Isolation, biochemical characterization and preparation of biofertilizers using Rhizobium strains for commercial use

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ABSTRACT
Rhizobium plays an important role in agriculture by including Nitrogen-fixing nodules on the roots of legume plants. The present study describes the characterization of Rhizobium strains isolated from root nodules of Vigna radiata. The Rhizobium strains were rod shaped, gram negative acid and mucous producing. They were found to be temperature and pH sensitive. They were unable to grow in the presence of 0.1% methylene blue and lactose. It utilizes glucose and starch as sole carbon source. Rhizobium strains were unable to hydrolysis the gelatin and showed sensitivity against different antibiotics such as tetracycline, kanamycin and streptomycin. The isolates were also showed demonstrable antibacterial activity against Streptococcus, E. coli and Pseudomonas. The Rhizobium can be further easily immobilized onto carrier like charcoal power which can be applied as biofertilizer.

Key Words: Rhizobium strains, characterization, antibiotics, antibacterial activity, immobilized, biofertilizer.

INTRODUCTION
Nutrient enrichment of soil by nitrogen fixing symbiotic bacteria present in legumes has been known for centuries. Scientific demonstration of this symbiosis was stared in 19th century and it established the fact that bacteria present in nodules on legume roots are responsible for fixing atmospheric nitrogen (Zsbrau, 1999). Rhizobium spp. are well known group of bacteria that act as the primary symbiotic fixer of nitrogen. These bacteria infect the root nodules of leguminous plants, leading to the formation of lumps and nodules where the nitrogen fixation takes place. The bacterium’s enzyme system supplies a constant source reduced nitrogen to the host plant and the plant furnishes nutrients and energy for the activities of the bacterium. This symbiosis reduces the requirement for nitrogenous fertilizer during the growth of leguminous crops (Dilworth and Parker, 1969; Olevera et al., 2007; Booling et al., 2007; Hunter et al., 2007).

In the present study, strains of Rhizobium were isolated from the root nodules. Further characterization was done by performing various biochemical tests and also determine if the rhizobial cells be efficiently immobilized on matrices to produce industrially important biofertilizer.

Materials and Methods
Isolation of Rhizobium from Vigna radiata Roots
The fresh and plump root nodules (Figure 1) of Vigna radiate were collected from the plants grown in the field. The collected nodules were surface-sterilized with 75 percent and 0.1 percent ethanol and mercuric chloride respectively and washed thoroughly with distilled water. Rhizobium strains were 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). After two days of incubation, Rhizobium colonies were obtained. Pure isolates were used for further analysis and all tests were performed in triplicates.

Identification and Phenotypic Characterization of Isolates
The morphological traits evaluated comprised colony morphology, mucous production and change in pH of medium during growth and growth rate. Mucous morphology analysis was based on type, elasticity and appearance, while colony morphology parameters were diameter, form, transparency and color (Aneja, 2003). Gram staining reaction was performed to evaluate type of strains.

NaCl Variation Assay
Rhizobium cultures were grown in triplicates on YEM medium having different concentrations of NaCl ranging from 0.4 to 0.6% (w/v). Growth was determined by measuring the optical density (O.D.) at 600 nm after 48 hours of inoculation.

pH Variation Assay
To analyze the effect of pH variation on the growth of the organism, media were prepared with pH 4.0 and 9.0. After incubation of 48 hours growth was determined by measuring the O.D. at 600 nm.

**Methylene Blue Treatment**

One percent methylene blue dye added to the growth medium and inoculated with *Rhizobium*. Incubation was given at 29.4°C temperature for 2 days and observations were made following the method of Gao et al., 1994.

**Glucose Peptone Agar (GPA) Assay**

GPA assay was performed to determine the capability of the *Rhizobium* strains to utilize glucose as the sole carbon source for its growth liquid. Glucose peptone broth (40 g/L glucose, 5 g/L peptone, 15 g/L agar, pH 7.0) was also used.

**Lactose Assay**

Lactose assay was performed to determine the capability of the *Rhizobium* to utilize lactose present in medium (10 g/L lactose, 5 g/L peptone, 3 g/L beef extract, 15 g/L agar, pH 7.0). Lactose broth was also used.

**Gelatin Hydrolysis**

The test was performed to determine capability of *Rhizobium* to produce gelatinase enzyme as use gelatin as media source. Degradation of gelatin indicates the presence of gelatinase enzyme (Aneja, 2003). The actively grown cultures were inoculated in nutrient gelatin medium (5 g/L peptone, 3 g/L beef extract, 12 g/L gelatin) and grown for 48 hours. On subjecting the growing culture to low temperature treatment at 4°C for 30 minutes to 60 minutes. The cultures which produce gelatinase remain liquefied while others due to the presence of gelatin become solid.

**Starch Hydrolysis**

The test was performed to determine capability of *Rhizobium* strains to use starch as carbon source (de Oliveira, 2007). Starch agar medium (5 g/L peptone, 2 g/L potato starch, 3 g/L beef extract, 15 g/L agar, pH 7.0) were inoculated with culture and incubated at 29.5°C temperature for 48 hours. In the presence of starch, the production of extra-cellular enzymes occurs indicating the potential of the organism to use starch as carbon source. Iodine was used to determine capability of *Rhizobium* to use starch. Drops of iodine solution (0.1N) were spread on 48 hours old culture grown on petri-plates. Formation of blue color indicated non-utilization of starch and vice-versa.

**Fluorescence Assay**

King’s medium (2 g/L peptone, 1.5 g/L MgSO4, 1.5 g/L K2HP04, 10 mL/L glycerol, 15 g/L agar, pH 7) was used to determine the ability of the isolates to fluorescence (King, 1954). The medium was inoculated aseptically with *Rhizobium* cultures and incubated at 29.4°C. Observations were made after 48 hours under UV-transilluminater.

**Triple Sugar Ion Agar Test**

The test was performed to determine the capability of isolates to use various carbohydrate sources e. g. sucrose, glucose, lactose etc as media for growth. Triple sugar medium consists of beef extract 3 g/L, yeast extract 3 g/L, peptone 15 g/L, NaCl 5 g/L, lactose 10 g/L, sucrose 10 g/L, dextrose 1 g/L, ferrous sulfate 0.2 g/L, sodium thiosulfate 0.3 g/L, phenol red 0.24 g/L, agar 15 g/L, pH 7.0 (Kligler, 1918; Hajnna, 1945). After inoculation and incubation, color was observed on the butt and the slant. On the basis of capability of organisms to use carbohydrates, three possible observations were made, first after 24 hours yellow slant and red butt, second red slant and yellow butt after 48 hours whereas third after 72 hours dark red slant and dark yellow butt.

**Detection of Antimicrobial activity**

Antimicrobial activity of the *Rhizobium* isolates against potential pathogenic bacteria such as *Bacillus subtilis* (NCDC 71), *Escherichia coli* (MTCC 40) and *Staphylococcus aureus* (MTCC 737) was tested by Agar well assay technique (Schillinger, and Lucke 1989).

**Detection of Antibiotic resistance**

Antibiotic resistance of the *Rhizobium* isolates was tested against kanamycin, streptomycin and tetracycline using Disk Diffusion method (National Committee for clinical Laboratory Standards, 1999).

**Catalase Test**

This test was preformed by adding 2-3 drops of 3% hydrogen peroxide in fresh YEM broth cultures of isolates. Transfer a colony of the organism on a microscope slide and add the drop of 3 percent H2O2. If catalase is present, the H2O2 is broken down into water and oxygen, which result in the immediate formation of gas bubbles.

**Urease Test**

The test was performed to determine capability of *Rhizobium* strains to produce urease enzyme or not. Urease is a hydrolytic enzyme that attacks the nitrogen and carbon bond in amide compounds such as urea and forms the alkaline end product as ammonia. The presence of urease is detectable when the organisms are grown in a urea broth medium containing the pH indicator phenol red. As the substrate urea is split into its products, the presence of ammonia creates an alkaline environment that causes the phenol red to turn to a deep pink color. This is a positive reaction for the presence of urease. Failure of deep pink color is evidence of negative reaction.
**Immobilization of *Rhizobium* cells**

*Rhizobium* cells were immobilized onto two matrices: agar-agar and agarose, Adinarayana *et al.*, 2005. Inoculums were prepared by adding five ml of sterile distilled water to 48-hold slant of *Rhizobium*. The cells were scrapped from the slant into sterile distilled water and the resulted cell suspension was transferred aseptically into 250 ml flasks containing 45 ml of sterile inoculums medium. The flask was kept in an incubator shaker at 185 rpm at 29.4°C. The content of the flasks was centrifuged at 3000 rpm for 10 minutes and the supernatant was decanted. The cells pellet was washed thoroughly with sterile potassium chloride (20.0 g/L) solution and sterile distilled water subsequently. Finally the cell mass was suspended in sterile sodium chloride solution (9.0 g/L). This cells suspension was used as inoculum for immobilization. A definite quantity of agar-agar was dissolved in 18 ml of 0.9% sodium chloride solution to get final concentration of 2% and sterilized by autoclaving. The cells suspension (2 ml) was added to the molten agar-agar maintained at 40°C, mixed well and poured into sterile Petri plates. The solidified agar blocks were cut into small equal size cubes. Cube of each agar and agarose were used as inoculums for fresh media. Inoculated flasks were kept in an incubator shaker at 185 rpm at 29.4°C for 2-3 days and assayed for immobilization was done.

**Bio-fertilizer preparation**

Once the pure culture of *Rhizobium* has been established and confirmed for its various activities, the next step was conversion of the rhizobia broth into a form which is easily used by farmers. *Rhizobium* cells were immobilized on carriers, which is an inert material used for mixing with broth so that inoculants can easily be handled, packed, stored, transported and used. The carrier (charcoal) was powdered and dried in sun to get 5% moisture level. Then it is screened through 100-200 mesh sieves and neutralized by mixing with calcium carbonate powder and sterilized by autoclaving. If the carrier is neutral there is no need of mixing calcium carbonate powder. The broth containing rhizobial cells were mixed with carrier and kept in trays or tubes. The moisture content was maintained to about 35-40%. After proper mixing, it is left for 2-10 days by covering the trays with polythene at 22-24°C. During this period *Rhizobium* cells multiplied, a process called curing. Thereafter, *Rhizobium* inoculants can be used directly or packed and stored.

**Results and Discussion**

Colonies of *Rhizobium* were obtained on YEM agar medium after incubation at 29.4°C for two days. The colonies were having sticky appearance showing the production of mucous though at lower levels (Fig. 2). General microscopic view of the isolates showed them to be rod cells and gram negative in nature. It is known that salt stress significantly reduces nitrogen fixation and nodulation in legumes. Hashem *et al.* (1998) have proposed that salt stress may decrease the efficiency of the *Rhizobium*-legume symbiosis by reducing plant growth and photosynthesis, and hence nitrogen demand, by decreasing survival and proliferation of rhizobia in the soil and rhizosphere, or by inhibiting very early symbiotic events, such as chemotaxis and root hair colonization, thus directly interfering with root nodule function.
To date, some rhizobial isolates have been shown to grow under high salt conditions 4-5 percent (Kucuk et al., 2006). Results indicated that cells were able to grow on 1 percent NaCl containing medium but unable to grow on higher concentrations, showing that the isolate was sensitive to the salt. pH is an important parameter for the growth of the organism. Slight variations in pH of medium might have enormous effect on the growth of organism. Superior growth of *Rhizobium* has been reported at neutral pH i.e. 7. Results showed that cells were able to grow only at pH 7.0 at 29.4°C temperature. No growth was observed in medium with pH 4.0 and 9.0. Similar observation were made by Gao et al., 1994; Kucuk et al., 2006; Baoling et al., 2007.

Methylene blue and gentian were used as an agent against the growth of the microorganisms. Rhizobial cells were unable to grow either on medium containing 0.1% methylene blue (Fig. 3) or on 0.1% gentian. Earlier studies have also indicated that rhizobial cells were unable to grow in the presence of the two dyes (Wei et al., 2003). Rhizobial cells were able to grow on the GPA media showing the utilization of glucose as the carbon source by the *Rhizobium* (Fig. 4). It is a confirmatory test for *Rhizobium* and these are able to utilize glucose as carbon source (Kucuk et al., 2006). However, pure *Rhizobium* isolates are unable to grow on lactose (Fig. 5). It was observed that rhizobial cells do not produce gelatinase enzymes as medium containing gelatin solidified when kept at 4°C for 30 as well as 60 minutes (Fig. 6). Negative gelatinase activity is also a feature of *Rhizobium* (Hunter et al., 2007). Positive results were obtained from the starch hydrolysis assay. On subjecting inoculated plates to iodine test, clear zones around the colonies were seen and the colonies turned yellow in appearance, whereas, blue color appears on no growth areas (Fig. 7). This indicates that the isolates have the potential to hydrolyze starch present in the medium. De Oliveira et al. (2007) also observed that *Rhizobium* strains can utilize starch obtained from different sources. *Rhizobium* cells grown on King’s Medium under the UV source show the absence of ability of the organisms to fluoresce (Fig. 8). Yellow slants and red butt (Fig. 9) were obtained showing the utilization of glucose and sucrose in the triple sugar agar medium (Hajnaa, 1945). No such studies have been conducted on *Rhizobium* strains.

*Rhizobium* isolates were showed antibacterial activity against *Streptococcus* and *E. coli* whereas it didn't showed antibacterial activity against *Pseudomonas* (Fig. 10). *Rhizobium* isolates found to be the most potent strain and showed maximum diameter of inhibition zone ranges from 15 mm to 13 mm against *E. coli* and *Staphylococcus aureus*, respectively. The *Rhizobium* isolates were sensitive to tetracycline, kanamycin and streptomycin antibiotics (Fig. 9). The diameter of zone of inhibition of bacterial growth was found to be 3.5, 2.5 and 2 cm for tetracycline, kanamycin and streptomycin, respectively. There are 3 known determinants of bacterial permeability to an antibiotic: hydrophobicity, electrical charge, and amount of the antibiotic (Hungaria et al., 2000) and the *Rhizobium* that showed a high level of resistance did not take up the antibiotics. *Rhizobium* isolates were showed catalase and urease negative test. The *Rhizobium* can be easily immobilized onto carrier like charcoal power which can be applied as biofertilizer (Fig. 11 and 12). Although we have also developed biofertilizers using charcoal as carrier; however the field trials are yet to be conducted.

REFERENCES


