacillus megaterium

The viable count of *B. megaterium* was done by plating technique using Hydroxy apatite Agar medium by confirming the formation of solubilizing zone around the bacterial colony. Serial dilutions of inoculant samples were plated on hydroxy apatite medium containing suspended insoluble phosphate compounds. The plates were incubated at room temperature for 3-5 days. The clearing zone was observed around the bacterial colony, thus confirming the ability of *B. megaterium* to solubilize the insoluble form of phosphorous. The population was calculated by the following formula and results were recorded.

$$\text{Number of colony farming unit (cfu) per gram of sample} = \frac{\text{mean number of Colony forming units X dilution factor}}{\text{Quantity of sample at dry weight basis}}$$

Enumeration of *Pseudomonas fluorescence*

The viable count for *P. fluorescence* was done by plating technique using Kigs B agar medium by confirming fluorescent colonies on agar plate. The results were recorded.

$$\text{Number of colony farming unit (cfu) per gram of sample} = \frac{\text{mean number of Colony forming units X dilution factor}}{\text{Quantity of sample at dry weight basis}}$$

3. Results and discussion

In the present investigation an attempts have been made to compare the role of vermicompot and lignite carriers in improving the shelf life on inoculated bacterial culture like *A. lipoferum*, *B. megaterium* and *P. fluorescens*. The population test was carried out both in vermicompost and lignite based inoculant ones in every 30 days after inoculation of culture for 12 months. The physicochemical properties of vermicompost and lignite were analyzed by standard method [12] and the results are presented in Table No.1.

Table 1. Physicochemical properties of vermicompost and Lignite.

S.No.	Properties	Vermicompost	Lignite
1	Colour	Brownish black	Brownish black
2	Water holding capacity (%)	140.0	150.0
3	pH	7.4	5.8
4	EC(m.mhl/kg)	2.0	3.1
5	Organic Carbon (%)	18.0	22.0
6	Nitrogen (%)	2.2	0.9
7	Phosphorous (%)	1.4	0.4
8	Potassium (%)	1.3	0.3
9	Copper (ppm)	4.0	0.9
10	Magnesium (ppm)	4.0	1.6
11	Zinc (ppm)	5.2	5.4
12	Iron (ppm)	36.0	44.0

Data represents mean value of three determinations

Survival of *Azospirillum lipoferum*

The survival capacity of *A. lipoferum* in sterilized vermicompost carrier recorded maximum value of 24.66 x 108 cfu/g of dry wt from 60th day to 120th day and then gradually declined to 00.61 x 108 cfu/g of dry wet on 360th day of storage. The similar pattern was recorded in lignite carrier. The survival capacity and growth gradually increased to 24.66 x 108cfu/g of dry wt on 60th day of storage (Table No. 2 and Fig.1).

Table No.2:Survival of *Azospirillum lipoferum* (Az 204) in sterilized Vermicompost and Lignite carrier materials (Population x 108 cfu/g dry weight basis)

Carriers	Days of storage											
	30	60	90	120	150	180	210	240	270	300	330	360
Vermicompost	14.46	24.66	24.66	24.66	14.46	6.60	5.38	3.07	2.15	0.86	0.50	0.61
Lignite	14.46	24.66	14.46	5.38	5.38	0.72	0.61	0.50	0.52	0.16	0.02	0.02

Table No.3: Survival of *Bacillus megaterium* (PB2) in sterilized Vermicompost and Lignite carrier materials (Population x 108 cfu/g dry weight basis)

Carriers	Days of storage											
	30	60	90	120	150	180	210	240	270	300	330	360
Vermicompost	28.33	43.33	50.00	61.00	71.00	69.20	61.50	47.60	38.40	27.60	12.30	7.60
Lignite	25.00	36.66	48.00	30.00	16.60	15.38	3.25	3.00	1.00	0.18	0.15	0.09

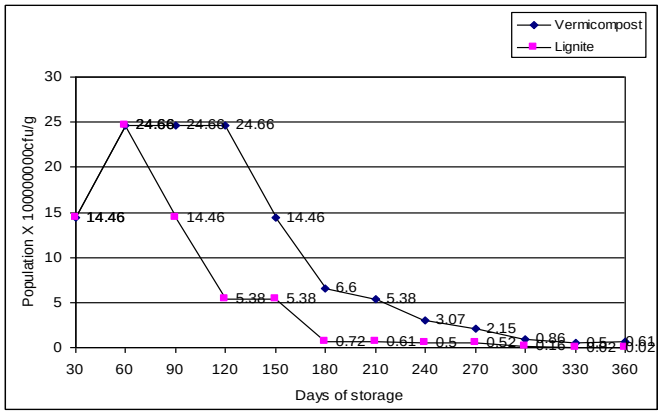
Data represents mean value of three determinations

Table No.4:Survival of *Pseudomonas fluorescence* (PF1) in sterilized Vermicompost and Lignite carrier materials (Population x 108 cfu/g of inoculant dry weight basis)

Carriers	Days of storage											
	30	60	90	120	150	180	210	240	270	300	330	360
Vermicompost	24.60	30.76	49.23	46.15	38.46	24.67	25.00	18.80	14.25	10.42	9.05	5.00
Lignite	23.00	32.21	43.00	22.70	15.42	4.12	0.61	0.24	0.17	0.06	0.04	0.02

Data represents mean value of three determinations

Fig.1. Survival of *Azospirillum lipoferum* in vermicompost and lignite Fig.2. Survival of *Bacillus megaterium* in Vermicompost and lignite carriers



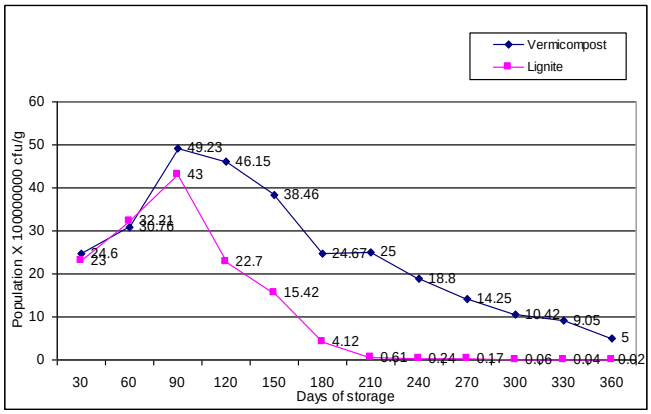
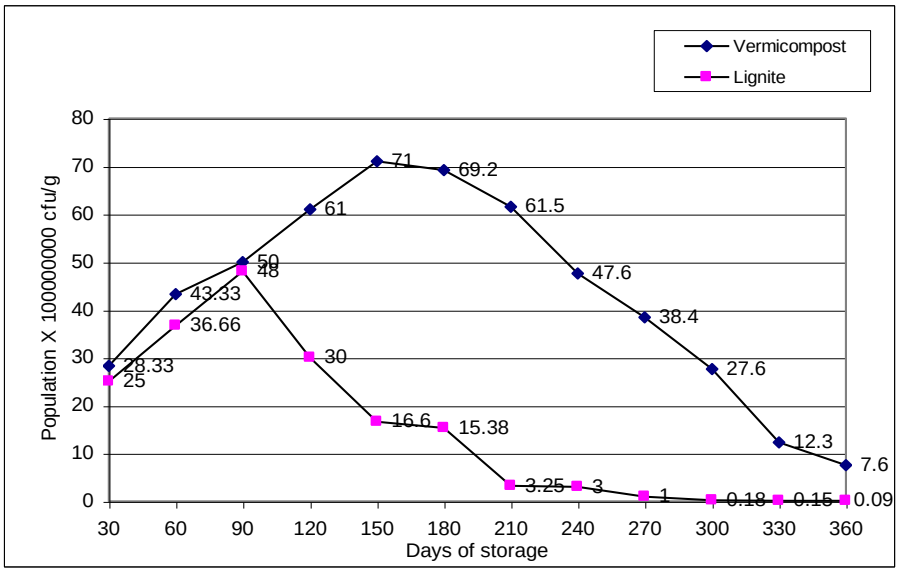


Fig.3. Survival of *Pseudomonas fluorescens* in vermicompost and lignite carriers



Survival of *Bacillus megaterium*

The survival capacity of *B. megaterium* on lignite carrier exhibited gradual increase of growth to 48.0 x 108 cfu/g of dry wt on 90th day and then declined gradually to the value of 00.09 x 108 cfu/g of dry wt on 360th day. But in the case of vermicompost carrier it was quite interesting that the growth rate increased gradually from 28.33 x 108 cfu/g of dry wt on 25th day to 71.0 x 108 cfu/g of dry wt on 150th day and then declined gradually to 7.60 x 108 cfu/g of dry wt on 360th day. Comparatively, the vermicompost carrier is considered as better ones for the survival of *B. megaterium* (Table No.3 and Fig.2).

Survival of Pseudomonas fluorescens

The P. fluorescens in lignite based carrier showed gradual growth up to 90th day (43 x 108 cfu/g of dry wt) and then declined gradually from 120th day to 360th day (22.70 x 108 cfu/g of dry wt and 0.02 x 108 cfu/g of dry wt respectively). Similar trend was also observed in the case of vermicompost carrier. The growth gradually increased up to 90th day (49.23 x 108 cfu/g of dry wt) and then decreased gradually to 5.00 x 108 cfu/g of dry wt on 360th day. Comparatively, irrespective of carrier material difference *B. megaterium* and *P. fluorescens* showed better survival capacity when compared to survival value of *A. lipoferum*. Among the vermicompost and lignite carrier based binoculants, the vermicompost carrier based bioinocults showed better shelf life period when compared to lignite carriers (Table No. 4 and Fig.3).

Lot of work has been done on the role of carrier material increasing the shelf life of bioinoculant. In the present study the shelf life of bioinoculants in vermicompost carrier was found to be more effective. Comparatively the physico chemical properties of vermicompost and lignite, it was found that the vermicompost carrier showed better properties like pH, nitrogen, phosphorous, potassium, copper, manganese and iron etc., when compared to the lignite. Since the vermicompost carrier is having high nutrient value which may influence the survival capacity of the cultures.

Generally the peat is an ideal carrier material for biofertilizer generally. Similarly charcoal wood charcoal and soil [13], filter mud from sugarcane[14], lignite and vermiculite[15] are all considered as best carrier material for biofertilizers. It was reported that the better survival of rhizobium in different carriers like charcoal, bagasse, filter mud and peat [14]. In the present study the same trend was found with vermicompost carrier on the survival and self life of *A. lipofreum, B. megaterium* and *P. fluorescens*.

It was found the survival of A. lipoferum , *B. megaterium* and *P. fluorescens* are better in vermicompost carrier. The population of these microorganisms increased upto 120th day and then declined. Comparatively among the bioinoculants the *Bacillus megaterium* showed better results in survival in vermicompost carrier. Gopalaswamy et al [16] reported vermicompost an alternative carrier material for biofertilizers like *Azospirillum* and phosphobacteria. Muthukumarasamy et al [17] found that vermicompost prepared from unused bagasee was found to be a better carrier for the *Azospirillum*. But in our study the vermicompost carrier prepared from vegetable waste was found to be better carrier for bioinoculants like *A. lipoferum , B. megaterium* and *P. fluorescens*.

Conclusions

In the present study the shelf life of Azospirillum lipoferum, Bacillus megaterium and Pseudomonas fluorescens in vermicompost carrier was found to be more effective than lignite carrier. The combined application of these bacterial inoculants showed better results in growth, yield and quality parameters of rice when compared to dual and individual form of application. Vermicompost carrier showed better properties like pH, nitrogen, phosphorous, potassium, copper, manganese, and iron etc., when compared to lignite. Since the vermicompost carrier prepared by using vegetable waste is having balanced nutrient value, which may influences the survival capacity of the cultures.

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