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ISOLATION AND CHARACTERIZATION OF *RHIZOBIUM* FROM ROOTS OF FENUGREEK AND ITS USE AS A BIOFERTILIZER

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Abstract: Fenugreek *(Trigonella foenum-graecum)* is an ancient and very well-known medicinal plant used in the preparation of traditional medicine in various parts of the world. India is one of the biggest cultivators of Fenugreek. Fenugreek is famous for its medicinal properties, the protein source in diet, *Rhizobium* associated symbiotic nitrogen fixation in root nodules. The present study focused on the isolation and characterization of a *Rhizobium* strain from fenugreek root nodules and its application as a biofertilizer. The characterized *Rhizobium* isolates are gram-negative, rod-shaped, mucous, and acid-producing. The isolates are found to be pH and temperature sensitive. As a sole carbon source, it usually utilizes sucrose, glucose, and starch. The *Rhizobium* species which are isolated from fenugreek roots are having the ability to synthesize industrially important enzymes; cellulose and amylase. Amylase is among the most important enzymes and is of great significance in present-day biotechnology.

Index Terms - **Fenugreek, *Rhizobium*, Biofertilizers, Enzyme production, Root nodules.**

I. INTRODUCTION

The word rhizobia come from the Ancient Greek ‘’rhiza ‘’, meaning ‘’root ‘’ and ‘’Bios ‘‘meaning life. The bacteria from the *Rhizobium* genus are reported so far as a physiologically heterogeneous and genetically diverse group of microorganisms. These bacteria are classified based on their ability to produce root nodules. They fall under nodulate group of plants from the family Leguminosae. Rhizobia are characteristically found to be gram-negative and hence cannot form Endospore [1]. Rhizobium species fall into two groups based on their characteristics of growth:

There is a new system that has been developed to classify rhizobia. This system helps to recognize three rhizobia genera. This genus-I and II contains Rhizobia whereas genus-III includes agrobacteria. Genus-I includes acid-producing and fast-growing Rhizobia, and Genus-II includes all alkali-producing and slow-growing Rhizobia (*Bradyrhizobium*) [2].

As compared to chemoorganotrophs which are predominantly aerobic, Rhizobia are easy to culture. They can grow in the presence of oxygen and utilizes amino compounds and simple carbohydrate. Optimum growth of most of the strains was observed at a temperature ranging from 25-30 ℃ and pH at 6-7. Rhizobia are among the unique soil microorganisms, which are having the ability to fix nitrogen-forming symbioses with legumes [3]. Fenugreek *(Trigonella foenum-graecum.)* is commonly known as “methi”. It is an annual crop. As the fenugreek crop is leguminous hence; its root nodules help in enriching the soil with the help of atmospheric nitrogen. In the field of agriculture, there is a huge increase in the use of fertilizers to improve crop yield and productivity. Excess use of fertilizers hurts the environment. The present study aims to investigate the isolation and characterization of *Rhizobium spp*. from roots of fenugreek and its use as a biofertilizer. Colonies of *Rhizobium* were obtained by growing on a YEM agar medium after the incubation at 29 ℃ for two days [4]. Colonies had a sticky appearance. The pH of media and broth during the growth of isolate was changed from pH 6 to 7 showing acid production which is a characteristic of *Rhizobium*. Testing involves GPA test, starch hydrolysis TSI test [5].

Pot culture was performed to see the effect of isolated rhizobia as inoculants on plants. The seeds used of fenugreek. Results showed that plants were healthy as compared to plants without-bio fertilizer [6].

II. Material and Methods

Sampling and Processing

Fresh and plump roots of fenugreek were collected from the Kamargaon village of Ahmednagar district, Maharashtra, India. The collected fenugreek root nodules were surface-sterilized using 0.1% mercuric chloride and 75% ethanol and then washed thoroughly using distilled water. The root nodules were then used for further studies.

Isolation of Rhizobium from Fenugreek Roots

Rhizobium is a nitrogen fixer and is found in the nodules of the roots of fenugreek. Thus, these nodules are used for the isolation of rhizobium.

To obtain *Rhizobium* strain the crushed root nodules suspension was streaked on YEM agar plates and incubated at 30°C for two days. After incubation, bacterial colonies were obtained. Further spreading, streaking, and characterization of colony morphology was done and pure colonies of *Rhizobium* were isolated. Pure culture of bacterial colonies was used for further analysis and isolated colonies were preserved on slants for further use [7][8].

YEM + Congo red test

This test was performed to distinguish between agrobacterium and rhizobium. agrobacterium absorbs Congo red strongly whereas rhizobium absorbs little producing pink colonies. YEM containing 0.1% Congo red was prepared and plates were streaked with rhizobium isolate [9].

YEM + Bromothymol Blue Test

This test was performed to see if the organism shows acid production. YEM containing 0.1% bromothymol blue was prepared. Plates were streaked with rhizobium isolate culture. After 48 hours color of the colony was observed [10].

NaCl Effect on the Growth of *Rhizobium* Strain -

YEM containing different concentrations of NaCl ranging from 1% to 6% was prepared. Plates were streaked with rhizobium isolate. Then observed for growth [11].

Temperature Effect on The Growth of *Rhizobium* Strain –

YEM agar was prepared and poured into three plates, streaked with isolate and each plate was kept at a different temperature. after 48 hours of incubation, growth was observed [12].

pH Effect on the Growth of *Rhizobium* Strain

To analyze the effect of pH variations on the growth of organisms, media were prepared with pH 4, pH7, and pH9. Plates were streaked with *rhizobium* and growth was observed after 48 hours of incubation [12].

Glucose Peptone Agar Test

GPA test was done to determine the capability of organisms to utilize glucose as the carbon source for its growth. GPA Medium (glucose 4g, peptone0.5g, agar 1.5g, pH0.7g, distilled water 100ml) with *rhizobium* isolate, and after incubation growth was observed [13].

Gelatine Hydrolysis

The test was done to determine the gelatinase enzyme-producing ability of the isolated organism. The degradation of gelatine indicates the presence of gelatinase. *Rhizobium* isolate was inoculated in nutrient gelatine medium (peptone 0.52g, beef extract 0.32g, gelatin1.2g, distilled water 100ml) and growth for 48 hours. The gelatinase enzyme remains liquefied by subjecting the growing culture of an organism to low temperature (4 ℃ for 30 mins), while others become solid [14].

Starch Hydrolysis Test

A test was done to check the ability to isolate to utilize starch as a carbon source. Starch agar media (potato starch 0.2g, peptone 0.5g, beef extract 0.3g, agar 1.5g, pH 7, distilled water 100ml) was prepared and spot inoculated with *Rhizobium* culture. The plate was incubated for 48 hours. Iodine was added to the plate. The formation of blue color indicated the utilization of starch. peptone 0.5g, potato starch 0.2g, beef extract 0.3g, agar 1.5g, pH 7, distilled water 100ml.

Triple Sugar Iron Test

The test was performed to check the capability of isolates to utilize various carbohydrates. The different carbohydrates used were sucrose, glucose, and lactose.

Triple sugar iron medium (beef extract 0.3g, yeast extract 0.3g, peptone 1.5g, NaCl 0.55g, lactose 1g, sucrose 1g, dextrose 0.1g, ferrous sulphate0.02g, sodium thiosulphate 0.03g, phenol red 0.02g, agar 1.5g, pH7, distilled water 100ml) was prepared and *rhizobium* culture was inoculated. After incubation for 48hours color of the butt and the slant was observed [15].

Cellulose Hydrolysis Test

Inoculate the isolate in cellulose agar and incubate at 29℃ for 48 hours. After incubation observes the zone of clearance.

Biofertilizer Preparation

Farmyard manure was taken and dried and the dried manure was powdered. The fine manure powdered was autoclaved. After autoclaving, 48 hours old culture was mixed with the powder aseptically. The mixture was packed into a sterile plastic bag aseptically. The plastic bag was kept for 10 – 20 days. The moisture contained was kept at 34 – 40 %. After 10 – 20 days the mixture is ready for use as a biofertilizer [7].

Pot culture technique

The pot culture technique was performed to see the effect of isolated Rhizobia as inoculants on plants. The seeds used of fenugreek.

The pots were filled with soil and the pots were packed with plastic. These pots were autoclaved so that the pots, as well as soil, were autoclaved.

In the first pot, the upper layer of the soil was mixed with the previously prepared biofertilizer. The seeds were sown, watered, and kept in a clean place where there was sufficient sunlight.

In the second pot, the seeds were mixed with the broth and then sown in the soil. The pot was watered and kept in a clean place where there was sufficient sunlight.

In the third pot, first, the seeds were coated with molten jaggery, then mixed with the biofertilizer, and then sown. The pot was watered and kept in a clean place where there was sufficient sunlight.

The fourth pot was kept in control. No fertilizer was applied only seeds were grown. In all pots, 15 seeds were sown.

Plants were allowed to grow and observe for nodule formation on roots and the appearance of the plant [16].

III. RESULT AND DISCUSSION

Rhizobium colonies were obtained by growing on a YEM agar medium and incubating the plates at 30 ℃ for two days. The colonies observed are having sticky appearance. Analysis of colony morphology indicated round colonies, while color turned creams after 4 days. The pH of media and broth during the growth of isolates was changed from pH 7 to pH 6 showing acid production which is characteristic of Rhizobium. General microscopic showed the isolate to be gram-negative and rod-shaped.

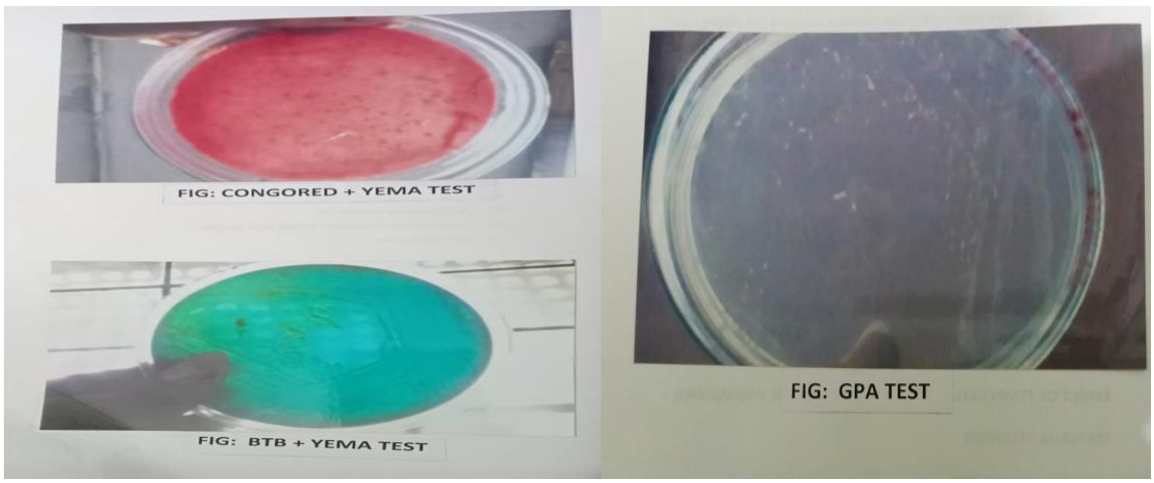

Figure 1. Congo red + YEMA Test and GPA Test

The isolate was above to grow on 1% NaCl concentration showing that the isolate was sensitive to higher salt concentration. For the growth of an organism pH of the media is an important parameter. Rhizobium isolate was observed to grow very well at pH 7 i.e., neutral pH

Rhizobium isolate was able to grow on GPA. *Rhizobium* isolate did not show gelatinase activity. *Rhizobium* isolate was able to hydrolyze starch which means it produces amylase also observed that *Rhizobium* strains utilize starch, *Rhizobium* isolate showed positive results for triple iron sugar test yellow slant and red butt was obtained.

Table 1. Morphological and Biochemical Characterization of Test Organism

Tests	New students
Colony Morphology	
Size	3-4 mm
Shape	circular
Colour	creamish
Pigmentation	No Pigment
Margin	regular
Elevation	Convex
Opacity	Opaque
Characterization	
YEM + CONGO RED	Pink-colored colonies were observed
YEM + BTB	Yellow-colored colonies were observed.
EFFECT OF NACL	The isolate was able to grow on 1 % NaCl concentration
EFFECT OF TEMPERATURE	The isolate was able to grow at 29℃
EFFECT OF pH	The isolate was able to grow at pH 7
GPA TEST	The isolate was able to grow on the GPA plate.
GELATIN HYDROLYSIS	The isolate was not able to hydrolyze gelatin
STARCH HYDROLYSIS	The isolate was able to hydrolyze starch.
TRIPLE SUGAR IRON TEST	Red butt and yellow slant were observed
CELLULOSE HYDROLYSIS	A zone of clearance was observed.
POT CULTURE TECHNIQUE	All the plants with biofertilizer were healthy and nodule formation was observed on roots compared to the control.

***YEMA- Yeast Extract Mannitol Agar, BTB- Bromothymol Blue, GPA- Glucose Peptone Agar**

Rhizobium isolate was able to produce light pink colored colonies on YEM containing conge red, showing only partial uptake of the dye. Yellow-colored colonies were seen on YEM containing bromothymol blue showing the production of acid by the organisms.

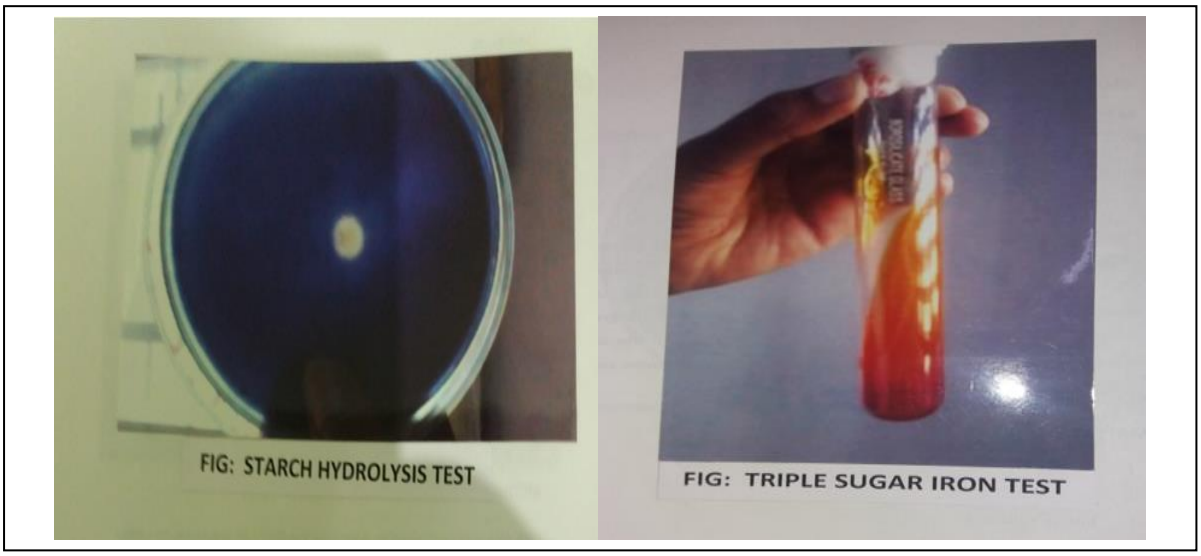

Figure 2. Starch Hydrolysis Test and Triple Sugar Iron Test

The *Rhizobium* was able to produce industrially important enzymes Cellulase and Amylase. Amylase is one of the most important enzymes having great significance in present-day biotechnology.

Figure 3. Pot Culture Methods to Test Biofertilizer Efficiency

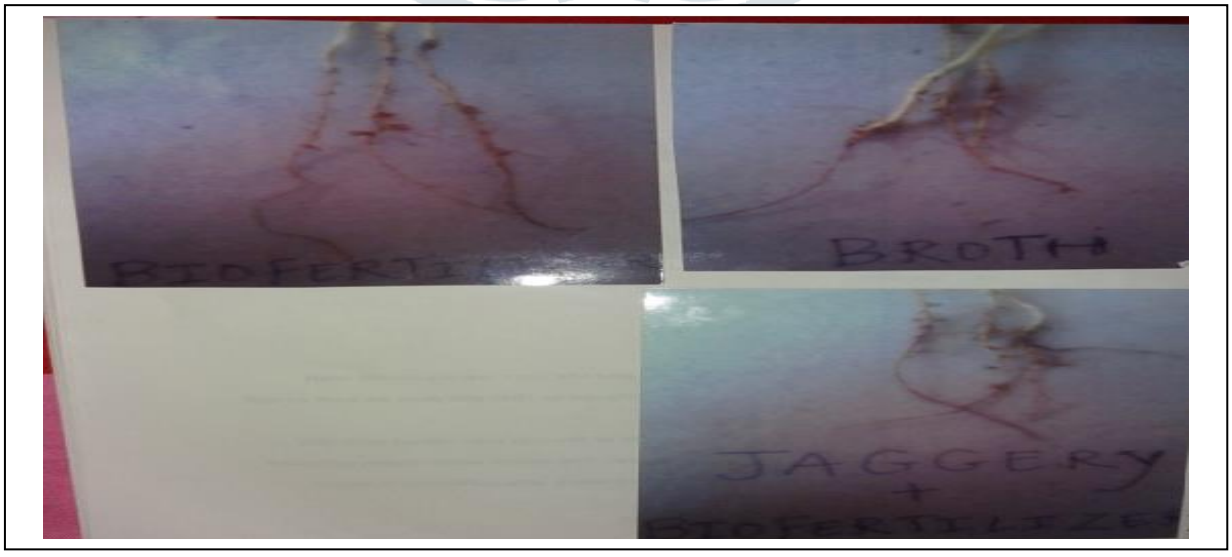

Figure 4 Nodule Formation on the Root of Fenugreek

Nodules were observed on the roots of the plants which were inoculated by the culture of the isolate as a biofertilizer. Moreover, the plants were healthy as compared to a plant without biofertilizers.

Thus, the *Rhizobium* isolate biofertilizer is effective and it helps the increasing crop yield by nitrogen fixation and also enhances the quality of the crop. Thus, the isolate can be used as an effective biofertilizer.

IV. Conclusion

As synthetic fertilizers have an adverse effect and a bad impact on environmental pollution, biofertilizer is a great and eco-friendly alternative to overcome the pollution. There are many plants and alternatives for the preparation of biofertilizers. Microorganisms have a great role in increasing the fertility of soil and plant yield. Microorganisms secrets many extracellular enzymes which are helpful for the growth of crops and plants. In the present study *Rhizobium* spp. isolates were obtained from fenugreeks root nodules for the preparation of biofertilizers. *Rhizobium* was found to be a good biofertilizer-producing microorganism. Hence the study was carried out on *Rhizobium*.

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