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Isolation identification and characterization of bacteria (Rhizobium) from chick pea (Cicer arietinum) and production of biofertilizer

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Abstract
The main purpose of this research paper is to isolate bacteria (Rhizobium) as a nitrogen fixing bacteria from root nodules of chick pea (Cicer arietinum) and its identification, characterization and hence the production of biofertilizer from it after growing it in the selective media and finally mass producing the bacteria and mixing it with carrier materials for further utilization in the soils to increase its nutrient quality naturally after combining it with the soil. Nitrogen is one of the important component which acts as a building blocks of most biomolecules but this inert nitrogen cannot be utilized by plants so the Rhizobium bacteria helps to fix the atmospheric nitrogen to ammonia which can be utilized by plants so the production of biofertilizer from this bacterial strains are the main focus in this paper.

Keywords: Rhizobium, Root nodules, Chick pea, Biofertilizer, Rhizoid soil, Carrier material, Methyl red test, Hydrogen sulphide test.

1. Introduction

Nitrogen the essential component which serves as the building blocks of proteins and nucleic acids is abundant in the earth's atmosphere but it cannot be utilized by plants because of the inert nature of the nature due to the presence of triple bonds between the nitrogen atoms. So for the nitrogen to be used by plants it must be fixed or converted to the form of ammonium or nitrite ions. There are certain microorganisms which are capable of converting the atmospheric nitrogen into ammonia or nitrite ions by a process known as nitrogen fixation. These nitrogen fixation can be done by certain soil microbes both symbiotically and as well as non-symbiotically. So among the symbiotic nitrogen fixing bacteria Rhizobium has been found to be having a greatest activity in fixing these atmospheric nitrogen for plants and also increasing the soil fertility. In this way the Rhizobium bacterial strains can be utilized for production of biofertilizers.

Biofertilizers are actually the natural mini- fertilizers which are responsible for providing safer plant nutrition and increasing the soil fertility through natural processes. These microorganisms actually can colonize the roots of different plants and the rhizobium can form root nodules in leguminous plants (Md. Zahrul islam *et al,* 2012) [1]. These rhizobial inoculants after forming the root nodules fixes the atmospheric nitrogen to ammonia which can be utilized by plants. Biofertilizers are the preparations of living cells of different strains of microorganisms that helps in enhancing the nutrient quality of soil by their interaction with the rhizosphere roots of plants when they are applied both on top soil and seed treatment. (Isolation of azotobacter and cost effective production of biofertilizer by Gomare *et al.,* 2013) [2]. Rhizobia bacteria that fix N2 (diazotroph) after becoming established inside root nodules of legumes (Fabaceae). The different genera of rhizobia, all of them belong to the Rhizobial, a probably-monophyletic group of proteobacteria and they are soil bacteria characterized by their unique ability to infect root hairs of legumes and induce effective N2-fixing nodules to form on the roots. They are rod shaped living plants which exist only in the vegetative stage. Unlike many other soil microorganisms, rhizobia produce no spores and they are aerobic and motile. (Matiru and Dakora, 2004; Dakora, 1995; Lhuissier *et al.,* 2001) [3]. The Rhizobial inoculants colonizes the roots of specific legumes to form tumor like growths called root nodules, which acts as factories of ammonia production. *Rhizobium* has ability to fix atmospheric nitrogen in symbiotic association with legumes and certain nonlegumes like *Parasponia*. Artificial seed inoculation is often needed to restore the population of effective strains of the *Rhizobium* near the rhizosphere to hasten N-fixation. Each legume requires a specific species of *Rhizobium* to form effective nodules (Mishra D.J *et al,* 2012) [4].

In this research paper the main focus is in the isolation of Bacteria (Rhizobium) from chick pea and then its characterization followed by the identification through different biochemical tests for the identification, its pure culture growth in selective and optimized media and then the mass production of the bacterial strain to be utilized as a biofertilizer.

2. Materials and methods

2.1 Collection of soil samples

Chick –pea plants with roots and rhizosphere soils which were uprooted from the soils were brought to the lab for the isolation of bacteria from it.

2.2 Extraction of Root nodules from the chick pea plants

The roots were first washed thoroughly with sterile distilled water for 10 seconds and small nodules which were obtained got surface sterilized in ethanol of 95% and again washed in

sterile distilled water for few seconds for about 7 times and then by the help of mortar and pestle the roots were smashed to extract more nodules and a milky white substances of bacteroids were obtained with the help of phosphate buffer solution dipping.

2.3 Serial Dilutions of the Extracted root nodules and Rhizosphere soil

After the extraction of bacteroids solutions from the chick pea serial dilution was done. To get the growth of the Rhizobium bacteria about 2gms of serially diluted nodule and root rhizosphere soil were diluted to 10 ml of water in a test-tube which served as stock solution. Remaining 9 test tubes were filled with 9 ml of water. Transferring of 1 ml of water from the stock solution to 9 ml of sterilized distilled water with the help of pipettes yielded 10^{-1} dilutions and the series continued upto 10^{-9} dilutions. Sterility is the hallmark of any bacteriological isolation so the entire process was carried in the Laminar Airflow.

2.4 Colony identification and External morphology study of bacterial strains

In the study of the colony identification of Rhizobium bacteria spread plate technique were used and as the Rhizobium bacteria grows only in selective media of Yeast Extract Congo Red Agar media so about four petridishes and YEMA Congo red agar media of about 100 ml were both autoclaved. After autoclaving the YEMA congo red agar media were poured in the four petriplates and allowed to solidify. Then the serial dilutions of 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} were chosen. About 0.1 ml of both root nodule and rhizosphere soilutions were spread on the four petriplated having yema congo red agar media and spread well by a spreader. The petriplates were then kept in the incubator at 37^{0}celsius for 3 days.

After the plates were taken out of the incubator colony morphology and identification was done.

2.5. Pure culture of isolated bacteria

After the growth of well-developed colonies in the petridishes were observed the petriplates were maintained in cool temperature for the isolation of pure culture. About 50 ml of YEMA CONGO RED Media were prepared and four test tubes were sterilized in autoclave. Then after sterilization the test tubes were poured with YEMACONGO Red media for slant preparation and pure culture isolation. With a help of inoculating needle the opaque translucent colonies were picked up in loopful and streaked in the four test tubes. The test tubes were then placed in incubator for the growth of pure culture at 37 degree Celsius for 2-3 days.

2.6 Gram staining of the bacterial strains for identification

The pure cultures of bacterial strains from the four test tubes were put for gram staining for more specific identification of the colonies. The gram staining was done in Laminar airflow hood. For this purpose the four slides were taken from slide rack. The slides were washed with ethanol. Then each colonies were marked on the slides. Then with the help of inoculating needle the loopful strains were picked from each test tubes and made a smear on the slides and heat fixed. The slides were then taken in the staining room for staining the smears. Then smears were stained in following steps a) First applied crystal violet on each six slides. Kept for 30 secs.b) Distilled water wash.c) Iodine on the slides as mordant (1 min) then 95% alcohol wash and then washed with distilled watrer.d) safranin was applied on the slides and then washed with distilled water and f) air dried the slides. All the gram staining technique was done following the Christian Gram technique and Collee JG, Miles RS Mackie. (1989) [1].

2.7 Preparation of production media of rhizobium bacteria as starter cultures

After the bacterial strains were identified to be as Rhizobium in gram staining techniques these strains were transferred to a liquid broth which may also be called as the production media and as well as the starter culture for the growth of cells. Production media may be defined as that media the number of viable bacterial cells of that particular bacteria increases because that bacteria is grown in that particular media only. Thus for Rhizobial bacterial culture to be made the strains were grown only in YEMA Congo media liquid broth.

Thus 100 ml of one conical flasks were taken and Yema Congo red media was prepared without putting agar in conical flask, then the media was autoclaved and sterilized. After cooling by the help of one inoculating needle a loopful of bacterial culture was transferred from the pure culture to the production media. Then the production media was kept inside the B.O.D Incubator for 1-2 weeks. After 1-2 weeks the viable cell counting was done and it was found that the population of bacterial strains are about 10^{9} CFU/ml. For the mass production of Rhizobium biofertilizer strains were transferred to larger conical flasks.

2.8 Mass production of Rhizobium Bacterial strains for the preparation of Rhizobium Biofertilizer.

The Rhizobium bacterial strains in the starter cultures were neded to be grown in large scale for which their mass production were urgently needed. So for that purpose larger conical flasks of 1000 ml were taken and then again starter cultures were transferred to these larger conical flaks containing the appropriate growth media in aseptic conditions for small scale production and for large scale production again 1 litre of the starter cultures were put into the fermenter. Continuous agitation and proper aeration was needed and it was done for about 1 week. The flasks were checked for time to time for the growth of the cell mass and that they were free of any contamination. After 1 week the cell population was measured and it came upto 109 cells/ml or 10^{9} Cfu/ml load in the larger conical flasks. Then the conical flasks were stored in cool temperatures so that they can be mixed with proper carrier materials. Moreover it is not advisable to keep the conical flasks for long time in storage because of the loss of cell load.

3. Carrier Material preparation for the Rhizobium Biofertilizer strains

Carrier materials are those materials which are used to encapsulate the biofertilizer bacterial strains so that the activity of the strains are not deactivated in the soil by maintaining the pH. The carrier materials have the following properties a) Must have high organic matter content b) Low soluble salts less than 1% c) High moisture content capacity.

Activated charcoal, cow compost and vermicompost was used as carrier material. There are many steps for preparation of the carrier material. The steps are discussed below-

A) Dried cow dung and black coal was brought from different areas. With mortar and pestle the entire coal was crushed to dried powdered form. After crushing also the remaining

pieces were further powdered by the help of mixer and grinder. The dust form of coal as charcoal was made and to it 1% calcium carbonate and wooden charcoal or activated charcoal was mixed and neutralized so that no contaminants are present

B) Similarly the cow dung was also crushed and powered with the help of mixer and grinder.

C) Some amount of vermicompost was also added as a carrier material.

3.1 Preparation of Inoculate (Mixing)

Large scale mass produced bacterial cell cultures of Rhizobium spp were taken out of storage and then the cell cultures were mixed with the carrier materials which were sterilized in individual beakers. The carrier materials and the production media were mixed in the ratio 2:1 where 1 part of production media was mixed with 2 parts of carrier material or in other words 30:60 ratio of both. It was done manually and under aseptic conditions. The cell count of that carrier mixed culture was found to be 10^8 CFU/gm. The Rhizobium biofertilizers were packeted in polythene bags which are advised to be of 250 gm. Then the packets were left in room temperature for curing.

4. Biofertilizer packeting and storage

The polythene packets containing Rhizobium bacterial biofertilizers mixed with carrier materials were stored in cool place away from direct sunlight. The biofertilizer packets were than taken to the hilly regions for applications inn different fields.

5. Reagents and media used

During the washing of roots to obtain the root nodules phosphate buffer was used which had Disodium Phosphate- 300.00 mM, Monopotassium Phosphate- 300.00mM. Yeast Extract Mannitol Agar media was used for the growth of Rhizobial bacteria which had its composition as Mannitol- 10gm/ml Dipotassium hydrogen phosphate- 0.5 gm/ml Magnesium sulphate- 0.2gm/ml Sodium chloride- 0.1gm/ml Yeast Extract- o.5gm/ml Distilled water- 1000ml Dissolved 250 mg of congo red in 100 ml water Agar-2gm. Yema Congo Broth- Mannitol- 10gm/ml Dipotassium hydrogen phosphate- 0.5 gm/ml Magnesium sulphate- 0.2gm/ml Sodium chloride- 0.1gm/ml Yeast Extract- o.5gm/ml Distilled water- 1000ml Dissolved 250 mg of congo red in 100 ml water.

Different Biochemical tests were done which included Methyl Red test, Voges Proskauer test, H_2S, Catalase test, and Nitrate Reduction test. In the gram staining techniques crystal violet, gram iodine and safranin were used. Other reagents included kovac reagent, alpha naphthol, Hydrogen sulphide, Gram iodine.

6. Results and discussions

In this Research paper Figure 1 shows the root nodules from the chick pea plants and Fifure2 shows the extraction of root nodules in the Phosphate buffer medium. Figure 3 shows the growth of Rhizobium bacteria on Congo red Yeast Extract Mannitol agar medium. Figure 4 is the bacterial strain observed under the microscope by gram staining and Figure 5 shows the Catalase test of the Rhizobium bacterial strain and Figure 6 is the production media for different bacterial strains for obtaining the desired colony count for making biofertilizers. Table 1 shows the external morphology study of the bacterial strains, Table 2 shows the gram identification and Table 3 shows the biochemical tests of the bacterial strains.

These Rhizobial biofertilizers are very important for mating the soil fertility when applied to different soils. They are finally packeted to different hilly regions for application in soils.

Table 1: External Morphology study of Rhizobial bacterial Colonies

Plates	Colony	Colour	Shape
10^{-2}	1	White, Translucent	Circular
10^{-4}	2	White ,Translucent	Granular
10^{-6}	3	White Translucent	Granular
10^{-8}	4	White, Translucent, Gummy	Granular

Table 2: Gram Staining Identification of Bacterial strains from Pure Culture slants.

Sl. No	Colour	Shape	Name
Strain1	Pink	Oval	Rhizobium spp
Strain 2	Pink	Oval	Rhizobium spp
Strain 3	Pink	Oval	Rhizobium spp
Strain 4	Pink	Oval	Rhizobium spp

Table 3: Biochemical Tests of the Rhizobium spp strains.

Tests	Rhizobium spp
Methyl Red	Positive(Strain1 ,2,3,4)
Vp test	Negative(Stain 1,2,3,4)
Catalase test	Positive(Strain1,2,3,4)
Starch Hydrolysis	Negative(Strain1, 2, 3, 4)
Hydrogen sulphide	Negative (Strain1, 2, 3, 4).
Triple sugar (Glucose, Sucrose, Maltose)	Positive (Strain1, 2, 3, 4).

Fig1: Root nodules of Chick pea

Fig 2: Root nodules extracted in buffer solution.

Fig 3: Growth of Rhizobium in Congored Yema Medium.

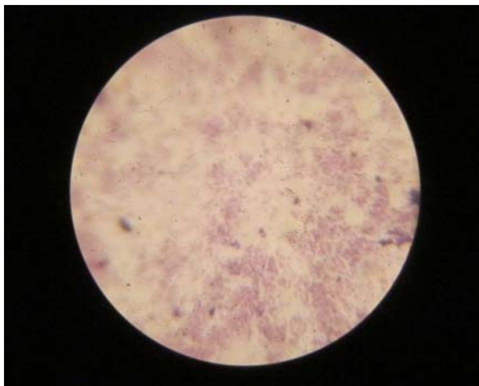

Fig 4: Gram staining of Rhizobium bacterial strain.

Fig 5: Catalase test for Rhizobium strain.

Fig 6: Production media of different bacterial strains for making biofertilizers.

6. Conflict of interest

No conflict of Interest

7. Acknowledgement

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