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Screening and Production of Phosphate Solubilising Bacterial Inoculants Using Different Carrier.

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ABSTRACT

Phosphate solubilising bacteria was isolated from rhizosphere soil. Bacterial population was more in number when compared to fungal population. The highest phosphate solubilising bacterial population was observed in bacillus species was the dominant organisms found almost in all the soil samples. This isolate was used to prepare biofertilizer. Vermicast and charcoal were used as a carrier material to prepare phosphate solubilising bacterial inoculants. The purpose of this research was to study the survival of *Bacillus megaterium* as one of phosphate solubilising bacteria on different carriers. In order to study the effects of different carriers on survival, after determination of their physical and chemical parameters, carriers were inoculated by bacteria and inoculants were maintained in controlled condition for 60 days. Bacterial populations were measured at times 0, 15, 30, 45 and 60 days by colony forming unit method. The results of bacterial count after 15 days incubation showed that bacterial population in vermicast containing samples was increased and it was decreased in charcoal treatments. Considering these results, the use of carriers containing high organic matter like vermicast could increase bacterial survival and led to efficiency improvement of biological inoculants.

Key words: *Bacillus megaterium,* Vermicast, Charcoal, Biofertilizer.

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INTRODUCTION

Agrochemicals which ushered the green revolution in the 1950-60's came as a mixed blessing for mankind. It boosted food productivity, but at the cost of environment and society. It dramatically increased the quantity of the food produced but decreased its nutritional quality and also destroyed the physical, chemical and the biological properties of soil over the years of use. It impaired the power of biological resistance in crops making them more susceptible to pests and diseases. Over the years it has worked like a slow poison for the farm soil and the society [10].

The use of biofertilizer nowadays known to bring out several benefits to soil, solubilisation of essential minerals, get hold of nutrients, offering micronutrients in more utilizable form for plants, and taking part in biological nitrogen fixation [7]. Biofertilizer are low cost, effective and renewable source of plant nutrients to supplement chemical fertilizers [1]. Biofertilizer consists of carriers and the microorganisms [12]. The soil acts as a reservoir for millions of microorganisms, of which approximately more than 85% are beneficial for plant life. Thus, the soil is a resilient eco system and soil microorganisms provide precious life to soil systems catering to plant growth. Soil microorganisms play a vital role in the evolution of agriculturally use full soil conditions and in stimulating plant growth [13].

Microorganisms of this group are generally known as plant growth promoting microorganisms (PGPMs) which include *Azospirillum*, *Azotobacter*, *Phosphobacteria, Rhizobia* and *Cyanobacteria.* The PGPMs are capable of putting forth advantageous properties on growth and yield characteristics of several cultivable crops in different parts of the world [7]. Phosphate solubilising microorganisms (PSM) have the potential to increase available phosphorus (P) for plant, especially in soils with large amounts of precipitated phosphate. These bacteria release bound phosphate by secreting a number of organic acids. PSM convert these insoluble phosphates into soluble form through the process of acidification, chelation, exchange reactions and production of gluconic acid. Many of the isolates are evidence for the presence of multiple organic acids. They are able to produce 13 kinds of organic acids including citric, gluconic, 2-keto-gluconic, succinic, glycolic, lactic, fumaric, formic, acetic, butyric, isobutyric, valeric and isovaleric acid.

These beneficial organisms are applied in the form of microbial inoculants [5]. The inoculants can be prepared from several types of carriers such as peat, charcoal, farmyard manure, lignite, alginate, etc., Nowadays; several types of agricultural waste like maize stubble, plant compost, mushroom waste, rice straw, oil palm and bunch can be composted and used as bioinoculant carrier. This system helps reducing the pollutants, saving energy, decreasing cost of production, and utilizing natural resources to the benefit [3]. Earthworms vermicompost give very high food productivity comparable to or even better than the chemical fertilizers with significantly higher nutritional quality which also improving the physical, chemical and biological properties of soil. Vermicompost is highly nutritive and a powerful plant growth promoter and protector and has scientifically proven to be a miracle plant growth promoters. It is rich in NKP, micronutrients, beneficial soil microbes and also contains plant growth hormones and enzymes secreted by earthworms. Vermicompost retains nutrients for long time and also protect crops from pests and diseases. It has high

moisture holding capacity and hence also reduces the use of water for farm irrigation by 40-50% [10].

Types of carrier and storage temperatures are important factors determining shelf life of bioinoculants in a warehouse without refrigerator in the range of -5 to 30^{0}C often causes reduction in microbial longevity. Many researchers have evaluated for suitable carriers from agricultural wastes and investigated effect of temperatures on shelf life in packages at various temperatures [3]. The present study has been undertaken with the objectives to isolate PSM from soil sample, to prepare biofertilizer using PSM with vermicompost and charcoal as a carrier material and to evaluate the shelf life of microbial inoculants in the carrier material at room temperature.

MATERIALS AND METHODS

Collection and identification of earthworms

Earthworms (*Perionyx excavatus*) are collected from Viralimalai, Pudukottai (dist), collected earthworms are identified in Jamal Mohamed College, Trichy.

Vermicast preparation

Layer (15-20 cm) of organic waste (coir waste, paddy husk) spreaded in the cement tank. Non-decomposable material separated first and then the waste is spread on the ground to expose into sunlight for two days. This will help to reduce insect population. Cow dung was collected and then added as a layer on top of the mixture. Soil filled on the cement tank. The organic waste mixed with cow dung in equal quantity and appropriate quantity of water poured over it so as to make a homogenous mixture. Earthworms inoculated on the cement tank and covered with jute bags to prevent birds from eating the worms (figure 1).

Figure 1: vermicast preparation

Harvesting of vermicast

The sacks are removed after two months the compost ready, it is black, quite light weight and has a pleasant, earthy smell. Excreta of the worms are scooped up manually from the surface of the bed after 60 days and sterilized it and used as carrier inoculums.

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Charcoal preparation

The charcoal powder was prepared and sterilized in an autoclave for 3 hours (figure 2).

Figure 2: Production of PSB inoculums

Analysis of carrier materials

Estimation of pH

25g carrier materials was mixed with 50ml of distilled water and kept on rotary shaker for 2 hours. Filtrate was obtained through Whatman filter paper under vacuum using a funnel. pH of filtrate is determined using pH meter.

Estimation of moisture

5 gm of carrier materials was taken on a dry petri-dish. It was heated in an oven for about 5 hours at 65^{o}C, constant weighing was done. Cooling is done in a dessicator and weigh. Percentage loss in weight was estimated as moisture content of the carrier materials. Calculation of moisture:

Moisture percent by weight=100(B-C)/B-A

Estimation of water absorbance

20g of different carrier materials was put into the separate funnel placed in the flask.100 ml of water added slowly. The water holding capacity of carrier materials and time taken to absorb water were noted.

Estimation of nitrogen

- 0.5gms of the sample was wrapped in aluminium foil and then was put in a kjeldhal flask. The catalyst mixture was added and digestion was carried out.
- Sample was heated on flame for 10-30 mins till charred. The flask was rotated until the organic matter destruction till gray coloured solution obtained.
- Digested sample was then diluted with 10ml of distilled water and 5 ml was taken in condensation flask. Flask was then heated till solution boils.
- At the end titration was carried out with 0.1 ml HCl by adding phenolphthalein indicator. End point – purple to pink.

Calculations:

% N = (A-B) x N of HClx1.4/wt. of the sample.

A = ml of HCl used, B = ml of the HCl used for blank

Phosphorous estimation

- 1 gm of dried sample was taken and 200ml of 0.002 N H_2SO_4 was added in it and mixture was stirred for half an hour.
- Solution was then filtered through Whatman filter paper no. 42.
- 5 ml of filtrate was taken and 2 ml of ammonium molybdate along with 05 drops of $Sncl_2$ was added in it.
- Total volume of the mixture was made to 100 ml with distilled water and absorbance was taken at 690 nm.
- Standard graph was plotted and readings were extrapolated.

Calculations:

% available phosphorous = mg P/ l of sample/50.

Estimation of potassium

- Preparation of standard curve: 0, 1, 2, 4, 6, 8 and 10 ml of stock solution is pipette out in 100 ml volumetric flask.
- The volume is marked up to the mark with addition of distilled water. It gives 0, 10, 20, 40, 60, 80, and 100 ppm of potassium respectively.
- The intensity of potassium at flame photometer is observed.
- The sample is aliquot directly with a flame photometer. Work out the ppm of potassium form the standard curve run a blank reading.

Calculations:

% Potassium=X/1000x100x100/0.2
=0.05 x X

X-ppm of potassium read from the standard curve

X-30 ppm

Collection of soil samples

Soil samples were collected from Semmedu-Kolli hill forest, Namakkal, Tamil Nadu.

Isolation of phosphate solubilising bacteria from soil sample:

Phosphate solubilising bacteria (PSB) were isolated from the soil sample by serial dilution as 1.0g of air dried samples was dissolved in 99ml of distilled water. The soil suspension was further diluted upto 10^{-6} level. The diluted soil suspension (0.1ml) was spread on the surface of pikovskaya's agar medium (PSK) which is a selective medium for isolating phosphate solubilising bacteria. The pH of the medium was adjusted to 6.8 with the help of 1N HCl/ 1 N NaOH. The plates were incubated at 28-30^{o}C for 48 hrs and the colonies were observed. Strains of PSB were picked out and purified by repeated streaking on PSK medium and were preserved as slant culture for further usage.

Identification of isolates

The isolate was identified by Gram staining and the biochemical characterization of the isolate was carried out by using standard method.

Production of PSB inoculum

Figure 3: vermicast (left) and charcoal (right) carrier after sterilization

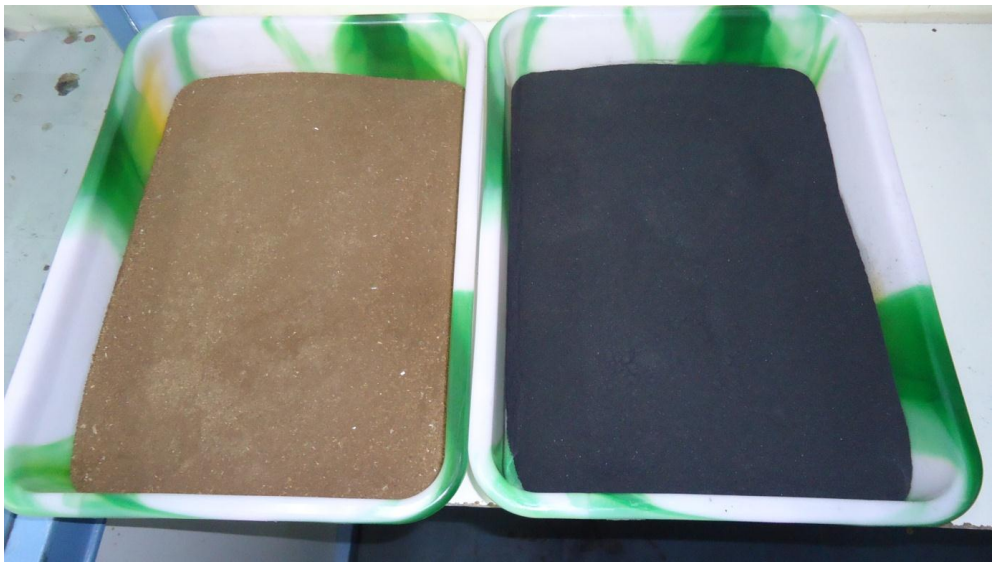

Figure 4: PSB fertilizer preparation

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Figure 5: Packaging of biofertilizer

A loopfull of PSB culture was transferred into 250ml Erlenmeyer flask containing 100ml of PSK broth and incubated at 28^{o}C on 120 rpm rotary shaker for 72 hours. After incubation, 10ml of the inoculums was transferred to 1000ml of respective broth and kept in shaking incubator for mass multiplication (figure 3). 750ml was mixed thoroughly with 1000g of each sterile carrier, adjusted the moisture content to75% water holding capacity (figure 4), packed in polyethylene bags, sealed and incubated under room temperature. The inoculums were repacked in sterile polyethylene bags (figure 5).

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Evaluation for survival of the PSB during storage at room temperature:

The number of PSB was after the inoculum was subjected to different carriers at room temperature. Ten grams of each sample was taken for estimating viable cells at the initial date, 15, 30, 45 and 60 days after storage using dilution plating method on PSK agar and incubated at 28^{o}C for 48 hrs. The numbers of apparent PSB colonies were counted calculated into viable cells.

RESULT

Isolation

Isolation was made from soil collected from Semmedu Kolli hill forest by the enrichment culture technique in PSK medium. All the isolates were subjected to various tests to confirm identity.

Identification

Colony morphology

Phosphate solubilisation zone was observed around the colony.

Microscopic observation

Isolates were further examined for their Gram's reaction and shape characteristically all the isolates were gram positive and rod shape.

Characterization of isolates on various biochemical tests

Table 1: Characteristics of *Bacillus megaterium*

Characteristics	Observations
Shape	Rod
Gram's reaction	Positive
Indole production	-
Methyl red	+
Voges proskauer	-
Citrate utilisation	-
Nitrate reduction	-
Urease	-
Catalase	+
Oxidase	+
Starch hydrolysis	+
TSI	H_2S –ve, Acid slant.

The confirmation of isolate was done through various biochemical tests. The isolate was showed positive results to MR, catalase, and oxidase where they expressed negative

result to indole, VP, urease, citrate and nitrate. The isolate was efficient in hydrolyzing starch (Table 1).

Physical and chemical properties of carriers

The pH of the vermicast of reared in agricultural waste (60 days) was nearly neutral (pH 7.15). The nutrients found in vermicast, such as total nitrogen, potassium, phosphorous indicate that the vermicast produced from agricultural waste using *Perionyx excavatus* were good in nutrients. Some physical and chemical properties of carriers are shown in the table 2. There was a significantly difference among the physical and chemical properties of the carriers.

Table 2: Physical and chemical properties of carriers

Parameter	Carrier materials	
	Vermicast	Charcoal
pH	7.15	7.45
Moisture (%)	18.18	18
Water uptake (mins)	18(mins)	28(mins)
N_{tot} (%)	8.5	0.83
P_{tot} (%)	6.0	0.39
K_{tot} (%)	5.0	0.24

Survival of PSB in the carriers at room temperature

After 6 months of inoculants maintenance, the population of the bacteria was determined through Colony Forming Unit method. At initial days the bacterial population was higher in charcoal. After 15 days of incubation, the bacterial population in the charcoal was intensively declined. While the population of bacteria in the vermicast increased after 15 days. As listed in table.3, the best carrier was treatment of vermicast. The weakest carrier was activated charcoal.

Table 3: Population of inoculants during storage

Days	Carrier	Colony forming unit/gram	
		10^{-4}	10^{-5}
0	Charcoal	268	128
	Vermicast	136	120
15	Charcoal	243	129
	Vermicast	142	128
30	Charcoal	210	93
	Vermicast	158	135
45	Charcoal	198	87
	Vermicast	169	140
60	Charcoal	120	56
	Vermicast	202	137

DISCUSSION

The study conducted by Padmavathiamma *et al*., reported that the enrichment generally had a significant effect on the nutrient contents, especially for N, P, K, Mg, and Mn. *Eudrilus* compost, when treated with *Azospirillum* and Phosphate-solubilising organisms, gave an Nitrogen-content of 2.08% which was significantly higher than the N-content of uninoculated *Eudrilus* compost (1.8%). The nitrogen was enriched appreciably by *Azospirillum*. The enrichment increased progressively when *Azospirillum* inoculation was supplemented with phosphate solubilising culture, a beneficial additive to obtain good quality compost rich in N.

Rasal *et al.,* study showed that the mechanisms of conversion of insoluble Phosphorous by Phosphate-solubilising organisms to available forms include altering the solubility of inorganic compounds to the ultimate soluble form by production of acids and H_2S under aerobic and anaerobic conditions and by mineralizing organic compounds, with the release of inorganic phosphate [11].

Tiunov and Scheu have shown that earthworms deprive easily available carbon to microorganisms and availability of carbon increases effective mobilization of Nitrogen and Phosphorous by earthworms. Earthworms are mainly responsible for fragmentation and conditioning of the substrate, increasing surface area for microbial activity, and significantly altering biological activity of the process. The vermicasts when used as carrier material for biofertilizers supported the survival rate for more than one year [14].

Sekar and Karmegam [9]reported that vermicasts from *E.euginiae* as a carrier material which supports the survival of more than $1x10^7$ g^{-1} viable cell of *A.chroococum*, *B. megaterium* and *R. leguminosarum* till the end of 10^{th} month which is longer than observed in lignite. Chao and Alexander [2] concluded that bacterial retention on activated charcoal and peat carrier at 25°C was more than 4°C. Mendez and Videira (2005) stated that bacterial maintenance at 28°C for 41 days caused an increase in number of viable bacterial cells on all carriers so that the population reached nearly 10^9 bacteria per gram of carrier.

CONCLUSIONS

The study revealed that PSB are more in rhizosphere region. The evident from the present study the vermicast as a carrier material supports the survival of viable cells for nearly 6 months. Vermicast alone are suitable materials can be used as carrier material for the long time survivability of the biofertilizers. Use of these PSB as microbial inoculants will increase the available phosphorous in soil, helps to minimize the phosphorous fertilizer application, reduces environmental pollution and promotes sustainable agriculture.

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