

Original Research Article

<https://doi.org/10.20546/ijcmas.2020.907.070>

Characterization of Microbial Inoculants and its Different Formulation for Mass Production

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ABSTRACT

Keywords

Carrier materials,
Additives,
Microbial
inoculants, Growth
pattern, IAA
production

Article Info

Accepted:
08 June 2020
Available Online:
10 July 2020

Biofertilizers are alternative source of nutrient inputs in sustainable agriculture. Plant growth promoting microorganisms such as *Rhizobium* spp. and *Bacillus* spp. can be used as biofertilizers. In our present study, different *Rhizobium* and *Bacillus* isolates were screened for the selection of suitable medium for its maximum growth at different incubation period. The Growth pattern of *Rhizobium* isolates were evaluated in two different media viz. Nutrient broth and YEM broth while of *Bacillus* spp. on four different media viz. LGD broth, Nutrient broth, NYSM broth and BMS broth. Our results revealed that the YEM broth supported better growth of *Rhizobium* isolates than Nutrient broth. However, the maximum growth of *Bacillus* spp. was observed in BMS broth while least in LGD broth at O.D_{620nm} after 72 hrs. But there was the problem to take optical density because of formation of black pellicle in BMS media by *Bacillus* spp. Therefore, we conclude that NYSM media is best media for the growth of *Bacillus* spp. *Rhizobium* and *Bacillus* spp. were evaluated for IAA production. *Bacillus* spp. was found more efficient than *Rhizobium* in terms of IAA production. For different carrier materials like Charcoal, Fly-ash and additives such as Glycerol and Trehalose were used over a period of 15 days of storage to assess the survivability of different *Rhizobium* and *Bacillus* isolates. Based on the finding of the study, the charcoal can be used as an alternate carrier material to prepare the microbial inoculants. However, the liquid inoculants were found to be better in supporting the population of inoculants than the carrier based inoculants.

Introduction

Biofertilizers assume a significant agent for enhancing the fundamental plant nutrients for sustainable agriculture because these are economically feasible and eco-friendly. The biofertilizers are preparation of living cells or latent cells of efficient strains of

microorganisms which enhance the uptake of nutrients by plants by interaction in rhizosphere soil (Motsara *et al.*, 1995). *Rhizobium* is a significant symbiont for legumes however it can be useful for nonlegume plants by delivering phytohormones. Phytohormone enhances the plant growth, lateral root formation, root

development, stem elongation and signal transformation. *Bacillus* spp. advanced plant development by various mechanisms, like- Phosphorus solubilization, Zinc solubilization, K solubilization, micronutrients uptake and translocation, suppressing of phytopathogens and secretion of phytohormones, for example, Indole Acetic Acid (Choudhary and Johri, 2009; Gutiérrez *et al.*, 2003).

Carrier based biofertilizers are more prevalent in India, in general, it is suffer from short shelf life, poor quality, high contamination and unpredictable field performance. Death of the organisms in the inoculated seed is one of the important factors contributing the failure of inoculation response in field condition. Much research is done in India on *Rhizobium* strain selection and formulation technologies are limited.

A break through is needed in the inoculants technology to improve the shelf life and field efficacy of bio-fertilizers in India to make them commercially viable and acceptable to farmers. One of the main problem in inoculants technology is the survival of micro-organisms during storage and several parameters such as culture medium, physiological state of the microorganisms when harvested (Alexander, 1977) the process of dehydrates, rate of drying the temperature storage and water activity (A_w) of the inoculums have an influence on their shelf life.

So, studies to increase the shelf life of inoculants or finding alternate formations for carrier based inoculants are important. Moisture retaining capacity of the CBBF is very low which does not allow the organism viable for longer period and the LBF facilitates the enhanced viability of the organism (Mahdi *et al.*, 2010). In the present study experiments have been conducted to

improve the shelf life and quality parameters of microbial inoculants of *Rhizobium* isolates and *Bacillus* spp. by the addition of suitable additives and also developing new formulation such as liquid inoculants. Therefore, present investigation was undertaken to Characterize *Rhizobium* and *Bacillus* inoculants morphologically and biochemically and assess the survival of microbial Inoculants in different carrier materials and liquid formulations.

Materials and Methods

Origin of isolates

Three isolates of *Rhizobium* were taken from different soil type of Bihar that is, from Tal land and *Bacillus* isolate was taken from Maize rhizosphere of diara soil. These isolates were selected to characterize and used to formulate liquid biofertilizer by the use of moisture protectants. All these isolates were obtained from Department of Microbiology in Faculty of Basic Sciences & Humanities, Rajendra Agricultural University, Pusa, Samastipur, Bihar.

Three promising *Rhizobium* strains and *Bacillus* isolate were selected for further morphological and colony characteristics. Growths of different isolates were measured by taking optical density after incubation of different time interval at $35 \pm 2^\circ\text{C}$. The Growth pattern and selection of suitable medium for optimum growth of different *Rhizobium* isolates were evaluated in two different media viz. Nutrient broth and Yeast extract mannitol broth while of *Bacillus* spp. on four different media. viz. LGD broth, Nutrient broth, NYSM and BMS broth by measuring O.D at 620nm after incubating them for 24 hours, 48 hours and 72hours at $35 \pm 2^\circ\text{C}$. These isolates were further studied for growth parameters, Indole Acetic Acid (IAA) production which acts as growth promoter.

Collection and analysis of carrier materials

Rhizobium and *Bacillus* isolates were obtained from Microbiology Department of Faculty The carrier materials like, flyash and charcoal were collected from department of soil science, Dholi and were used in the present study. All the carrier materials were dried in shade, powdered through 60 micron sieve. These materials were analysed for their physical and chemical characters viz., water holding capacity (Emmanuel *et al.*, 2010), bulk density (Emmanuel *et al.*, 2010), pH (Piper, 1966), organic carbon content (Walkley and Black, 1934) and electrical conductivity (Piper, 1966).

Preparation of carrier based formulations of different *rhizobium* isolates and *bacillus* spp.

These formulations were developed as described by Vidhyasekaran and Muthamilan, (1995) by using a mixture of 30 ml of different microbial inoculants with 100 g of carrier. The carriers were autoclaved for 1 hour on each of two consecutive days. *Rhizobium* and *Bacillus* strain were grown on YEM broth and BMS broth for 48 h as a shake culture in rotary shaker at 150 rpm. at room temperature ($25\pm 2^{\circ}\text{C}$) containing 9×10^8 colony forming units (CFU) per ml was added to 100g of the carrier material i.e charcoal and flyash and mixed well under sterile conditions. Then packed in polythene bags, sealed and stored at room temperature ($25\pm 2^{\circ}\text{C}$) and with a moisture content of 35%. In each carrier, the population of bacteria was estimated by using serial dilution technique.

Preparation of liquid inoculants

The YEM broth inoculated with different *Rhizobium* isolates in 250ml conical flask. It was allowed to multiply by incubating at

$30\pm 2^{\circ}\text{C}$ in a incubator cum shaker at 100rpm for 72 hours. The broth containing approximately 25×10^{11} CFU/ml was used as a starter culture for the production of liquid bioinoculant. The broth was mixed with sticking and spreading agents viz., Glycerol and Trehalose were added at the rate of 2 ml, / 100ml. They were packed in 100ml lot in opaque low density grade polypropylene bags of thickness 75 micron as per the procedure followed by Somasegaran. The liquid inoculants of *Rhizobium* isolates and *Bacillus* spp. were analyzed initially for their survival. The estimation of population was done by serial dilution and plating method.

Results and Discussion

Source of isolates

Rhizobia are known to supply biologically fixed nitrogen directly to the legume plant and also have great relevance in improving pulse productivity. Accordingly in the present study, three different *Rhizobium* spp. were selected from Chickpea, lentil and gram nodule of Tal land of Bihar and *Bacillus* spp. were selected from Maize rhizosphere of Diara soil of Bihar.

Growth of different *Rhizobium* spp. and *Bacillus* spp.in different media

Different *Rhizobium* isolates having origin from different soils were screened for the selection of suitable medium for its maximum growth. Growth pattern of different *Rhizobium* isolates were measured at OD_{620} after incubation of 24hrs, 48hrs and 72hrs at $35\pm 2^{\circ}\text{C}$. The optical density of *Rhizobium* isolates were obtained on Nutrient broth and YEM broth. The perusal of data in (Table 1) reveals that maximum growth of Chickpea, Lentil and Pea isolates were observed as 1.770, 2.047 and 0.875 respectively in YEM broth and 1.495, 1.268 and 0.612 in Nutrient

media broth at the end point of 72 hours. It indicates that YEM broth supports better growth of isolates than Nutrient media broth for different *Rhizobium* isolates. The growth was increasing with time of incubation and at the endpoint of 72 hours the growth was highest.

Similarly, the *Bacillus* spp. was evaluated for growth potential in four different Growth media viz. LGD broth, Nutrient broth, NYSM broth and BMS broth. Growth pattern of *Bacillus* spp. were measured in four different media viz. LGD broth, Nutrient broth, NYSM broth and BMS broth at OD_{620nm} after incubation of 24hrs, 48hrs and 72 hrs at 35±2°C. The data suggested maximum growth was observed in BMS broth i.e 1.321 while least growth in LGD broth i.e 0.899 at O.D_{620nm}, after 72 hrs (Table 2). But there was the problem to take optical density because of formation of black pellicle in BMS media by *Bacillus* spp. The growth was increasing with time of incubation and at the endpoint of 72 hours the growth was highest.

Morphological characterization

Colony morphology of different *Rhizobium* and *Bacillus* isolates were also examined in different media. Both isolates were studied for morphological and Culture characteristics which comprised of the appearance, pigmentation, shape, size, margin, and elevation, of the colonies on standard Medium that is, YEMA for *Rhizobium* isolates and NYSM for *Bacillus* sp.(Table 3). In case of *Bacillus* spp., the Morphology and Colony Characteristics on the standard medium was round shape, entire margin, drop like elevation, wet gummy colony appearance and slightly whitish pigmentation. In case of *Mesorhizobium* and *Rhizobium leguminosarum* spp., the morphology and Colony Characteristics on the standard

medium was circular shape, entire margin, convex elevation, wet gummy colony appearance and white pigmentation .

IAA production

Numerical strength of microorganism plays an important role in sustaining the soil health and plant growth. Bihar soil is rich in *Mesorhizobium*, *Rhizobium leguminosarum* and *Bacillus* spp. populations. The role of *Rhizobium* spp. as a PGPR was also evaluated and isolates exhibit IAA production with values ranging from 0.294 to 0.964 µg/ml in (Table 4). This suggests that *Mesorhizobium* was poor in IAA production. The role of *Bacillus* spp. as a PGPR was also evaluated and isolates exhibit IAA production with values of 20.04 µg/ml. This suggests that *Bacillus* spp. was excellent in IAA production. Role of different *Rhizobium* and *Bacillus* isolates as plant growth promoter has been documented (Bhasan *et al.*, 2004).

Physico-chemical properties of carrier materials

The physico-chemical properties such as organic matters, pH, EC, Bulk density, and water holding capacity were estimated for the carrier materials like Charcoal and Flyash (Table 5).The organic matter content was maximum in Charcoal (5.30 %). Flyash recorded minimum organic matter content of (0.36 %). The pH of charcoal was 7.1 followed fly ash 6.4. The Low electrical conductivity value was recorded in charcoal (0.49 dS/m) than fly ash (0.65 ds/m).The Bulk density of 0.75 g/cm³ and 0.47g/cm³ was recorded in Charcoal and flyash. The flyash was observed to show the maximum water holding capacity (63.0 %), whereas Charcoal recorded (52.6 %). The physico-chemical characters of carrier materials have got profound influence on the survival of inoculants.

Table.1 Growth of different *Rhizobium* spp.in different media composition at different time interval

S.NO	Isolates	O.D ₆₂₀ (24hr)		O.D ₆₂₀ (48hr)		O.D ₆₂₀ (72hr)	
		N.M	YEM	N.M	YEM	N.M	YEM
1	Chickpea	0.921	1.509	1.328	1.658	1.495	1.770
2	Lentil	0.684	1.456	0.866	1.553	1.268	2.047
3	Pea	0.141	0.251	0.453	0.616	0.612	0.875

Table.2 Growth of *Bacillus* spp. in different media composition at different time interval

Media	O.D ₆₂₀ (24hr)	O.D ₆₂₀ (48hr)	O.D ₆₂₀ (72hr)
BMS	0.430	0.721	1.321
LGD	0.123	0.460	0.899
NYSM	0.301	0.612	1.012
NM	0.212	0.472	0.952

Table.3 Morphology and Colony Characteization of different isolates

S. No	Isolates	Shape	Margin	Elevation	Colony appearance	Size	Pigmentation
1	<i>Bacillus</i> spp.	round	entire	drop like	wet gummy	tiny	slightly whitish
2	<i>Rhizobium</i> (Chickpea)	circular	entire	convex	wet gummy	small	White
3	<i>Rhizobium</i> (Lentil)	round	entire	convex	moist wet	small	slightly whitish
4	<i>Rhizobium</i> (Pea)	round	entire	convex	moist wet	tiny	slightly whitish

Table.4 IAA production of different Isolates

S.No.	Isolates	IAA Production
1	<i>Bacillus species</i>	20.04
2	<i>Rhizobium</i> (Chickpea)	0.967
3	<i>Rhizobium</i> (Lentil)	0.294
4	<i>Rhizobium</i> (Pea)	0.456

Table.5 Physico-chemical properties of charcoal and fly-ash carrier material

S.No	Carrier	pH	organic matter content (%)	EC (ds/m)	Bulk density (g/cm ³)	Water holding capacity (%)
1	Charcoal	7.1	5.30	0.49	0.75	52.6
2	Flyash	6.4	0.36	0.65	0.47	63.0

Table.6 Population of different isolates in different formulation

Formulation	<i>Bacillus spp.</i>	<i>Rhizobium</i> (Chickpea)	<i>Rhizobium</i> (Lentil)	<i>Rhizobium</i> (Pea)
	$\times 10^9$			
Charcoal	5.0	10.2	12.0	8.5
Flyash	4.5	9.0	10.2	8.0
Liquid	12.3	15.6	17.0	18.2



Plate.1 Pigmentation and colony morphology of *Rhizobium* and *Bacillus spp*

The ideal characteristics of an inoculant carrier include more surface area, rich in organic matter, high water holding capacity, neutral pH, easy availability and inexpensiveness.

The Population of different isolates in different formulations viz., charcoal, flyash and liquid were estimated under sterile condition over a period of 15 days at room temperature.

The result is presented in (Table 6). The population of different *Rhizobium* and *Bacillus* isolates over a 15 days of storage reveals that liquid inoculants supported maximum population in Pea isolates i.e (18.2×10^9) followed by Lentil (17.0×10^9), Chickpea (15.6×10^9) and *Bacillus spp.* (12.3×10^9).

Among the different formulations tested the liquid inoculants supported the maximum population of 15 days of storage followed by charcoal and Flyash.

Based on the finding of the study, we conclude that YEM broth supported better growth of *Rhizobium* isolates while, NYSM media is best media for the growth of *Bacillus spp.* The charcoal can be used as an alternate carrier material to prepare the microbial inoculants, whereas, the liquid inoculants were found to be better in supporting the population of inoculants than the carrier based inoculants.

Hence, It has been recorded that the liquid formulation supported the maximum population compared to other formulation. However, further studies are needed for Screening different concentration of

additives to standardize liquid formulation and to enhance the shelf-life of microbial inoculants -viz- validation of its efficiency under field conditions.

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How to cite this article:

Geeta Kumari, Deepak Kumar, Devendra Singh and Navnit Kumar. 2020. Characterization of Microbial Inoculants and its Different Formulation For Mass Production. *Int.J.Curr.Microbiol.App.Sci.* 9(07): 621-627. doi: <https://doi.org/10.20546/ijemas.2020.907.070>