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ISOLATION OF *Rhizobium* AND COST EFFECTIVE PRODUCTION OF BIOFERTILIZER

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ABSTRACT

The aim of present study was the production of cost effective biofertilizer using optimized media for *Rhizobium* to meet increasing nutritional food requirements with biotechnology to increase crop yields by reducing use of chemical fertilizers to maintain ecological balance for sustainable production in minimum cost to increase percent of proteins, vitamins, nitrogen containing products which helps to increase yield, physical and chemical profile of soil to evaluate the fertility status has been observed during study that optimized inoculants with coal powder added is more beneficial when compare with laboratories activated charcoal powder for germination rate and growth of plants. After cultivation of experimental plants it is noted that 20-60 kg nitrogen remain back which benefits for further plantation of leguminous plants.

KEYWORDS : *Rhizobium*, Biofertilizer Production, Symbiotic Nitrogen Fixing Bacteria, Carrier Material

The term biofertilizer can be more appropriately called 'microbial inoculants' are commercial preparation of microorganisms by using which nitrogen, phosphorous level and growth of plants increased. *Rhizobium* inoculant was first made in USA and commercialized by private enterprise in 1930s. (Smith, 1992) The beneficial micro-organisms, especially PGPR (Plant Growth Promoting Rhizobacteria), are grown in the simple, cheap media and they are mixed with the appropriate carriers to produce biofertilizers. Such PGPRs also fix nitrogen legume crops like red-gram, black-gram, groundnut, cowpea, and soyabean which help in saving 20-40 kg chemical nitrogen. 50-300 kg nitrogen fixation from soil and helps in saving about 25-100 kg chemical nitrogen i.e. 55-220 kg urea per hectare. *Rhizobium* is a soil habitat Gram-negative bacterium, which can able to colonize the legume roots and fixes atmospheric nitrogen symbiotically. There is symbiotic association between plant and the *Rhizobium* is initiated when bacteria in the soil attach to root hairs. This highly specific attachment process is mediated by plant proteins, the lectins that bind the bacteria to the surface of the root hairs, that is then penetrated by the microbes. The infected root cells divide and form a nitrogen fixing nodule which provides the anaerobic environment necessary for nitrogen fixation.

The effect of thymidine concentration on the inhibition was less support the growth of thyf and thy- strains of bacteria and the role of thymidine is in the biochemical modulation of antimetabolites (Stephens and Rack, 2000) which used during design of optimized media. The morphology and physiology of *Rhizobium* will vary from free-living condition to the asteroid of nodules. In addition to fixing the atmospheric nitrogen through nodulation, it shares many characteristics with other PGPRs including hormones production and solublization of organic and inorganic phosphate (Russell et. al., 1982). Through plant growth promoting substances, it helps in root expansion, improve uptake of plant nutrients, protects plants from root diseases and most important improves biomass production of fast growing at wasteland.

IDENTIFICATION OF BACTERIAL CULTURE BY MORPHOLOGICALAND BIOCHEMICAL TESTS

The bacterial cultures were classified mainly to their generic level. Organism is isolated from root nodules and morphological identification of the isolated strain was done by gram's staining and motility test. Bacterial colonies grown on YEMA are streaked over CRYEMA after incubation of these plates at 28-30^{0}C for 7 days it has been observed that *Rhizobium* utilizes Congo red slowly and form white, circular, translucent, glistening, elevated and raised colonies for further confirmation stained with carbol fuschin for microscopic observation where PHB of it stained and proceed to biochemical glucose-peptone agar (GPA) test in which a master plate prepared using colonies on YEMA and replica plating from master plate is done on GPA medium colonies fails to grow where as 2 % salt containing YEMA medium has inability to grow colonies then *Rhizobium* confirmed at preliminary base and send for

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identification by using automation system at Indrayani laboratories. This culture is used for mass production of *Rhizobium*.

Culturing of Microorganisms

The selective and optimized mediums used for mass culturing of *Rhizobium* biofertilizers are as follows:

Selective Yeast Extract Mannitol Broth

Components	**Quantity (g L^{-1})**
Mannitol	10.0
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.1
Yeast extract	0.5
Distilled water	1 L

Optimized Broth

Components	**Quantity (g L^{-1})**
Mannitol	10.0
K_2HPO_4	0.45
$MgSO_4.7H_2O$	0.2
NaCl	0.1
Yeast extract	0.1
$FeCl_3.6H_2O$	0.02
$Cacl_2.7H_2O$	0.04
Thymidine	Trace
Congo red	0.001
Distilled water	1 L

MATERIALS AND METHODS

For mass production of *Rhizobium* bacterial strain isolated from various regions and grown on slants for preservation as per need culture from slant were transferred to liquid broth of selective as well as optimized medium in the rotary shaker for 4 days to prepare starter culture. Later on the starter cultures is transferred to the fermenter in batch culture mode for 4-9 days for bacterial growth when cell count reached to 108- 109 cells/ml, the broth used as inoculant. For easy handling, packing, storing and transporting broth is mixed with an inert carrier material which contains sufficient amount of rhizobial cells. In present study broth is mixed with unsterile soil: Activated charcoal, A. R. (RM 1332-100g) in a ratio of 1:3 where as other set prepared by using unsterile soil: crude coal powder (crude) in same ratio. After proper mixing carrier containing inoculant is left for 4-10 days by covering with polythene at 22-24^{0}C which helps to multiplies *Rhizobium* in the remaining broth and above formulated microbial inoculant used as biofertilizer.

Use of Biofertilizer

There are different methods of applying biofertilizers

- Coating on seed
- In soil at root system of plant
- Mixing with soil
- By dissolving into water

Used 50 ml water with 1% sucrose, boil for 10 min then add 1% gum arabic allow to cool to form slurry which called as sticker solution. In sticker solution add 20 gm of biofertilizer mix it properly. Seeds were added to the slurry to form uniform coat of the *Rhizobium* culture around the seed without damaging seed coat. The inoculated seeds were kept on gunny bags away from sunlight for drying under shade and sown immediately.

Determination of NPK and Organic Carbon

Determination of total Nitrogen by Kjeldahl method (Page and Keeney., 1989), Olsen method for phosphorous (Olsen, et al., 1954) and Ammonium acetate extractable method for Potassium content was done as per in (Ghosh et al., 1983., and Page and Keeney., 1989). Organic matter and carbon was determined by Ignition method (Jackson, 1967).

NPK, organic matter and carbon was calculated by the following formulae.

$$\text{Available Phosphorus (kg/ha)} = \%totalN \quad (x \quad y)X(0.014)X\frac{100}{Wt.of\_ovendry\_soil}$$

$$\text{R-from std curve} \quad R \quad \frac{Total\_vol\_of\_extract}{vol\_of\_aliquot\_taken} \quad \frac{1}{wt\_of\_soil} \quad \frac{2.24 \quad 10^6}{10^6}$$

$$Available\_K \quad R \quad \frac{Vol\_of\_the\_extract \quad 2.24 \quad 10^6}{wt\_of\_soli\_taken \quad 10^6}$$

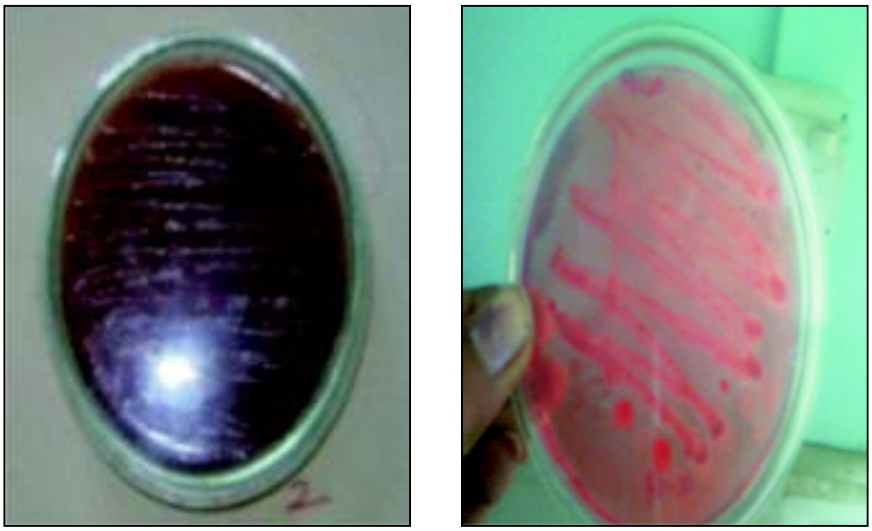

Figure 1: Isolated bacterial Colonies of *Rhizobium* on selective and optimized media

Figure 2: Growth of experimental legume and non-legume crops

Organic matter and carbon determined by

%Organic matter = 100-(Z+%moisture)(oven dry basis)

% Organic carbon % organic matter x 0.58* oven dry basis

organic matter is assumed to contain 58%organic carbon.

RESULTS AND DISCUSSION

Rhizobium forms white, translucent, glistening elevated and comparatively small colonies on selective medium where as on optimized medium bacteria utilizes Congo red slowly slightly pinkish colonies (Figure, 1). For experimental trials bags with 1 kg soil capacity were used. Biofertilizer applied to seed, root and soil were function to mobilize the availability of nutrients especially NPK by their biological activity in particular area and help to enhance soil health by building up micro flora to supply different kind of nutrients in the soil in this study generally all parameter were significantly showed favorability more for seed coating with biofertilizer than the mixing with soil and dissolving in water over control (Figure, 2).

Experimental site have inadequate NPK for plantation initially however, after use of biofertilizer it enhance nitrogen fixation thus disease free high growth of fenugreek was resulted and economically it is better to use biofertilizer of *Rhizobium* alone for leguminous plants by using mass inoculums of optimized media. This study not only showing improved growth potentiality with optimized media also considering seed germination found early almost 100% when compared with control. At maturity data regarding plants height, nodule formation, root weight, shoots weight and grain yield were recorded using standard procedures. During study N influences vegetative and reproductive phase of plant growth by biofertilizer improve the soil fertility by aggregation and adding nutrients to soil it has been found that growth of plant inoculated by crude coal with optimized media of fenugreek was more whenever compare to selective media along with activated charcoal and crude coal powder as carrier materials. Soil samples from each treatment were collected and analyzed for NPK content and organic matter according to under the laboratory condition after the use of appropriate biofertilizer soil profile is potentially improved (Table 1a & 1b). ANOVA analysis in Table, 2 gives calculated F value is greater than tabulated F crit value therefore the values are significant at 5 % level after treatment of biofertilizer. For one liter of bacterial culture 5 kg of carrier material is required for biofertilizer preparation (Hyness et al. 2001).

CONCLUSION

The productivity and quality of fenugreek than gram seed can be substantially increased by Rhizobial seed coating inoculation for legume crops whereas un inoculated control and non-leguminous plants has shown less growth of subsequent crops so this type of biofertilizer was showing more potentiality during growth of legume crops to get more benefit from Rhizobial symbiosis than non-legume crops. In this research other important parameter considered for physical and chemical characteristics profile of soil showed at start of experiment inadequate NPK for plantation and after the use of appropriate biofertilizer profile is potentially improved. In further study media optimization for growth of new improved inoculant may contain Rhizobia and PGP organisms for design of biofertilizer.

Table 1. Physical and chemical characteristics of experimental soils

(a) Before inoculation of Biofertilizers

Soil parameter	Used for set preparation
pH	4.1
Available N	18.55
Available P	5.12
Available K	60.58
Organic matter (%)	1.424

(b) After 60 days inoculation of Biorfertilizers

Soil parameter	Selective	Optimized	Control
pH	5.4	5.7	4.3
Available N	32.55	42.22	20.55
Available P	7.88	8.52	6.20
Available K	68.80	106.00	64.80
Organic matter (%)	1.880	2.292	1.698

Table 2 : ANOVA

Source of Variation	SS	df	MS	F	F crit
Rows	12860.02	4	3215.006	32.45996	3.837853
Columns	479.9072	2	239.9536	2.422666	4.45897
Error	792.3621	8	99.04526		

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