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Optimization of production parameters and evaluation of shelf life of *Rhizobium* biofertilizers

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ABSTRACT

One of the major concerns in today's world is the pollution and contamination of soil. The use of chemical fertilizers and pesticides has caused serious health diseases. Many studies revealed a microbial preparation (biofertilizer) does not harm to the environment. *Pisum sativum* (Garden Pea) is known for its dietary protein source, fodder for animals and symbiotic nitrogen fixation by *Rhizobium* present in its root nodules. This study revealed that organism cannot grow on Glucose-Peptone Agar plate but able to grow on Yeast Extract Mannitol Agar medium (YEMA) plates and detains pink colour of YEMA medium. Different biochemical tests were done to identify *Rhizobium* like urease, nitrate reduction, oxidase, catalase and salt tolerance test. The present study describes the optimization of production parameters and evaluation of shelf life of *Rhizobium* biofertilizer inoculated in multani mitti and coal ash. It utilizes sucrose as sole carbon source, different amount of yeast extract and salts such as Epsom salt (Sendha namak), Halite (Kala namak), NaCl, $NaNO_3$, KI. The *Rhizobium* isolated from *Pisum sativum* roots has potential to produce industrially important biofertilizer. Quality control tests were done at the stage of mother culture, production culture and purity of carrier materials. Utilization of coal ash and multani mitti as carrier in biofertilizer formulations emerged as safe and effective alternatives. Shelf life of Rhizobium biofertizer in coal ash was found to have better growth for two months. The *Rhizobium* can be easily immobilized onto carriers like coal ash and multani mitti hence the best way to control waste like coal ash.

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Introduction

Biofertilizers are ready to use live formulates of beneficial microorganisms which on application to soil mobilize the availability of nutrients by their biological activity in particular and helps build up the micro flora and in turn, the soil health in general. It's also affordable and does not cause eutrophication and perturbation of soil (Scheirr, 1999). Biofertilizer is a substance added to soil to improve plant's growth and yield. First used by ancient farmers. Modern synthetic fertilizers are composed mainly of Nitrogen, Phosphorous and Potassium compounds with secondary nutrients added. The use of synthetic fertilizers has significantly improved the quality and quantity of crop, although their long-term use is doubted by environmentalists. Petroleum is one of the composite of chemical fertilizers; its long term use leads to soil pollutions. Chemical fertilizers will cost 2400 ₹ /acre, but biofertilizers merely cost 1.8 ₹ /acre. Excessive use of chemical fertilizer are damaging the environment e.g., about 10% of the ground water samples in Punjab contained more NO_3^- than the maximum permissible limit prescribed by World Health Organization (WHO) (Milkha *et al*., 2010). In comparison to chemical fertilizers biofertilizers are cost effective, eco friendly, increase crop yield by 20-30% and activate soil biologically. Chances of contaminants will be high is one of the disadvantage of biofertilizers. *Rhizobium* bacteria are a group of soil based bacteria (SBB's) which establish symbiotic relationships with legumes. These SBB's form nodules on the roots of the legumes and provide nitrogen to the plants. The bacterium's enzyme system supplies a constant source of reduced nitrogen to the host plant and the plant furnishes nutrients and energy for the activities of the bacterium. This symbiosis reduces the requirements for nitrogenous fertilizers during the growth of leguminous crops (Dilworth and Parker, 1969). In turn, the plants provide carbon and energy for the SBB's. *Rhizobium* rods are 0.5-0.9x1.2-3.0 µm commonly pleomorphic under adverse growth conditions. Usually contain granules of poly-ß-hydroxy butyrate which are refractile under phase contrast microscopy. Non-spore forming, gram negative, motile by one polar or sub-polar flagellum or two to six peritrichous flagella. Optimum temperature and pH for rhizobia is 25-30°C, 6-7 respectively. Colonies on Congo Red Yeast Extract Mannitol Agar (CR+YEMA) are circular, convex, semitransluscent, raised and mucilaginous, usually 2-4 mm in diameter within 3-5 days. Analysis of colony morphology indicated round colonies, white colored till 3-4 days of growth and turning yellowish in colour after 4 days. Typical colonies had a diameter of 5-7 mm. The pH of the medium and broth during growth of isolates was changed from 7.0 to 6.0, thus showing the production of acid which is the characteristic of *Rhizobium* to produce acid during growth (DeVries *et al.*, 1980; Baoling *et al.*, 2007). Genetically studied about nodule forming species of *Rhizobium* consists of an extremely, large plasmid known as 'megaplasmid' in cell which

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possesses numerous genes coding for nodulation *(nod* genes) and nitrogen fixation *(nif* genes) (Rosenberg *et al.,* 1981). Both *nod* genes and *nif* genes are closely located. However, a physiological map of 135 Kb segment of megaplasmid was established which contained *nod-nif* regions. *Rhizobium meliloti*, contains *fixL* and *fixJ*, which are positive regulators of symbiotic expression of diverse nitrogen fixation (*nif* and *fix*) genes. *nif* gene regulation is shown to consist of a cascade: the *fixLJ* genes activate *nifA*, which in turn activates nif HDK and *fixABCX*. Like *nifA, fixN* can be induced in free-living microaerobic cultures of *R. meliloti*, indicating a major physiological role for oxygen in *nif* and *fix* gene regulation. Microaerobic expression of *fixN* and *nifA* depends on *fixL* and *fixJ*. The FixL and FixJ proteins belong to a family of two-component regulatory systems widely spread among prokaryotes and responsive to the cell environment. FixL, which has features of a transmembrane protein, senses an environmental signal and transduces it to FixJ, a transcriptional activator of *nif* and *fix* genes (David *et al.,* 1988). Currently some of the biofertilizers are in more demand such as, Nitrogen fixing bacteria (*Azotobactor, Beijerinckia, Clostridium, Nostoc, Anabena, Rhizobium, Frankia, Azolla, Anabena, Azospirillum lipoferum* etc.), Phosphorus solublizing bacteria (Pseudomonas spp. Bacillus megaterium, Bacillus subtilus, Bacillus megaterium, Bacillus phosphoticum), Fungi biofertiluzers (Arbuscular mycorrhiza, Ectomycorrhiza, Ericoid mycorrhiza, and Orchid mycorrhiza). The objects behind the application of Biofertilizers to seed, soil or compost pit is to increase the number and biological and metabolic activity of useful microorganisms that accelerate certain microbial processes to augment the extent of availability of nutrients in the available forms which can be easily assimilated by plants. The need for the use of biofertilizers has arisen primarily due to two reasons i.e. though chemical fertilizers increase soil fertility, crop productivity and production, but increased intensive use of chemical fertilizers has caused serious concern of soil texture, soil fertility and other environmental problems, use of Biofertilizers is both economical as well as environment friendly. Therefore, an integrated approach of applying both chemical fertilizers and biofertilizers is the best way of integrated nutrient supply in agriculture. Chemical fertilizers are more effective in poor soil condition and makes plants healthier. These fertilizers support in resisting bugs and disease. One of the benefits of chemical fertilizers is that it is made for farmer's choice. Particular types of deficiency sort out by rich in single fertilizers, as for example if the soil is deficient of sulphur one can use sulphur fertilizer. Chemical fertilizers have an NPK ratio of nearly 60% while organic fertilizers have a low NPK ratio merely 16%. They help in increasing the crop yield by 10-25%. Some carrier materials are much proficient in seed inoculation like peat , lignite, coal, filter mud, peaty-soil, polyacrylamide, charcoal-bentonite vegetable oils, mineral soils, vermiculite, coal ash and multani mitti (commonly known as "Calcium Montmorillonite"). Industrial Microbiology gained a wide area of research when first Rhizobium biofertilizer were produced under the brand name "Nitragin" for 17 different legumes by Nobbe and Hiltner in 1895**.** Later rhizobia were from preserved nodules of various leguminous plants During 1951 and 1952 various leguminous plants from the grounds, gardens, and greenhouses of Vassar College were collected and examined nodules of leguminous plants can be preserved by freezing for as long as 2 years; they cannot be preserved by storage under mineral oil at room or refrigerator temperatures (Potter, 1954). To know the maximum production in different types of media compositions, selected rhizobia strains were characterized and monitor isolated from tree legumes (Manassila *et al.*, 2007). In the other studies *Rhizobium* strain characterized the isolated from the roots of *Trigonella foenumgraecum* (fenugreek) (Singh *et al.,* 2008). By this study revealed that the nitrogen fixtation bacteria and phosphate solublization bacteria not only found in rhizosphere or farming soil. It can be also isolated from Saline Belt of Akola and Buldhana district of Maharastra (India) (Tambekar *et al.,* 2009). In this comparative study showed shelf life of fly-ash based bio-fertilizers vs perfected chemical fertilizers in wheat (*Triticum aestivum*) (Kumar *et al.,* 2010). The present study has an objective of isolation, optimization and shelf life of *Rhizobium* biofertilizer in two cheapest carrier materials (Calcium Montmorillonite (Multani mitti) and Coal ash). Rhizobia isolated from root nodules of *Pisum sativum* have shelf life of 3 months on lab scale production. In view of this, there is a need to search for new easily available and cheapest carrier materials for *Rhizobium* biofertilizers storage.

The present study entitled "Optimization of Production Parameters and Evaluation of Shelf life of *Rhizobium* Biofertilizer" was carried out in the Department of Microbiology and Fermentation Technology, Jacob School of Biotechnology and Bioengineering, Sam Higginbottom Institute of Agriculture, Technology and Sciences, (Deemed-to-be-university) Allahabad.

Materials and methods

Sampling site and collection of root nodules

The root nodules of *Pisum sativum* (Garden pea) were taken from Agriculture farm of Sam Higginbottom Institute of Agriculture, Technology and Sciences. Nodules were taken by uprooting the pea plant and collected in alluminium foil sheet.

Isolation of *Rhizobium*

Processing of sample

Colonies were isolated from the root nodules of *Pisum sativum* (Garden pea) by serial dilution method. Root nodules of *Pisum sativum* were located on the roots with a pink colour.

Processing of root nodules

The collected nodules were surface-sterilized with 75% ethanol for 10 sec and 0.1% mercuric chloride for 5 min and washed thoroughly with distilled water. *Rhizobium* strain was obtained by streaking the crushed root nodules on YEM (yeast extract mannitol, pH 7.0) agar plates and incubated at 29.4°C (Aneja, 2003).

Serial dilution method

The pink colour nodules were crushed in 1ml of sterile water with a sterile glass rod. A uniform suspension was made of nodules and made serial dilution of nodules extract. This method is based on the principle that when materials containing bacteria is cultured, every viable bacterium develops into a visible colony on a nutrient agar medium (Aneja, 2003). The root nodule extract was serially diluted and made a dilution up to 10^{-9} with sterile distilled water and 1 ml of diluted characteristics are used to identification of sample was inoculated into sterile Petri plates and poured 10^{-4}-10^{-7} dilutions for *Rhizobium* colonies.

Starter culture of *Rhizobium*

YEMA media was prepared and autoclaved by transferring in a flask. Thereafter pure *Rhizobium* colonies were transferred into sterilized CR+YEMA petri plate. Streaked YEMA plates were incubated in a cooling incubator at 28-30°C. After 4-6 days, sufficient numbers of cells were grown on YEMA plates. This culture is called as "Mother culture of *Rhizobium*".

Procurement of microorganism and its maintenance

The bacterial species *Rhizobium* was isolated from root nodules of *Pisum sativum* was subcultered on YEMA plates on regular interval of 7 days.

Tests for purity of mother culture

Before mass production of *Rhizobium* biofertilizer, the mother culture was checked by the following methods:

A. Growth
B. Purity
C. Gram stain

A. Growth

By streaking a mother culture on Yeast mannitol+Congo red agar (CRYEMA) plates, were checked growth of rhizobia. YEMA is selective media for *Rhizobium*. The incubation temperature was 29.4°C. After 2 days of incubation, *Rhizobium* colonies were obtained. Further streaking, spreading and visual characterization of colony morphology helped in isolation of pure cultures of *Rhizobium*. Pure isolates were used for further analysis and tests.

B. Purity

Streaking of *Rhizobium* culture was done on glucose peptone agar plate for checking purity, and incubated for 24 hours at 29.4ºC. No growth or poor growth was obtained on GPA. Good growth and colour change was expected from contaminants.

C. Gram stain

The Gram stain, a differential stain was developed by Dr. Hans Christian Gram, a Danish physician, in 1984. It is very useful stain for identifying and classifying bacteria into two major groups; the gram-positive and gram-negative. In this process, different reagents used were crystal violet (primary stain), gram's iodine (mordant), alcohol (decolorizing agent) and safranin (counter stain) for 1 min, 1min, 20-30 seconds and 30 seconds respectively. The bacteria which retains the primary stain (appear dark blue or black) are called gram-positive, whereas those that lose the crystal violet and counter stained by safranin (appear red) are reffered to as gram-negative (Aneja, 2003). A loop of mother culture was checked by Gram staining and observed under microscope was found gram-negative.

Biochemical Tests: Following biochemical tests were carried out to identify Rhizobium.

Urease test

Urease test was performed by growing the test organism on YEMA media containing the indicator phenol red. Urease broth was used to differentiate medium and tests the ability of an organism to produce an exoenzyme, called urease, that hydrolyzes urea to ammonia and carbon dioxide. The broth contains two pH buffers, urea, a very small amount of nutrients for the bacteria, and the pH indicator phenol red. All the inoculated as well as uninoculated (control) tubes were incubated at 37°C for 48 hours. Phenol red was turned to yellow in an acidic environment and fuchsia in an alkaline environment. If the urea in the broth was degraded and ammonia was produced, an alkaline environment was created, and the media urea broth formulated to test for rapid urease-positive organisms. If colour of phenol red is turned to pink, the test is posititive for *Rhizobium*.

Nitrate reduction test

The isolated strains were inoculated into nitrate broth media. All the inoculated as well as uninoculated (control) tubes were incubated at 37°C for 48 hours. After incubation period, two reagents added solution A (sulfanilic acid) which was followed by solution B (α-napthanol). Development of cherry red colour indicates positive test and no change in colour indicates negative test.

Oxidase test

Oxidase test was performed to determine the presence of oxidase enzyme in bacterial isolates (Steel, 1961). Kovac's reagent (1% N, N, N.N-tetramethyle-pphenylene diamine) was dissolved in warm water and stored in dark bottle. A strip of filter paper was dipped in this reagent and air-dried. With the help of sterile wire loop, one-day-old grown colonies from agar plates were transferred on this filter paper strip. If the organism changes to pink to dark purple then it shows positive test.

Catalase test

This test was performed to study the presence of catalase enzyme in bacterial colonies. Grown colonies (24 hrs old) were taken on glass slides and one drop of H_2O_2 (30%) was added. Appear of gas bubble indicated the presence of catalase enzyme (MacFaddin, 1980). Release of free 0_2 gases in the form of bubbles is a positive catalase test.

Salt tolerance test

Salt tolerance test was done to check the growth of *Rhizobium* on 2% salt containing YEMA media. In this test 2% salt is added to the normal YEMA medium instead of 1% salt concentration keeping all the constituents same. *Agrobacterium* is able to grow on YEMA medium containing 2% NaCl, whereas *Rhizobium* cannot grow on such medium.

Production Media Optimization to Scale up Culture

Components are established to be best suited for the growth of the organism on laboratory scale. But in order to grow the *Rhizobium* on fermenter scale or other production purposes, the option of production media optimization cannot be left out. So, the various components of the YEMA media were varied or replaced and used at different concentrations for media optimization for *Rhizobium*. The production media optimizations were mainly focused on:

To optimize different level of carbon source (sucrose) were taken, such as 2.5 gm, 5 gm, 7.5 gm, 10gm and 12.5gm in place of mannitol keeping the other constituents of the normal to YEM broth same.

To optimize different level of yeast extract 1 gm, 2 gm, 3 gm, 4 gm and 5 gm were taken, keeping the other constituents of the normal to YEM broth same.

To optimize different sources of salt such as halite (Rock salt also known as "sendha namak"), $MgSO_4.7H_2O$ (Epsom salt also known as "kala namak"), KI and $NaNO_3$ were taken in place of NaCl.

Table 1. Composition of various media formulation used for media formulation used for production media optimization

Media name	YEM medium	Other components
	Sucrose	
SR1	2.5 g/L (25%)	**Yeast extract 4.0 g/L**
SR2	5.0 g/L (50%)	**K2HPO4 0.5 g/L**
SR3	7.5 g/L (75%)	**$MgSO4.7H_2O$ 0.2 g/L**
SR4	10.0 g/L (100%)	NaCl 0.1 g/L
SR5	12.5 g/L (125%)	
	Yeast extract	
YE1	1.0 g/L (25%)	**Mannitol 10 g/L**
YE2	2.0 g/L (50%)	**K2HPO4 0.5 g/L**
YE3	3.0 g/L (75%)	**$MgSO4.7H_2O$ 0.2 g/L**
YE4	4.0 g/L (100%)	NaCl 0.1 g/L
YE5	5.0 g/L (125%)	
	Salt concentration (instead of NaCl)	
S1 (Epsom salt)	↓	**Mannitol 10 g/L**
S2 (Halite)		**K2HPO4 0.5 g/L**
S3 (NaCl)	0.1 g/L	**$MgSO4.7H_2O$ 0.2 g/L**
S4 ($NaNO_3$)		Yeast extract 4.0 g/L
S5 (KI)	↑	

Mass production of Rhizobium

A Media composition that contains higher growth according to optical density was taken for mass production of *Rhizobium*. For mass cultivation of rhizobial inoculants, broth medium was prepared in 1 litre quantity each for two carrier materials (multani mitti and coal ash) and transferred in a large conical flask. The YEM broth contains (per 1000 ml) Sucrose 12.5 gm, K_2HPO_4 0.5 gm, $MgSO_4 .7H_2O$ 0.2 gm, Yeast extract 4.0 gm and KI 0.1 gm. The pH of the medium was adjusted to 6.8-7.0 by using HCl or KOH solution. Temperature was 28-30°C and incubation time were 4-9 days in shakers incubator. For the good bacterial growth aeration was needed. After mass production media were taken for further tests.

Test for purity of biofertilizer

The following qualities of the broth samples were checked to make sure that the broths are in good conditions:

A. pH

Fast–growing rhizobia such as rhizobia for peas, gram, and mungbean produce a little acidic compound. After incubation, the pH was decreased. (Example, pH before growing = 7.0, after growing pH = 6.4 – 6.6). If broth pH increases, it means some contaminants will occur, higher pH indicates presence of contaminants.

B. Staining (Gram stain)

Rhizobial cells were stained for observation of shape and size of the cells. Cells of rhizobia were rod–shaped, with one or two cells sticking together. They were not appeared in long–chain. Gram-stained cells were appeared red.

C. Optical Density

Broth culture with active rhizobial growth was become turbid in 4-9 days. Broth turbidity or optical density was checked by using spectrophotometer (at 540 nm). Values of O.D. correlate to number of cells. If O.D. values will be high then cells number also will be high. O.D. should be 0.3 to 1.0 shows optimum growth.

D. Viable count

The numbers of living cells were counted by spread plate or drop plate methods. Doing spread plate by making serial dilutions from $10^1 – 10^7$. Then three replicates of 0.1 milliliter of broth from 10^7, 10^6 and 10^5 were spread over the YMA + CR (Congo red) plates. Plates were incubated in cooling incubator (28–30°C) or at room temperature for 7 days. Colonies of rhizobial cells were round, opaque and have smooth margin. They were white and not absorb red colour as well as the other bacteria. Viable counts of rhizobia were checked by following formula:

Viable counts (N) = (y / d) x X

Where, N = no. of cells,

y = no. of colonies,

d = dilution,

X = volume of dilution plated

Tests for purity of carrier material

The carrier materials (coal ash and multani mitti) were powdered to a fine powder so as to pass through 212 micron IS sieve. pH, moisture content, viable number were tested for purity of carrier materials. The pH of multani mitti was 7.0 but coal ash had 7.5. The pH of the carrier material was neutralized with the help of calcium carbonate (1:10 ratio). The neutralized carrier materials were sterilized in an autoclave to eliminate the contaminants. Moisture content was checked with the help of tissue paper and 1gm of each carrier material were taken and poured on the nutrient media for checking any type of possible contaminants.

Processing of carrier material

The neutralized and sterilized carrier materials were spread in a clean, dry, plastic tray. The bacterial cultures were drawn from the shaking incubator added to the sterilized carrier and mixed well by manual (after wearing sterile gloves). The culture suspension was added to a level of 40-50% water holding capacity depending upon the population. The inoculants were allowed for curing for 2-3 days at room temperature (curing can be done by spreading the inoculants on a clean floor/polythene sheet or by keeping in trays with polythene covering for 2-3 days at room temperature) (Mishra *et al.,* 2010). During this period *Rhizobium* cells multiplied, a process called 'curing'. Thereafter, *Rhizobium* inoculants can be used directly or packed and stored (Singh *et al.,* 2008).

Packaging of biofertilizers

The polythene bags were of low density grade. Thickness of bags was around 50-75 micron. Each packet were marked with the name of the manufacturer, name of the product, the crop(s) to which recommended, method of inoculation, date of manufacture, date of expiry, full address of the manufacturer and storage instructions etc. The polythene packets were prepared 200gm for multani mitti and 100gm for coal ash. Thus, *Rhizobium* biofertilizers were produced in quantity of 2 kg, 1Kg each for multani mitti and coal ash.

Shelf life of *Rhizobium* biofertilizer and testing of possible contaminants

The packets were stored in a cool place away from the heat or direct sunlight. The packets were stored in cold storage conditions. The shelf lives of inoculants in the carrier inoculants packets were determined in 15 days interval. There were more than 10^9 cells per gram of inoculant at the time of preparation and 10^7 at the time of expiry. Contaminants were analysed while checking shelf life. The possibilities of contaminants were depended on the favorable condition for microorganisms in storage environment. Storage conditions for *Rhizobium* were at refrigerator temperature (4°C). The contaminations were checked on YEMA, glucose peptone agar (GPA) and nutrient agar (NA) plates.

Statistical Analysis

Data obtained in the optimization of *Rhizobium* biofertilizer produced in YEM broth were analysed to calculate significant differences in growth under different media concentration by using one way classification and conclusion was drawn on the basis of analysis of variance technique(ANOVA) (Fisher and Yates, 1968) at 5% level of significance. Tabulated value of F from the table at 5 % level of significance for V_1 and V_2 degree of freedom obtained. If the calculated value of F is less than the tabulated value F, i.e., $F_{cal} < F_{tab,}$ the null hypothesis, i.e., $H_0 : \mu_1 = \mu_2 = \mu_k$ is accepted and it is inferred in difference in means is insignificant otherwise it is considered as significant (Mittal, Sharma and Kumar, 1981).

Results And Discussion

In the present study, *Rhizobium* were isolated by serial dilution method from the root nodules of *Pisum sativum* collected from the different part of crop field of the agricultural farm of SHIATS, Allahabad. In YEMA medium, 54 colonies were observed in which 21 colonies retains congo red colour of the media but 33 of them were not retained congo red colour. The colonies were not retained congo red colour detected as rhizobia by different biochemical tests. Colonies of *Rhizobium* were obtained on YEM agar medium after incubation at 29.4°C for two days. The colonies were having sticky appearance were taken and inoculated on YEMA plates. Analysis of colony morphology indicated round colonies, white coloured till 3-4

days of growth and turning yellowish in colour after 5 days. Typical colonies had a diameter of 5-7 mm. General microscopic view of the isolates observed as rod cells and gram negative in nature. Rhizobia were isolated on yeast-extract mannitol agar (YEMA) using standard procedures. Single colonies were marked and checked for purity by repeated streaking on YEMA medium and verifying a single type of colony morphology, absorption of Congo red (0.00125 mg kg^{-1}) and a uniform Gram-stain reaction (Vincent, 1970). Colony morphology (color, mucosity (mucoid), border, transparency and elevation) and acid/alkaline reaction were evaluated on YEMA containing bromothymol blue (0.00125 mg kg^{-1}) as indicator (Alberton *et al.,* 2006). Poor growth on glucose peptone agar (GPA) medium, good growth on CR+YEMA media and microscopic view of *Rhizobium* as Gram negative were showed purity of mother culture. The confirmation of *Rhizobium* was made by the specific tests *viz,* morphological identification, microscopic observation, growth on yeast mannitol agar media, glucose peptone agar plates, biochemical tests (urease, nitrate reduction, oxidase and catalase test and salt tolerance test) (Table 1). The main aim of production media optimization was to define a media which shows the optimum conditions for the growth of the organism at cheap cost as compared to normal media. *Rhizobium* strains were able to utilize glucose and sucrose more efficiently than normal YEM medium (Kucuk *et al*., 2006). Once the production media have been optimized, this can be used to scale up biomass yields using shakers incubator on lab scale. Fermentors/bioreactors used for large scale production. The results indicated that isolated Rhizobia cells were able to grow in higher sucrose concentration (125%), higher yeast concentration (125%), and shows maximum growth in 1% KI containing medium.

Higher sucrose and yeast extract concentration showed maximum activity at A_{540} were 1.69, 0.97 respectively. Activity was found 0.69 for KI showing highest growth compared to halite (0.39), epsom (0.41), $NaNO_3$ (0.40) and NaCl (0.58) (Table 3). The growth of *Rhizobium* biofertilizer were studied on the basis of different concentration of sucrose keeping other composites of the YEM broth same. It was observed in the present study that the maximum growth of the the rhizobia found to be maximal in SR5 (12.5 g/L) at the 29.4°C for 7 days.The effect of sucrose concentration was found to be statistically insignificant (F_{cal} 0.007 < F_{tab} 3.89) at 5% level of significance (Figure 1).

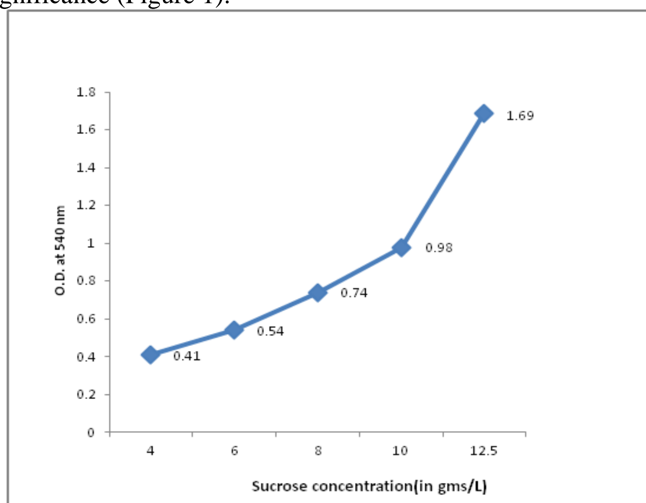

Figure 1: Effect of Sucrose Concentration on Production of *Rhizobium* Biofertilizer

Table 2: Morphological, Microscopic and Biochemical tests for *Rhizobium*

Morphology	**Watery, gummy, circular convex with smooth edges, glistening translucent or white having 5-7mm in diameter**	
Microscopic Identification	**Gram negative, rod shaped with one or two cells sticking together.**	
Biochemical Identification	Positive	Negative
1. Gram staining	×	√
2. Urease test	√	×
3. Nitrate reduction test	√	×
4. Oxidase test	√	×
5. Catalase test	√	×
6. Salt tolerance test	×	√

Table 3: Effect of Sucrose Concentration on Production of *Rhizobium* Biofertilizer

Media name	Sucrose concentration (in g/L)	Rhizobial growth(O.D. at $A_{540\ nm}$)
SR1	4	0.41
SR2	6	0.54
SR3	8	0.74
SR4	10	0.98
SR5	12.5	1.69

F_{cal} = 0.121 < F_{tab} (5%) = 3.89 Note: - SR = Sucrose

The growth of *Rhizobium* biofertilizers were studied on the basis of different concentration keeping other constituent of YEM broth same. Growths were observed in ascending order according to yeast extract concentration ranges (1-5 g/L). The effect of yeast extract concentration was found to be statistically insignificant (F_{cal} 0.045 < F_{tab} 3.89) at 5% level of significance (Figure 2).

Table 4: Effect of Yeast Extract concentration on Production of *Rhizobium* Biofertilizer

Media name	**Yeast extract concentration (g/L)**	**Rhizobial growth (O.D. at $A_{540\ nm}$)**
YE1	1	0.41
YE2	2	0.56
YE3	3	0.69
YE4	4	0.78
YE5	5	0.97

F_{cal} = 0.045< F_{tab} (5%) = 3.89 Note: - YE= Yeast Extract

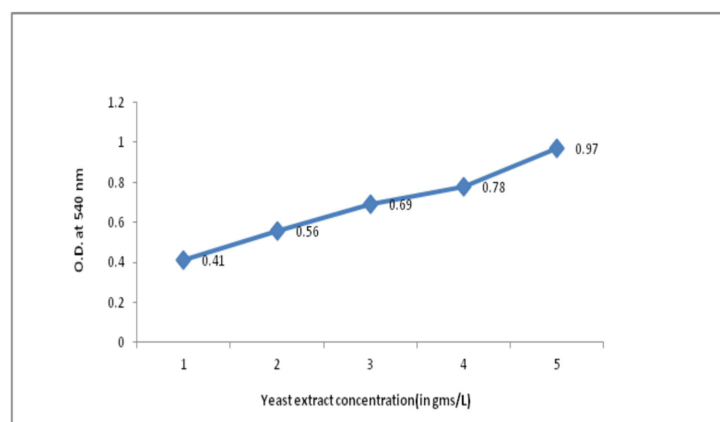

Figure 2: Effect of Yeast Extract concentration on Production of *Rhizobium* Biofertilizer

The growth of *Rhizobium* biofertilizer were studied on the basis of different salt concentration (0.1 g/L) keeping other constituents of the YEM broth same. It was observed in the present study that the maximum growth of the the rhizobia

found to be maximal in S5 (KI) at 29.4°C for 7 days. To date, some rhizobial isolates have been shown to grow under high salt conditions (4-5%) **(Kucuk *et al.,* 2006)**. In other experiment indicated that cells were able to grow on 1% NaCl containing medium but were unable to grow on higher concentrations, thus showing that the isolate was sensitive to the salt **(Singh *et al.,* 2008)**. The effect of salt concentration was found to be statistically insignificant (F_{cal} 0.111 < F_{tab} 3.89) at 5% level of significance (Figure 3).

Table 5: Effect of Salt concentration on Production of *Rhizobium* Biofertilizer

Media name	Salt concentration (0.1 g/L)	Rhizobial growth (O.D. at $A_{540\ nm}$)
S1	Halite (Kala namak)	0.39
S2	$NaNO_3$ (Sodium nitrate)	0.40
S3	Epsom (Sendha namak)	0.41
S4	NaCl (Sodium chloride)	0.58
S5	KI (Potassium iodide)	**0.69**

F_{cal} = 0.111< F_{tab} (5%) = 3.89 Note: - S= Salt Concentration

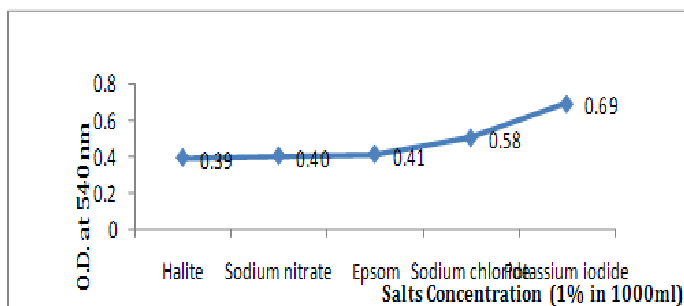

Figure 3: Effect of Salt concentration on Production of *Rhizobium* Biofertilizer

Mass Production of *Rhizobium* Biofertilizer

Mass production media was established for higher activity of *Rhizobium* biofertilizer instead of normal YEM media for storage in multani mitti and coal ash. Large-scale production of *Rhizobium meliloti* on whey showed good growth **(Bissonnette *et al.,* 1986)**. Purity of production media was checked after 7 days of incubation at 29.4°C. Purity of the production media was checked by pH, Gram stain, optical density and viable counts. pH of the medium showed acid production i.e., 7.0 during inoculation and after incubation period it was 6.0-6.4. The pH of the medium and broth during growth of isolates was changed from 7.0 to 6.0, thus showing the production of acid which is the characteristic of *Rhizobium* to produce acid during growth **(DeVries *et al.,* 1980; Baoling *et al.,* 2007)**. Rhizobia showed growth in 3 days and turned the yeast mannitol agar media containing bromothymol blue to yellow colour showing that all were fast growers and acid producer **(Shah *et al.,* 1995)**. Purity of mass production media were checked by Gram staining identified as Gram negative. Optical densities (O.D) were 0.69 to 1.69 and viable counts were count as 3.4×10^5. Plate count methods are sufficiently reliable for assessing high quality inoculants with higher than 10^8 rhizobia per gram of carrier **(Date, 1962; Vincent, 1970)**. The minimum numbers of rhizobia required to guarantee good nodulation are considered to be 2 x 10^7 cells/g inoculant, or 300 cells per seed. However, numbers as high as 10^4-10^6 rhizobia per seed are preferred, especially when there is substantial competition with native strains **(Centro Internacional de Agricultra Tropical, 1988)**. The standard required of the sterilized peat products in Australia, Canada, and New Zeland is 109 rhizobia per gram, with less than 0.1% contamination **(Thompson, 1980; Roughley, 1982)**. Nonsterile carriers are used almost exclusively in the United States, with the best quality inoculants containing rhizobial numbers of 10^8-10^9/g. The absence of quality control regulations allows some very poor quality products to be sold **(Hiltbold *et aI*., 1980)**.

Test For Purity of Carrier Materials (Multani mitti and Coal ash)

Carrier selected for storage of *Rhizobium* biofertilizer were multani mitti and coal ash. Both carriers are environment friendly in nature and good replacement of synthetic fertilizer. Purity of carrier materials was checked by viable number, pH and moisture content. 50% moisture level was good for curing process. Curing was done for multiplication of *Rhizobium.* Several research workers used different materials like garden soil **(Madhok, 1934)**, peat **(Iswaran *et al.*, 1969)**, cellulose powder **(Pugashetti *et al.*, 1971)**, coir rice husk and sand **(Khatri *et al.*, 1973)**, peat and bagasse **(Graham *et al.*, 1974)**, coal **(Dube *et al.*, 1975)**, sugarcane pressmud and coffee waste **(Kumar *et al.*, 1982)**. Many species of *Rhizobium* can be easily inoculated in some carrier material and they are known as better carrier materials like, survival of *Rhizobium phaseoli* TAL 241 and cowpea *Rhizobium* TAL 309, in three different carrier materials namely peat soil, lignite and soil compost mixture **(Jayaprakash, 1980)**. Charcoal and vermiculite as carrier for the preparation of inoculants **(Sparrow and Ham 1983)**.

Shelf Life of *Rhizobium* Biofertilizer

Survival was best for *Rhizobium* in both carriers at 4°C. The shelf life of both carriers based inoculants were successfully checked for 2 months and growth were observed in 10^{-9} rhizobia per gram. The choice of a peat acceptable for inoculant production can be made only on actual tests of its suitability for growth and survival of rhizobia **(Roughley and Vincent, 1967)**. Rhizobia can grow in peat without nutrient supplements, as evident from the multiplication and survival when added as diluted cultures **(Somasegaran, 1985; Somasegaran and Halliday, 1982)**.

Thus, rhizobial growth was observed for 2 months in both carrier materials and this can be stored up to 6 months at 4°C cold condition. During checking of shelf life no contaminants were observed. Chances of contaminants were denied after observed single type of colonies on YEMA plates. Contaminants were also checked on GPA (Glucose-Peptone Agar) and NA (Nutrient Agar) plates. Shelf life was checked regularly and observed 10^{-9} cells per gram and 10^{-7} cells per gram at the time of expiry.

Comparison between Both Carriers (Multani mitti and Coal ash)

Numbers of colonies were observed during checking of shelf life in both of the carrier shows decreasing number of colonies after continuous 15 days of intervals. The shelf life was found to be statistically significant (F_{cal} 5.14 > F_{tab} 3.89), (4.66 > F_{tab} 3.89), (5.99 > F_{tab} 3.89) at 5% level of significance **(Table 6)**.

Table 6: Number of colonies obtained during shelf life of both carriers

Carrier name	Dilution	1st 15days	2nd 15days	3rd 15days	4th 15days
Multani mitti	10^{-7}	65	58	42	37
	10^{-8}	45	39	35	24
	10^{-9}	23	22	20	16
Coal ash	10^{-7}	78	72	67	56
	10^{-8}	63	59	48	37
	10^{-9}	38	33	32	24

F_{cal} = 5.14 > F_{tab} (5%) = 3.89, 4.76 > F_{tab} (5%) = 3.89, 5.99 >F_{tab} (5%) = 3.89,

Colonies number observed in both carrier materials and multani mitti was less effective than coal ash. Survival of rhizobia indicated both the carrier were better for seed inoculation. The populations of Bradyrhizobium japonicum were larger in perlite carriers containing sugar cane bagasse and malt residue and were capable of maintaining an acceptable *B. japonicum* population for at least 180 days which was most likely due to higher nutrient contents of those two carriers (**Khavazi and Rejali, 2002**).

Conclusion

In the present study, biofertilizers produced by *Rhizobium* was found to be potent since it could be active at a high concentration of sucrose and yeast extract but shows good results in KI compared to other salts. Therefore it was found that biofertilizer produced on lab scale might be beneficial tool for agriculture and environment. In the future, emphasis can be placed on the proper and economical utilization of abundantly present *Rhizobium*. Deep knowledge of molecular and cloning in suitable expression vectors can be second major target for improving the production of biofertilizers. Much progress is needed to coin a new word "Bioferticides". It enhances the mechanism of biofertilizers and biopesticides. A bioferticides will act as a fertilizer as well as a pesticide. Therefore, this concept will lead towards a new area of Industrial Microbiology and Genetic Engineering.

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