INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 18, No. 4, p. 22-28, 2021

RESEARCH PAPER OPEN ACCESS

Isolation, identification and characterization of *Rhizobium* from lentil root nodule

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Key words: *Rhizobium*, Root nodules, Red gram, nitrogen fixation, Biofertilizer.

http://dx.doi.org/10.12692/ijb/18.4.22-28 Article published on April 29, 2021

Abstract

The use of some microorganisms are capable of fixing atmospheric nitrogen can reduce chemical (nitrogen) contamination in the soil. The main purpose of this research paper is to isolate *Rhizobium* as a nitrogen fixing bacteria from root nodules of lentil (*Lens culinaris*) and its identification, characterization and hence the production of biofertilizer. In culture initiation ssurvival rates were higher using 2-3% chlorox with 70% ethanol as a disinfection medium and the contamination rate was also lower. The primary inoculation of *Rhizobium* was effected by cutting and smashed nodules at 30-40 days after sowing of lentil plant. Gram staining, biological microscope and scanned electron microscope observations showed that the bacteria were gram negative, rod shaped, the length to width ratio is 3:1 and even smooth edges. *Rhizobium* was also positive in all the biochemical tests. *In vitro* culture initiation, microscopic reviews and biochemical testing for specific microbial isolates are essential for microbial fertilizer production.

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Introduction

The root nodules of leguminous plants are born by nitrogen-fixing bacteria associate with the genus *Rhizobium*. Nodules are found at the base of the roots of the legume plants and produce symbiosis with nitrogen-fixing bacteria. Under nitrogen-limiting conditions, capable plants form a symbiotic relationship with a host-specific strain of bacteria known as rhizobia. Nodules are mainly developed on the lateral root and sometimes the main root. Nodules first become visible on capable plants in the upper portions of the primary root under the hypocotyls about two weeks after seedlings emerge. On woody plants they may persist for four or five years and look like a string of beads (Somasegaran and Hoben, 1994).

Rhizobium is a genus of Gram-negative soil bacteria that fix nitrogen. *Rhizobium* species form an endosymbiotic nitrogen-fixing association with roots of legumes and *Parasponia* (Wikipedia). The bacteria colonize plant cells within root nodules, where they convert atmospheric nitrogen into ammonia using the enzyme nitrogenase and then provide organic nitrogenous compounds such as glutamine or ureides to the plant and the plant in turn, provide the bacteria with organic compounds made by photosynthesis, Sawada, Kuykendall, Young (2003). This mutually beneficial relationship is true of all of the rhizobia, of which the genus *Rhizobium* is a typical example.

Rhizobium microbes usually reside in the nodules of legume plants and provide nitrogen nutrients to the plant. Inoculation of these biofertilizer with seeds usually speeds up the formation of nodules and does not require additional application of nitrogen fertilizer (Wagner 2011). Therefore, these microorganisms are used as an alternative to inorganic nitrogen fertilizers. The organism can be grown by artificially culturing in a sterile and controlled environment. This requires sterilization from nodules and inoculation through artificial nutrition in accordance with certain rules. Which is important for the subsequent increase in the number of microorganisms. *Rhizobium* is very important bacteria in agriculture. Isolation of *Rhizobium* is very important for its use in sustainable agriculture. Isolation of *Rhizobium* is important to observe the morphological and biochemical characteristics of the isolates. Species of *Rhizobium* are also specified in different species so isolation and identification is required to know the isolates species. By isolating the bacteria, it can be stored for further laboratory and field experiments. Nitrogen is often a rare nutrient in the soil. That is why it is used more than any other fertilizer in agriculture. Excessive use of synthetic fertilizers can lead to serious environmental problems and so much research is being done on *Rhizobium* bacteria. Researchers hope to find ways to increase plant productivity and reduce reliance on artificial fertilizers.

The objectives of this experiment were isolation of *Rhizobium* strain from lentil (*Lens culinaris*) root nodule, identification and characterization of isolated strain and optimization of culture conditions.

Materials and methods

Isolation of Rhizobium

Collection of root nodules

Lentil plants were collected from the experiment field of the department of Agronomy and Agricultural Extension, Rajshahi University, Rajshahi. 45 days old lentil plants were carefully uprooted with the help of grubber for the isolation of bacteria (*Rhizobium*). The roots were first washed thoroughly under running tap water to remove soil particles and soaked into blotting paper.

Surface sterilization of root nodules

For experiment healthy unbroken brown nodules were selected. Guidelines was followed for collecting nodules and preserving according to Date and Halliday (1987) and Somasegaran and Hoben (1994). The nodules were immersed in sterilizing agent Sodium hypochlorite (1%, 2%, 3% and 4%) for 2 minutes and then washed repeatedly with sterile distilled water. After that sterilized in ethanol of 70% and again washed in sterile distilled water for 2 minutes for about 7 times to remove all sterilants. The

nodules were cultured by three types; (i) whole nodule (ii) small piece of nodule (iii) Smashed nodule (crushed each nodule with a sterile glass rod in a test tube and added sterile water). The sterilized nodules were cultured in YEMA plate and incubated at 25˚C for 2-3 days and checked for possible microbial growth.

Preparation of culture media

Yeast Extract Mannitol (Vincent, 1970) Agar plates were prepared and All culture media were sterilized at 121˚C for 20 minutes by autoclaving. Prepared nodules were cultured in YEMA plates. The samples were inoculated throughout the YEMA plates and were incubated for 2-3 days at 25ºC. Then isolated *Rhizobium* was sub cultured in YEMA plates for further experiments.

Identification and External morphology study of isolated bacterial strains by Gram staining

The pure cultures of isolated bacterial strains were taken for gram staining for more specific identification of the colonies. The gram staining was done in laminar airflow hood. For this purpose the six slides were taken from slide rack. The slides were washed with ethanol. Then each colony was marked on the slides. Then with the help of inoculating needle the loopful strains were picked from each test tube and made a smear on the slides and heated to fix. Then the slides were taken in the staining room for staining the smears. Then smears were stained in following steps (i) First applied crystal violet on each six slides and has kept for 30 second. (ii) Washed using sterile distilled water. (iii) Put Iodine on the slides as mordant (1 min) then 95% alcohol washed and then washed with sterile distilled watrer. (iv) Safranin was applied on the slides and then washed with distilled water. (v) Finally air dried the slides. The entire gram staining technique was done following the Christian Gram technique and Collee, Mackie (1989).

Biochemical characteristics of isolated bacteria

Catalase test

This test was performed to study the presence of enzyme Catalase which hydrolyzes H_2O_2 into H_2O and O_2 in bacterial strain. Rhizobial colony (2-3 days old) were taken on glass slide and one drop of H_2O_2 (30%) was added. Appearance of gas bubble indicated Catalase enzyme presence.

Lipase test

Lipase presence around bacterial colonies was detected by supplementing YEM with 1% (w/v) Tween 80.

Bromothymol blue test

It selectively identifies fast and slow growing isolate of Rhizobium. In this test sample were allowed to grow YEMA media contains BTB. After incubation for 48 hours at 25˚C positive sample showed yellow color due to acid production.

Starch hydrolysis

This test was performed to determine the capability of *Rhizobium* to use starch as a carbon source19.Starch Agar Medium was inoculated with *Rhizobium* and analyzed for starch utilization. Iodine Test was used to determine the capability of microbes to use starch. A drop of iodine (0.1N) was spread on 24 hour old culture and clear zone of inhibition were formed.

Oxidase test

Oxidase test was performed to determine the presence of oxidase enzyme in different isolates of *Rhizobium* spp. Kovac's reagent (1% N, N, N.N-tetramethyl- p-phenylenediamine) was dissolved in warm water and stored in dark bottle. A strip of filter paper was dipped in this reagent and air-dried and put into one-day-old Rhizobial colonies from agar plates.

Citrate utilization test

Citrate utilization as a carbon source was examined by adding sodium citrate and Bromothymol blue (25 mg/L) instead of mannitol in YEMA medium. Isolates of *Rhizobium* spp. were streaked in sodium citrate added YEMA medium plates with bromothymol blue as an indicator. Then plates were incubated for 24 - 48 hours.

Results and discussions

Isolation of rhizobium

Influence of surface sterilization method on growth and contamination of isolated bacteria

To entirely reach authentic bacteria (endophytic) from inner root tissues of *Lens culinaris*, epiphytic microorganism and other contamination must be dispelled, through surface sterilization method. For this root samples were treated by the different combination of a chemical disinfectant. Sterilizing agent 1% of NaOCl+70% ethanol was not effective individually as a high percentage of contamination was observed along with the growth of endophytes.

Table 1. Influence of sterilizing agent on culture incubation of *Rhizobium* from isolated root nodules.

Sterilization methods	Contamination rate	Survival rate
1%NaOCl*	65%	60%
2% NaOCl	20%	75%
3% NaOCl	0%	72%
4% NaOCl	0%	21%

*70% ethyl alcohol was common in every method.

Table 2. The cell morphology of isolated *Rhizobium* cells from the root nodules.

Morphological characters	Observation
Gram stain	Negative
Shape	Quadrate rods
Axis	Smooth
Ends	Regular
Length: Width	3:1
Group mate	Paired, systemic and bunch

Whereas as root samples treated with 2%, 3%, 4% of sodium hypochlorite and 70% ethanol to achieve a satisfactory result. Using 2% of NaOCl was effective survival percentage of bacterial endophytes but there was observed contamination. In 3% and 4% NaOCl and 70% ethanol treated had no contamination but 4% of NaOCl treated method had poor bacterial growth.

Table 3. Colony features of *Rhizobium*.

Colony morphology	Observation
Shape	Circled
Size	2-4 mm diameter
Color	Colorless, same as the agar
End	Smooth
Surface	Soft
Smell	Sweet musky
Consistency	Sticky/slimy
Structure	Amorphous

The results of the optimization process for the surface sterilization are shown in the Fig.1. Vigorous agitation of the nodules in a dilute sodium hypochlorite solution eliminated the need for a wetting agent and effectively sterilized nodules with highly convoluted surfaces such as those formed by *R. leguminosarum* bv. phaseoli (Beattie and Handelsman,1989). 3% sodium hypochlorite and 70% ethanol for 10 minutes was found effective for surface sterilization of *Rauvolfia serpentina* root tissues, with high percentage survival and no contamination (Shruti Shukla and Verinder Wahla, 2019) as we found in table 1.

Table 4. Cell Morphology of *Rhizobium* cell from *Rhizobium* colony on YEM agar plate incubated at 25˚C for 4days.

Cell morphology	Observation
Gram Stain	Negative
Shape	Quadrate rods
Ends	Regular
Axis	Smooth, bent
Length: Width	3:1
Groupment	Paired, systemic and bunch

Table 5. Effects of various Biochemical tests.

Serial No	Tests Performed	Results
1	Catalase Test	+ve
2	Lipase Test	-ve
3	Bromthyl blue Test	+ve
4	Starch hydrolysis Test	-ve
5	Oxidase Test	+ve
6	Citrate Utilization Test	-ve

*Growth is signified by "+" and poor growth is signified by "-".

Inoculation of root nodules in the culture media (YEMA)

After the sterilization process is completed, bacterial colonies become visible 24 hours after the transfer to nutrients media by three types of nodule in petri dish. In this case, the contaminated petri plates show an abundance of random colonies and microorganisms with different color variations (Figure 1.). The colonies of specific ribosomes are of definite and off-white color.

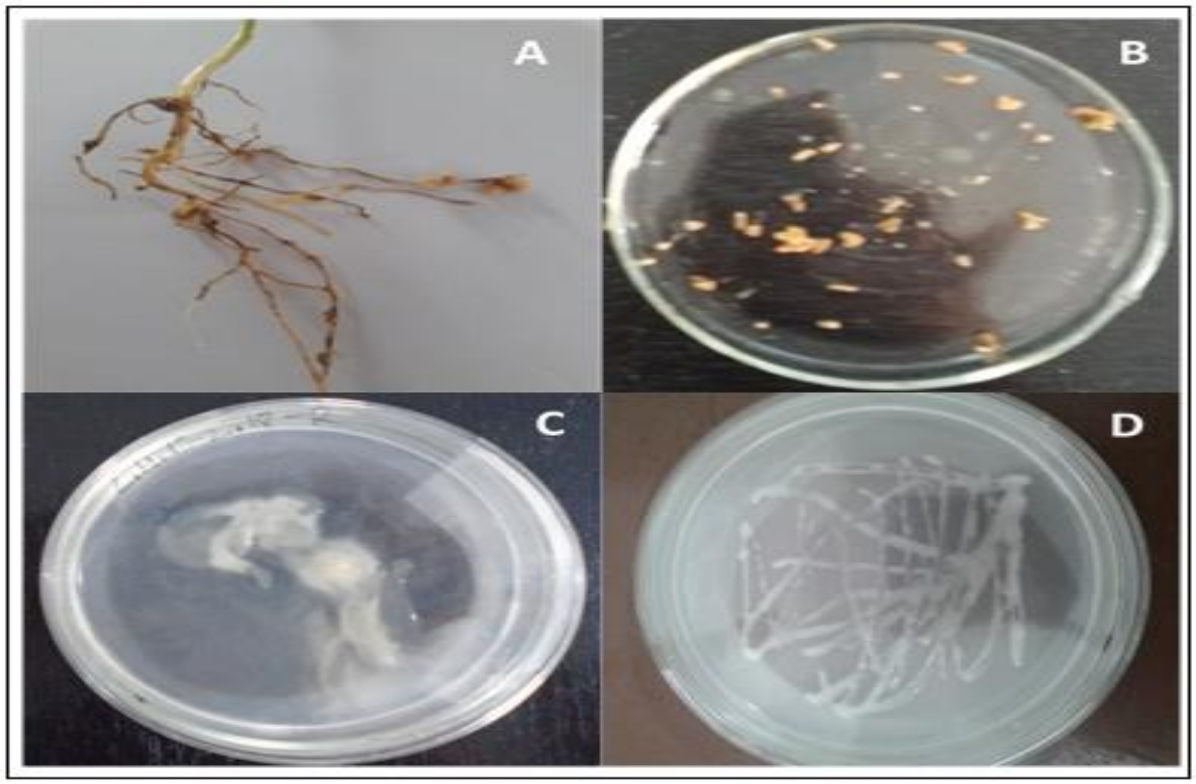

Fig. 1. Isolation of *Rhizobium* from lentil root nodule and *in vitro* culture initiation. A-Lentil root with nodule, B- Isolated fresh legumes, Co-Culture initiation of *Rhizobium* in YEMA media, D-Fresh culture of isolated bacteria (*Rizobium leguminosarum*).

Identification and external morphology study of isolated bacterial strains by Gram staining

Isolated bacteria were prepared by gram staining for microscopic study and morphological observation were shown in Figure 2, Table 2, 3, 4 and 5. The cell and colony morphology of isolated *Rhizobium* cells from the root nodule displayed slimy and colourless or yellowish with diameter range <2-4mm after incubation for 4 days. The shapes of the colonies were circled. The margins were smooth/bent and soft surfaced. The general microscopic characteristics of the selected isolates (*Rhizobium*) cells showed rod shaped and gram negative in nature and also similar to those reported earlier (Gauri *et al.*, 2011).

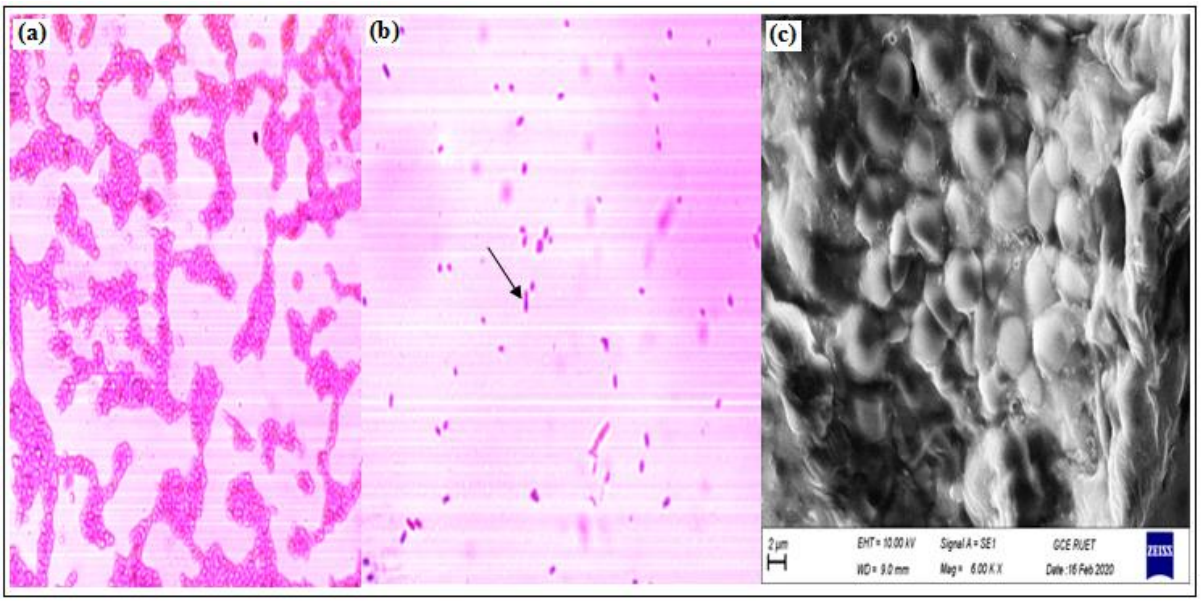

Fig. 2. Microscopic study of isolated bacteria. A= gram negative growth response of *Rhizobium*, B= Arrow indicates the gram negative rod shaped bacteria observed using Inverted microscope. C= Aggregation of *Rhizobium* inside the root nodule by SEM.

Biochemical characteristics of isolated bacteria

On the basis of biochemical observations, isolates were designated as *Mesorhizobium* sp. Singh *et al.*, (2013) also observed positive results for catalase and oxidase activities. Wani and Khan (2013) and Gauri *et al.*, 2011, also reported that *Mesorhizobium* isolates were positive for catalase, oxidase and citrate utilization and were negative for lipase. Datta *et al.*, 2015, observed that caseinase test is negative in *Rhizobium leguminosarum* strain. In our findings, the oxidase test showed positive where the colonies turned dark purple to black in color within 5 minutes in the test isolates. In the present study catalase test was found to be positive due to bubble formation around bacterial colonies. Datta *et al.*, 2015 also observed bubble formation around bacterial colonies of all four strains. Javed and Asghari *et al.*, (2008) also characterized the *Rhizobium* from root nodule with the same biochemical tests.

Conclusion

The isolated bacterial culture appeared as Gram negative rods and they are motile. It gives negative results to methyl red, voges-proskauer, citrate utilization, urease, Catalase, gelatin hydrolysis and ketolactose test. It gives positive result to starch hydrolysis and acid from glucose test. All this biochemical tests confirmed that the isolated bacterial culture is *Rhizobium*. In this experiment we used a rapid, inexpensive method for isolating and identifying *Rhizobium leguminosarum* strains from lentil root nodules. The method can be used for other small or medium-sized nodules producing legume plants.

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