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M Meharaj Beham
PG Scholar, Department of Agricultural Microbiology, Agricultural College and Research Institute, TNAU, Madurai, Tamil Nadu, India

K Kumutha
Professor and Head, Department of Agricultural Microbiology, Agricultural College and Research Institute, TNAU, Madurai, Tamil Nadu, India

R Subhashini
Associate Professor, Department of Agricultural Microbiology, Agricultural College and Research Institute, TNAU, Madurai, Tamil Nadu, India

P Kannan
Assistant Professor (SS&AC), Department of Soils & Environment, Agricultural College and Research Institute, TNAU, Madurai, Tamil Nadu, India

Corresponding Author:
K Kumutha
Professor and Head, Department of Agricultural Microbiology, Agricultural College and Research Institute, TNAU, Madurai, Tamil Nadu, India

Comparison of different formulation of *Rhizobia* for cell viability

M Meharaj Beham, K Kumutha, R Subhashini and P Kannan

Abstract
In today's sustainable farming increasingly used phrase is "biofertilizers." Existing approaches are improving, and numerous new strategies have emerged to enhance their effects on plant systems. Bioformulation is one of the most important areas of research since it impacts the efficacy of inoculants. An effort has been made to improve bioformulation in the hopes of decreasing contamination and extending shelf life. *Rhizobium* is a symbiotic Nitrogen fixing bacterium that fixes atmospheric N_2in legume root nodules while also benefiting its host plants. In this research, different technologies were employed to produce the biofertilizer formulation. The purpose of this research was to establish inoculant formulations with higher population using acceptable carrier material. Rhizobial culture has been utilized as model organism and water soluble powder formulation were made using freeze drier and fluid bed drier. Among different carriers (Mannitol, Maltodextrin and Dextrose) evaluated. Mannitol based carrier formulation showed the best result than other two carriers in terms of viable cell count. These formulation were compared with the commercial lignite based formulation and liquid formulation, the results showed that freeze dried formulation resulted significantly higher population (log14.30cfu/g) than the other formulations.

Keywords: Biofertilizers**,** *Rhizobium,* formulation, freeze dry, fluid bed dry

1. Introduction
Green revolution boon the Agricultural production, with the of use of high inputs. Agriculture with tremendous input seems to satisfy the food security, simultaneously has its adverse effect on the soil richness. As we know that the soil is the largest buffer of nutrients, it needs to be replenished in order to meet the required productivity. The chemical fertilizers usage resulted in certain known side effects, it cannot alone bring sustainable nutrition. Considering the organic farming as an alternative, it is much ahead of practicality. A substantial amount of agrochemicals can be replaced by potential microbes, which can give high yield with sustainability. Microbes in the form of biofertilizers have a tremendous ability to support plant growth and development. Biofertilizers also serve as Plant growth Promoting Rhizobacteria (PGPR) and their usage can play important role in developing sustainable systems for crop production. *Rhizobium* is a Gram-negative soil bacterium that colonizes legume roots and fixes atmospheric nitrogen in a symbiotic manner (Gomare *et al*.,2013) [1]. It shares several properties with other PGPRs, including hormone synthesis and phosphate solubilization, in addition to fixing atmospheric nitrogen by nodulation. It aids in root expansion, enhances plant nutrient uptake, protects plants from root infections, and most importantly, improves biomass output and rapid growth at wasteland through plant growth boosting chemicals. The formulation of PGPR may improve plant growth characteristics which can replace the use of chemical fertilizers. The bioformulation is the biologically active products containing one or more beneficial microorganisms and an economical carrier materials. A good carrier is essential in making bioformulation to deliver the right number of viable cells in good physiological conditions at right time. Selection of formulation is very crucial as it determines the success of a PGP microbe. Formulations can be dry or liquid and also microencapsulated. A good formulation should be easy to handle and apply, and to provide more suitable micro environment that prevents the rapid decline of an introduced PGP microbe in the soil (Brahmaprakash and Sahu 2012) [2]. This has the ability to sustain microbial development, as well as a long shelf life and bioefficacy. Many alternative carriers and forms have been attempted, with varying degree of effectiveness. Every flaw in an existing technique points to a development of new technique.

In such a way, for making easily applicable product freeze dried formulation and Fluid bed dried formulations have been developed in this research to address the shortcomings of existing formulations.

2. Materials and Methods

2.1 Assay of PGP characteristics of Rhizobial culture

In this study *Rhizobium legminosarum* has been utilized for making formulations. The various plant growth promoting characters *viz*., IAA production, Gibberllic acid production, Nitrogen fixation ability, Phosphate solubilization, EPS production and siderophore production were evaluated for the rhizobial culture. Indole-3-Acetic Acid production was determined using the method developed by Glickmann and Dessaux (1995) [3]. Gibberllic acid production was examined according to Desai, (2017) [4]. To estimate the Nitrogen fixing ability of the culture Acetylene reduction assay was carried out as per Hardy *et al.,* (1968) [5]. Phosphate solubilization of the isolate was quantified by following Murphy and Riley (1962) [6] protocol. The *Rhizobium* culture has the capacity to produce exo polysaccharide that was assessed by the method of Albalasmeh *et al*., (2013) [7]. Siderophore producing ability of the isolate was determined using the CAS assay developed by Schwyn and Neilands (1987) [8].

2.2 Growth curve estimation

A 40ml of Yeast Extract Mannitol Broth was taken in 100ml side arm flask and incubated with 0.4 ml (1%) of actively growing Rhizobial culture aseptically. The flasks were incubated in the incubator - shaker at room temperature and the optical density (OD) values of the culture was measured at varying time interval using spectrophotometer at 600 nm wavelength. The values were plotted against time in semi log graph to find the maximum growth of the *Rhizobial* culture used.

2.3 Preparation of *Rhizobium* cells

The starter culture was prepared in 100ml quantity with incubation of 4 days at 28° ± 1°C in rotary shaker and transferred to 1lit broth in a 3 lit flask and incubated for growth at room temperature till the population reaches 10^9 cell/ml broth. Fermentation broth (YEMA) was prepared in 50 lit fermentor vessel (Scigenics India pvt. ltd) and the inoculation was done @ 1% and the fermentation was carried out at 30 °C with a 52 rpm agitation speed. A ring sparger was used to sparge air on the tank's bottom, and the head pressure was kept at 0.2kg $cm^{-2.}$ The pH was maintained at 7.0±0.2. After 4 days of fermentation, the fermented culture broth was then put through a tangential flow filter column (TFF) to concentrate the cells (Pall, India).

2.4 Freeze dry formulation

Lyophilization is a process of freeze drying bacterial cells that can be used for long term storage.The cells concentrated through TFF was used for freeze drying. The concentrated cells were mixed with phosphate saline buffer @ 5:1. Before mixing with both the buffer was mixed separately with three different carriers *viz*., Mannitol, Maltodextrin and dextrose. The concentrated broth was then mixed thoroughly with phosphate saline buffer added with different carriers and loaded in trays @ 1:10.The trays were kept in freeze drier and the process was initiated. Freezing, primary drying, and secondary drying are the three steps of the freeze drying process. The shelf temperature was maintained between -15°C to -30°C for 3 to 4 hours and 900 mtorr vaccum was employed in freezing stage. In primary stage -20°c to 30°C temperature and 300 mtorr vaccum was maintained for 17 hr. During secondary stage of freezing, the temperature was maintained at 30°C and 200 mtorr vacuum were used for 20 hours. The final product was obtained after 72 hours were assessed for the viable cell count (Jennings 1999) [9].

2.5 Fluid bed dryer formulation

It's a type of dryer where the material was kept suspended in an upward-flowing air stream against gravity. Electrical heaters warm the air. This heated air expands the material bed at a given speed, causing turbulence in the product (fluidization), and providing greater surface area for drying. Fluidization causes complete agitation of solid particles, resulting in a large amount of heat transmission and even drying. Different types of carriers were mixed with the TFF concentrated cells. The three types of carriers such as mannitol, maltodextrin and dextrose were used. The carriers were sterilized completely before being employed in the subsequent steps. 10 kg of sterilized carriers were combined with 1 lit of concentrated broth. This microbial cell and carrier mixture was fluidized in a sterile fluid bed dryer (FBD) tank. The FBD is a semiautomated machine that requires a time-temperature setting. This is dependent on the culture used, the carrier utilised, the moisture level of the carrier-inoculant mixture, the size of the clumps generated on the FBD tank, and other factors. The samples were fluidized at 40°C for 1hr cycle. After completion of the process the product was collected in the bigger size stain less steel bowel. Then the viable cells were counted in the final products (Lavanya 2014) [10].

2.6 Comparison of cell viability in different formulation

The population in the developed formulation was compared with the existing formulation by plate count assay. Rhizobial cell load of four different formulations were tested: lignite based formulation, liquid based formulation, freeze dried formulation, and fluid bed dryer formulation. One gram of each designed product was dispersed in 9ml of sterile distilled water and serially diluted up to the 15^{th} dilution. To count live bacteria in the formulation, the spread plate method was used. Colony forming units (CFU) were counted after 3-4 days in Congo Red Yeast Extract Mannitol Agar at room temperature (CRYEMA). Simultaneously the commercial cultures available in the department (ie, lignite based formulation and liquid formulation) were obtained and the population was assessed. Population was compared in all four formulation. Three replicated plates were maintained for each dilution.

3. Results and Discussion

3.1 Bioassay of PGP characters of Rhizobial culture

Effective microorganisms have an important role in plant growth, development, and yield. Plant growth promotion is defined as the production of plant hormones and other growth-related activities such as providing nutrients, releasing volatile organic compounds in the rhizopshere, and suppressing plant disease-causing pathogens and insect pests such as whitefly, either directly or indirectly, according to scientific understanding (Backer *et al*., 2018) [11]. The results of the PGP traits of the *Rhizobium* was given in the table 1

Table 1: Plant growth promoting traits of *Rhizobium*

PGP characters	Values
IAA production	3.0 (µg ml^{-1})
Gibberellic acid production	11.79 (µg ml^{-1})
Nitrogen Fixation	12 (µmoles min^{-1} g^{-1} nodule)
Exopolysaccahride Production	9.7 (µg ml^{-1})
Total P release	64.5 (mg ml^{-1})
Siderophore Production	28.8 (psu)

The *Rhizobium* has the ability to synthesis various plant growth hormones as well as other growth enhancing substances which have the potential to influence plant growth and development (Bashan *et al.*, 2005) [12].

3.2 Growth behavior of Rhizobia

Growth behaviour of the selected rhizobial culture was studied in YEMA broth. The results showed that the culture reached the maximum growth on the day 4 and thereafter it reaches stationary phase and declining phase (fig 1). In formulation development it is essential to make the formulation, when the cells are at actively growing stage with higher population. It is reported that (Lupwayi *et al.*, 2000) [13], the number of live cells in the inoculum determined the quality of the formulation. Based on the population, rhizobial culture was taken for formulation on day 4

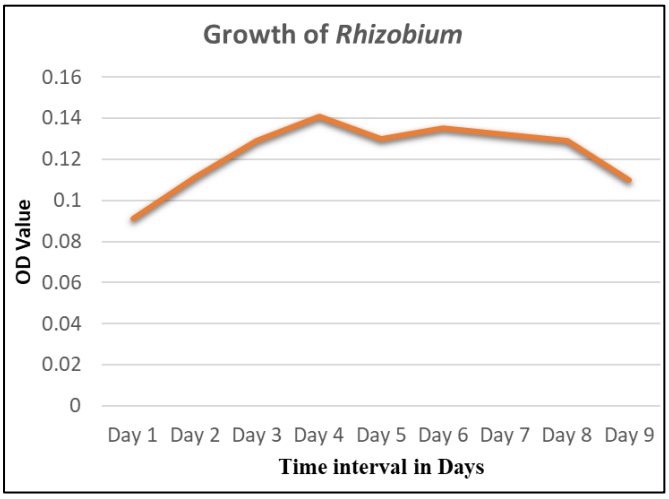

Fig 1: Growth curve of *Rhizobium*

3.3 Formulation development and cell viability

Rhizobial culture was taken for formulation development at exponential growth phase (4^{th} day) from the fermentor and made into different formulations such as fluid bed dried formulation, freeze dried formulation using different carriers *viz*., Mannitol, Maltodextrin and dextrose. Two different drying process as listed above (freeze drying and fluid bed drying) were used in this experiment. After preparation, the rhizobial population was assessed by serial dilution and plate count method. The results are presented below

The survival of *Rhizobium* varied depending on the carriers, in two different formulation, the maximum population was recorded in mannitol based product in both methods. In freeze dried product the cfu is log 14.30/g and the fluid dried mannitol based product contains log 9.40 cfu/g which was followed by maltodextrin based product and the population is log 12.00 cfu/g in freeze dried product and in fluid bed dried product it is log 8.52 cfu/g**.** The lowest viable cells was observed in dextrose based product. The population of log 9.67 cfu/g was recorded in freeze dried formulation and log 7.05 cfu/g was observed in fluid bed dry formulation when dextrose was used as carrier. In both the formulation mannitol based product shown the best result. Since the mannitol serve as the best matrix forming agent which is highly helpful in generating a frozen sample that keeps its shape as water is removed so that the survival rate was higher in the mannitol.

3.4 Comparison of the survival rate of *Rhizobium* in different formulation

The two different developed formulation was compared to the commercial formulation *viz*., the lignite based formulation and the liquid based formulation of *Rhizobium*. The survivability of the inoculant in the formulations was assessed by standard plate count method, which showed, the significantly higher population of *Rhizobium* in freeze dried formulation (log 14.30^{a}± 1.62 cfu/g) than other treatments (Table 2). Next to freeze dried formulation, fluid bed dried formulation showed the maximum value of 9.40± 1.06 log cfu/g. But it is at par with other products. During freeze drying mannitol serve as good cell protectant, which leads to higher population. In fluid bed drying process, the temperature used for drying process may be higher and also the cells were moved upwards by airforce, which could cause mechanical injury that may results in lesser population. This can be improved by reducing/ Optimizing the temperature for drying process to each organisms. Lignite and liquid formulation had lesser population and they have its own merits and demerits. As a whole the freeze dried formulation is less labour intensive and give good amount of viable cells. This powder can be recommended for use as such or else it can be used for making biotablets.

Table 2: Population of *Rhizobium* in different formulation.

Different formulation	Population in log cfu
Lignite based formulation	9.30^{b}± 1.05 /g
Liquid formulation	10.33^{b}± 1.17ml
Freeze Dried formulation	14.30^{a}± 1.62 /g
Fluid Bed Dried formulation	9.40^{b}± 1.06/g
SEd	0.8864
CD(0.05)	1.9314

Values are mean of three replicates (± standard error) (n=4) and column values followed by different letters are significantly different from each other at p=0.05 with LSD

4. Conclusion

Freeze dried powder formulation had higher cell population and shelf life, easy for application, less labour intensive. Similarly fluid bed dried formulation may also be used after improvement of cell population by slightly altering the protocol. Still these two methods can have scope in formulation Industry.

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