

Original Research Article

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Isolation and Characterization of *Rhizobium* from Chickpea (*Cicer arietinum*)

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ABSTRACT

Nutrient paucity in the soil poses confront to global production of food. The use of synthetic nitrogen fertilizers to boost crop yield is a recurrent farming practice, despite of its unfavorable effects and hazard to the environment and human population. *Rhizobium* is gram negative bacterium which associates symbiotically with the roots of leguminous plants. Screening and selecting the rhizobial strain is important for biological nitrogen fixation. The present study was aimed to isolate and identify *Rhizobium* from *Cicer arietinum* (chickpea) root nodules by using CRYEMA medium. The result indicated that a bacterium from root nodules of chickpea does not absorb red color on YEMA medium and the milky white colony with spherical convex surface was isolated. Many biochemical tests of the isolated strain like oxidase, catalase and bromothymol blue were positive, while starch hydrolysis, lipase test, lysine decarboxylase and caseinase were negative which revealed that the strain isolated from chickpea plant belongs to *Rhizobia* species. The study indicated that all the strains grew well at pH 6 and 7, temperature 28° C to 30° C and at salt concentration of 1%. The strains showed resistance to the antibiotics, metal salts and salinity.

Keywords

YEMA, Congo red,
Chickpea, *Rhizobia*,
Legumes and bacteria.

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Introduction

With increased concern about the production of adequate amount of food to feed the constantly increasing human population which is going to touch a mark of 9 billion by 2050 has forced us to reinforce the importance of sustainable increase in crop productivity. One of the methods for sustainable agricultural includes the use of beneficial microorganisms for plants as they are able to promote plant growth by growing endophytically on plants, in symbiotic association with plants or as free-living cells

in soil. The requirement for good agricultural practices is revitalizing the interest in biological nitrogen fixation and *Rhizobia*-legumes symbiosis, particularly those involving economically important legume crops in terms of food and forage.

Legumes are agronomically and ecologically important symbionts that lead to the development of new plant organ (legume nodule) in response to nitrogen fixing bacteria (Datta *et al.*, 2015). In developing countries

like India and other South Asian countries, chickpea is an important source of protein for millions of people. Other than having high protein content (20-22%), chickpea is rich in fiber, minerals (phosphorus, calcium, magnesium, iron and zinc) β -carotene and large amount of unsaturated fatty acids (Gaur, 2010). Besides playing an important role in human diet it also improves soil fertility by fixing the atmospheric nitrogen (Siddiqi and Mahmood, 2001; Kantar *et al.*, 2007). Maximum nitrogen requirement (4-85%) of chickpea as a legume is obtained through symbiotic Nitrogen fixation in association with compatible *Rhizobium* strain (Chemining and Vessey, 2006). The genus *Mesorhizobium* includes species with high geographical dispersion and able to nodulate a wide variety of legumes, including important crop species, like chickpea. It has been estimated that 1g of soil may contain a community of 10^9 microorganisms with *Rhizobia* representing around 0.1% of soil microbes or 10^6g^{-1} soil.

Rhizobia are one of the most efficient bacterial symbionts of legumes that fix atmospheric nitrogen by the process of biological nitrogen fixation (BNF).

Rhizobia are able to metabolize atmospheric nitrogen and convert it into plant usable form in specialized structures called nodules where aerobic condition are maintained by leghaemoglobin. In return, *Rhizobia* utilize the carbon substrates derived from the plant photosynthesis. In agriculture, perhaps 80% of the biologically fixed nitrogen comes from symbiosis involving leguminous plants and bacteria of family *Rhizobiaceae*.

The biological nitrogen fixation because of *Rhizobia*-legume symbiosis benefits not only the host crop but also the subsequent crops in that field. Besides this, it may also act as a non-symbiotic PGPB as in the case of certain

non-legume crops such as rice or wheat, which are the best-studied examples that benefit from *Rhizobia* as endophytes (Biswas *et al.*, 2000). For all these reasons, the *Rhizobium*-legume symbiosis has been widely studied as a model of mutualistic associations and as a beneficial association for sustainable agriculture. With increasing use of *Rhizobium* and other beneficial microbes as bio fertilizers, reduction in the need for chemical fertilizers can be observed. Therefore, bio fertilization has great importance in decreasing environmental pollution and deterioration of nature (Vessey, 2006; Erman *et al.*, 2011). The inoculation of seeds with *Rhizobium* is known to increase nodulation, N uptake, growth and yield parameters of legume crops (Erman *et al.*, 2011). Keeping in view the importance of *Rhizobia* in legume plants, the present study was undertaken to shed some light on different morpho-physiological and biochemical properties of *Rhizobial* strain isolated from chickpea plant.

Materials and Methods

Collection and extraction of root nodules from the chickpea plants

The experimental material for the present study was collected from Hisar district. Plants possessing healthy nodules with pink color were selected and transported to the lab (Fig. 1). Root nodules of chickpea plant (*Cicer arietinum*) were used as study material for isolation and further morphological and physiological characterization of *Rhizobium* strain. The roots were first washed thoroughly with sterile distilled water and nodules were surface sterilized by washing with 95% ethanol for 10 seconds and again washed in sterile distilled water for about 5 times. Roots were mashed with pestle mortar to obtained nodules and milky white substances of bacteroids by dipping in phosphate buffer solution.

Serial dilutions of the extracted root nodules

After the extraction of bacteroid solution from the chickpea root nodules, serial dilution was made. 2 ml of sterilized root nodule bacteroids solution was taken in 90 ml sterilized distilled water and serially diluted up to 10^{-6} dilution. For identification of the colonies, 10^{-4} to 10^{-6} dilution of nodule extract were plated on YEMA Congo red agar media plates. The petri plates were then kept in the incubator at 37°C for 3 days. After the plates were taken out of the incubator colony morphology and identification was carried out. All bacteriological isolation and the entire process of biochemical tests were carried in the laminar airflow to maintain the sterility.

Gram staining of the bacterial strain

The pure cultures of bacterial strains were put for gram staining for more specific identification of the colonies. The gram staining was done in laminar air flow hood.

The slides were firstly washed with ethanol and colonies were marked on the slides with the help of inoculating needle and were heat fixed. Then smears were stained in following steps a) First applied crystal violet on each slide and kept for 1 min. b) Distilled water wash. c) Iodine on the slides as mordant (1 min) then 95% alcohol wash (30 sec.) and then washed with distilled water. d) Safranin was applied on the slides and then washed with distilled water and f) air dried the slides. The entire gram staining technique was done following the Christian Gram technique and Collee JG, Miles RS Mackie (1989).

Effect of salt

The salinity tolerance was assessed by culturing the bacteria on YEMA medium

containing different salt concentration 1%, 2%, 3%, 4% (w/v) NaCl.

pH variation assay

The ability of *Rhizobial* isolate to grow at different pH was tested in YEMA medium by adjusting the pH to 5.0, 6.0, 7.0, 8.0 and 9.0 with NaOH and HCl.

Temperature tolerance

Temperature Tolerance was investigated by assaying the growth of bacterial cultures in YEMA medium at different temperature viz. 5°C , 28°C , 35°C and 40°C .

Effect of metal salts

The isolate was tested for their sensitivity to metal by amendment of freshly prepared YEMA plates with metal salts i.e. HgCl_2 and ZnSO_4 at 1 % (w/v) concentration. Effect of metal salts was determined by assaying *Rhizobial* growth after incubating the plates at 30°C for 48 hours.

Intrinsic Antibiotic Resistance (IAR) spectra

IAR was carried out to identify sensitivity or resistance of *Rhizobia* strain to different antibiotics. Antibiotics discs were used to assay the antibiotic resistance on YEMA media containing bacterial culture evenly spread across the surface. Petri plates containing the disc were incubated at 30°C for 3-7 days.

The presence or absence of inhibition zones around different antibiotics discs was noted (Bauer *et al.*, 1966). Filter paper discs containing different antibiotics viz. kanamycin, vancomycin, chloramphenicol and gentamycin were used in the present study.

Biochemical studies

Catalase test

This test was performed to study the presence of enzyme catalase in *Mesorhizobium* spp which hydrolyzes H₂O₂ into H₂O and O₂ in bacterial strains. Firstly, smear of strain was made on a clean and dry glass slide, then a few drops of H₂O₂ were added to the slide. Production of gas bubbles and effervescence showed a positive test.

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 YEM agar medium. Isolates of *Mesorhizobium* spp. were streaked in sodium citrate added YEMA medium plates with bromothymol blue as an indicator. Then plates were incubated for 24 - 48 hours.

Bromothymol blue test

It selectively identifies fast and slow growing isolate of *Rhizobium*. For this test, bacteria were inoculated on YEM agar medium containing 0.025% bromothymol blue and incubated for 3 to 10 days for acid or alkaline reaction^[32]. In this test sample were allowed to grow YEM media contains BTB. After incubation for 48 hours at 28 ° C positive sample showed yellow color due to acid production

Lipase test

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

Oxidase test

Oxidase test was performed to determine the presence of oxidase enzyme in different

isolates of *Mesorhizobium* spp. Kovac's reagent (1% N, N, N.N-tetramethyl-phenylene diamine) 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.

Starch hydrolysis

This test was performed to determine the capability of *Rhizobium* to use starch as a carbon source. For starch utilization Starch Agar Medium was inoculated with *Rhizobium* then iodine was added to determine the capability of microbes to use starch. A drop of iodine (0.1N) was spread on 24 hours old culture that showed clear zone of inhibition around bacterial colonies.

Lysine decarboxylase test

In this test *Rhizobium* strains were streaked on Bromocresol Purple Falkow medium (peptone 5 g, yeast extract 3 g, glucose 1g, Bromocresol purple 0.02 g, distilled water 1 liter). Then *Rhizobial* strains were streaked on the media and were kept for incubation at 34 °C for 24 hours.

Results and Discussion

Morphological characteristics

On the basis of morphological characters, the isolates were circular in shape with entire margin and milky to watery translucent appearance on CRYEMA medium. It was found that the strain grown showed the convex elevation in Yeast Extract Mannitol Agar medium. The colonies were 2.5 mm, translucent, whitish pink and glittering (Fig. 2). Roychowdhury *et al.*, (2015) showed the growth of *Rhizobium* bacteria on Congo red Yeast Extract Mannitol agar medium. *Rhizobial* isolates were tentatively assigned to genera *Mesorhizobium* on the basis of

morphological characters. Similarly, Rai *et al.*, (2013) and Gauri *et al.*, (2012) also characterized mesorhizobial isolates on the basis of their colony shape, colour and texture. Datta *et al.*, (2015) also found that *Rhizobium* was Gram negative, motile, rod shaped and were fast growers as they showed convex elevation in Yeast Extract Mannitol medium. Table 1 represents the morphological and cultural characteristics of strains indicating *Rhizobium*.

Microscopic observations

Gram's staining of the isolates was confirmed by microscopic observations and the Mesorhizobium spp. was found to be gram negative. Gauri *et al.*, (2012) also reported that microscopic examination of *Rhizobium* revealed the isolates to be gram negative. Roychowdhury *et al.*, (2015) observed his strain under the microscope by gram staining which showed white pinkish color and rod shaped bacteria.

Effect of salt, pH and temperature on *Rhizobium* growth

Symbiotic nitrogen fixation is restricted by many factors. One such factor is salinity as *Rhizobium* grows at higher salt levels as compared to their host plant species. Furthermore, chickpea is sensitive when it comes to salt tolerance and thus salinity is considered as one of the most important limiting factors for BNF of chickpea in arid and semiarid regions. Several studies conducted on chickpea (Singla and Garg, 2005; Garg and Singla, 2009) showed that NaCl salinity resulted in decreased plant growth, less N₂ fixation, decreased nodule numbers and reduced percentage of tissue nitrogen. Whereas, *Rhizobia* isolated from chickpea nodules and are comparatively tolerant to salt than their host (Vanderlinde *et al.*, 2010). However, these bacteria differ in

their capability of NaCl tolerance as some strains may grow at salt concentrations as high as 500 mM NaCl, others may not grow even at low NaCl concentration (100 mM) (Kucuk and Kivanc, 2008). In the present study, it was found that strain of *rhizobia* showed different growth rate at different concentration of NaCl, maximum growth rate was observed at 1% (w/v) NaCl and minimal at 4% (w/v) NaCl (Fig. 3; Fig. 9). Jida and Assefa (2012) also reported high tolerance to NaCl where 75% of the tested *rhizobia* could grow well with 1% NaCl. However, at higher concentrations, the percentage of tolerant isolate decreased with increasing salt concentration as only 11.1% of the isolates tolerated 5% NaCl. Berrada *et al.*, (2012) observed that 31% of the strains were tolerant to 2% NaCl when grown at different NaCl concentrations (0.5, 1, 1.5, and 2%).

Temperature is one of the major factors affecting rhizobial growth, survival in the soil and the symbiotic process itself (Niste *et al.*, 2013). High soil temperature in tropical regions is one of the major constraints for BNF in legume crops. High temperature may affect symbiotic relationships, nitrogen content, formation of roots, binding of rhizobia to root hairs, nodule development and function, and dry matter production (Boboye *et al.*, 2011). Low temperature affects nodulation, as the process of nodule initiation is completely inhibited under low 13 temperature (Sadowsky, 2005). High temperature also alters the pattern of cell surface components (EPS and LPS) secreted by rhizobia, which in turn negatively affects the process of nodulation and N₂ fixation Yadav *et al.*, (2010). The optimum temperature for growth of root nodulating bacteria ranged from 25°C - 30°C, however in both saprophytic and symbiotic life *rhizobia* often encounter temperature variation. Isolates of *rhizobia* were grown in YEMA medium different temperature viz. 5°C, 28°C,

35°C and 40°C for evaluating thermo tolerance. In the present study, optimum temperature for *rhizobial* growth was found to be 28°C whereas moderate growth was observed at 35°C and minimal growth of isolates was exhibited at 5°C and 40°C (Fig. 4). Alexandre *et al.*, (2009) reported that temperature greatly affected the growth and symbiotic performance of *Rhizobia*. Maatallah *et al.*, (2002) reported maximum growth of *Mesorhizobia* between 20 to 30 °C.

To observe pH tolerance of the rhizobial isolates, they were grown in YEMA medium

by adjusting pH to 5-9 and the plates were incubated at 28 °C for 36 hours. In the present study, optimum pH for rhizobial growth was between 6-7. Minimal growth of isolates was exhibited at pH 8 and pH 9 (Fig. 5).

Deora and Singhal, 2010 have proposed that slight variation in the pH of medium might have an enormous effect on the growth of *Rhizobium*. Rhizobial isolates were observed to be more sensitive to low pH than their host and this affects the establishment of the symbiosis, limiting the survival and persistence of the *Rhizobia* (Zahran, 1999).

Table.1 Morphological study of *Rhizobial* strain

| S. No. | Strain characteristic | <i>Rhizobial</i> strain |
|--------|-----------------------|-------------------------|
| 1 | Shape | Circular |
| 2 | Size | 2.5mm |
| 3 | Color | White |
| 4 | Opacity | Transparent |
| 5 | Bacterium shape | Rod shaped |
| 6 | Gram nature | Gram negative |
| 7 | Motility | Mobile |

Table.2 Effect of metal ions on growth of *Rhizobia*

| Metal Salt | Growth |
|-------------------|--------|
| HgCl ₂ | - |
| ZnSO ₄ | + |

Growth is signified by “+” and poor growth is signified by“-”.

Table.3 Effect of various biochemical tests

| S.No. | Biochemical Test | Growth |
|-------|----------------------|--------|
| 1 | Bromothymol blue | + |
| 2 | Caesinase | - |
| 3 | Catalase | + |
| 4 | Citrate | - |
| 5 | Lipase | - |
| 6 | Lysine decarboxylase | - |
| 7 | Oxidase | + |
| 8 | Starch hydrolysis | - |

Growth is signified by “+” and poor growth is signified by“-”.

Fig.1 Chickpea plant root nodules



Fig.2 Isolated single colonies of rhizobia on CRYEMA plate

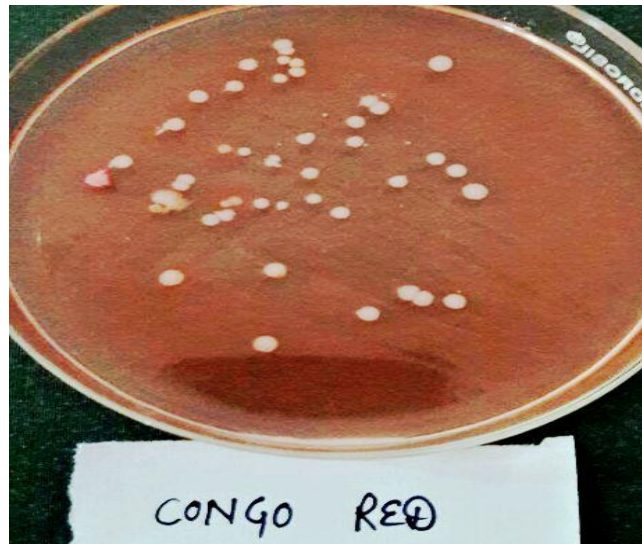


Fig.3 Effect of different concentration of NaCl on growth rate of *Rhizobium*

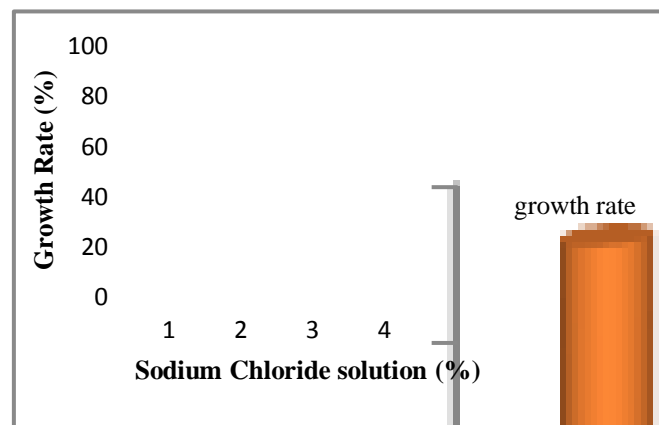


Fig.4 Effect of temperature on growth rate of *Rhizobium*

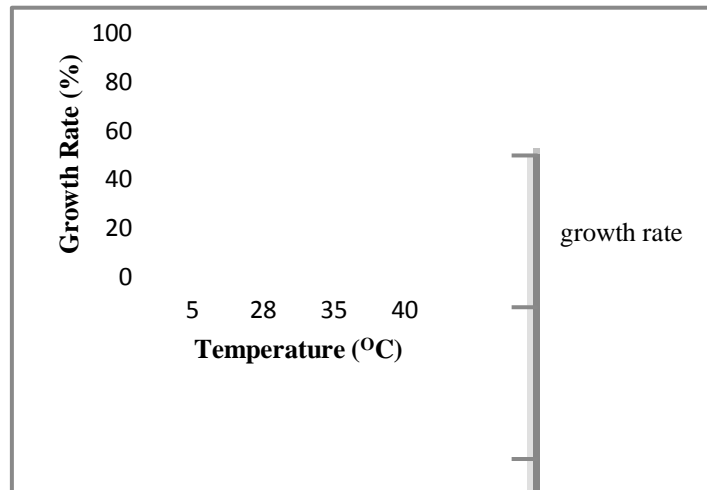


Fig.5 Effect of pH on growth rate of *Rhizobium*

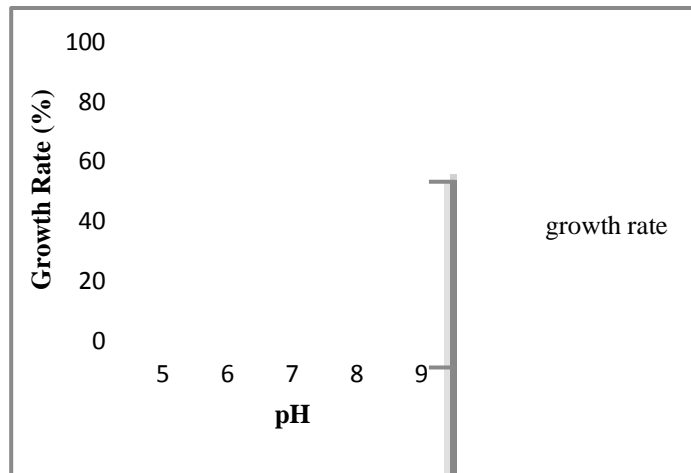


Fig.6 Effect of metal salts on growth of *Rhizobium* strain

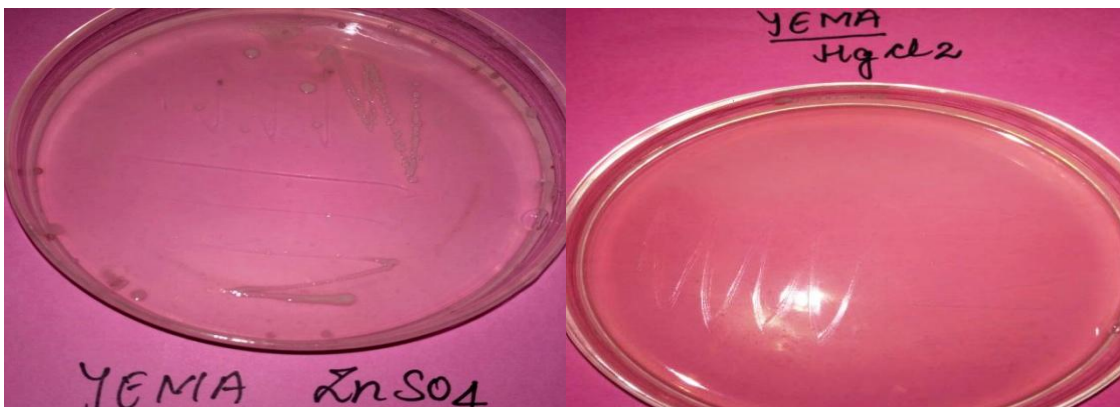


Fig.7 Effect of antibiotics on *Rhizobium* strain

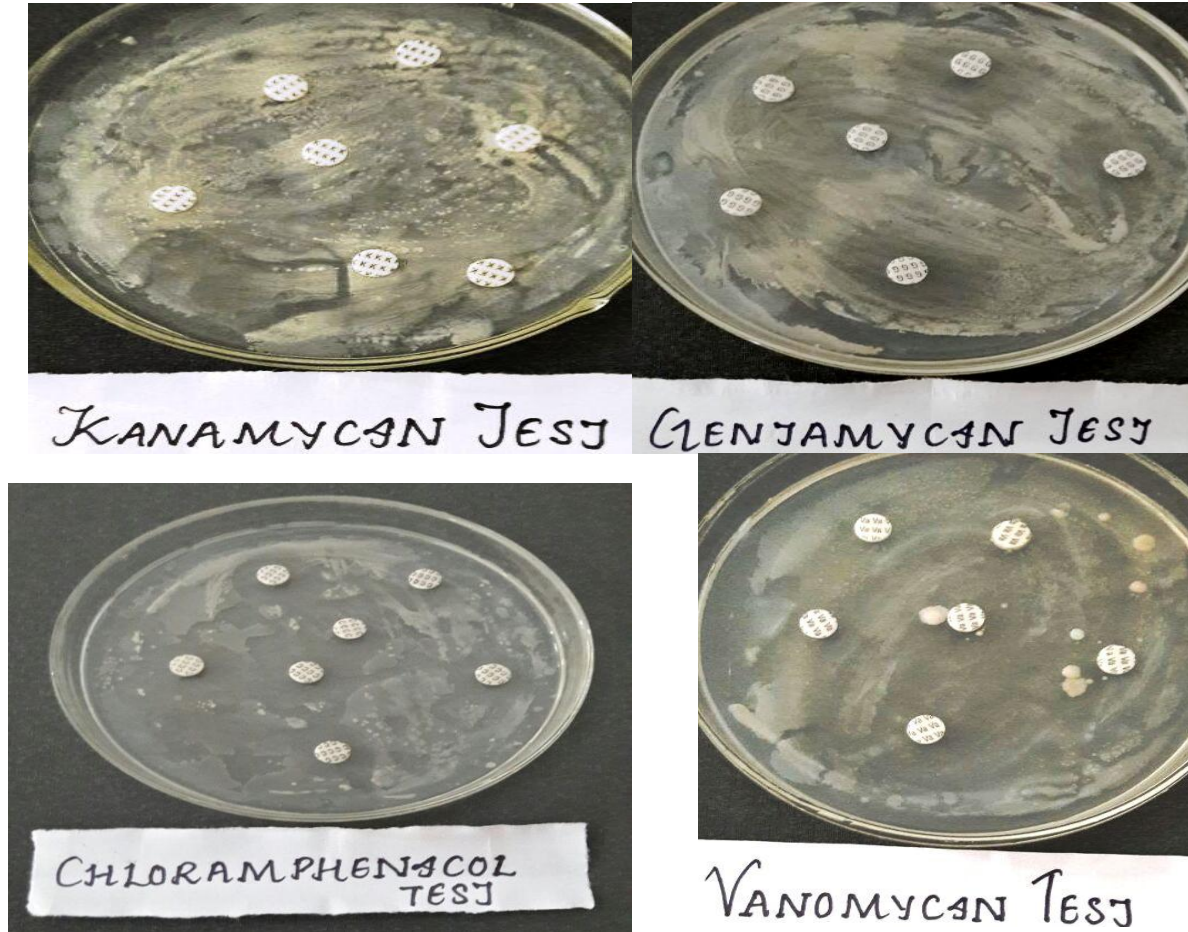


Fig.8 Effect of various biochemical test on *Rhizobium* growth

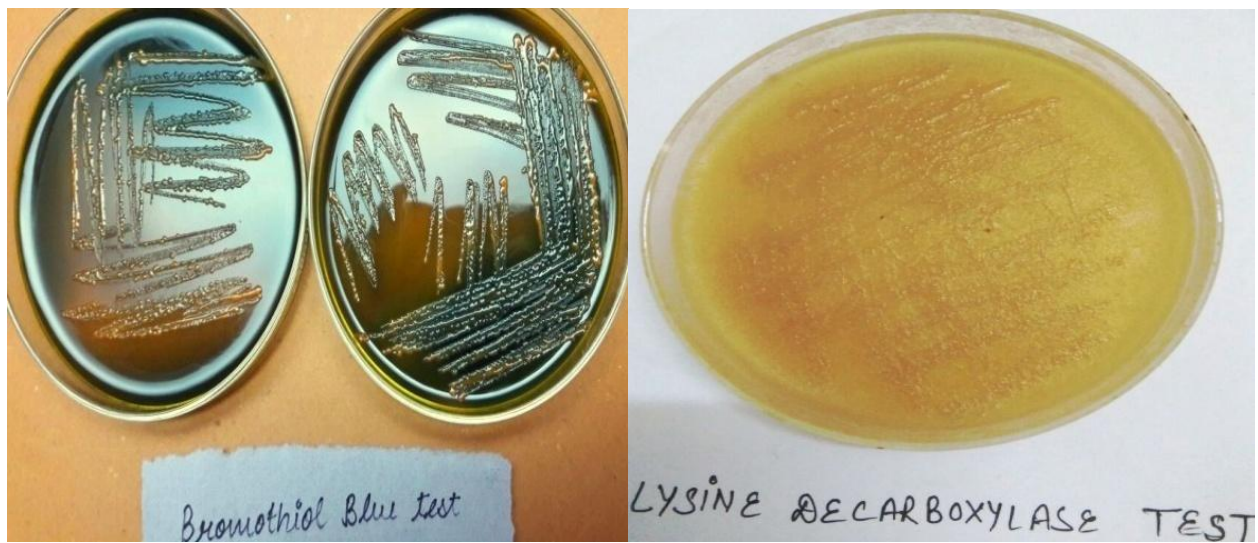
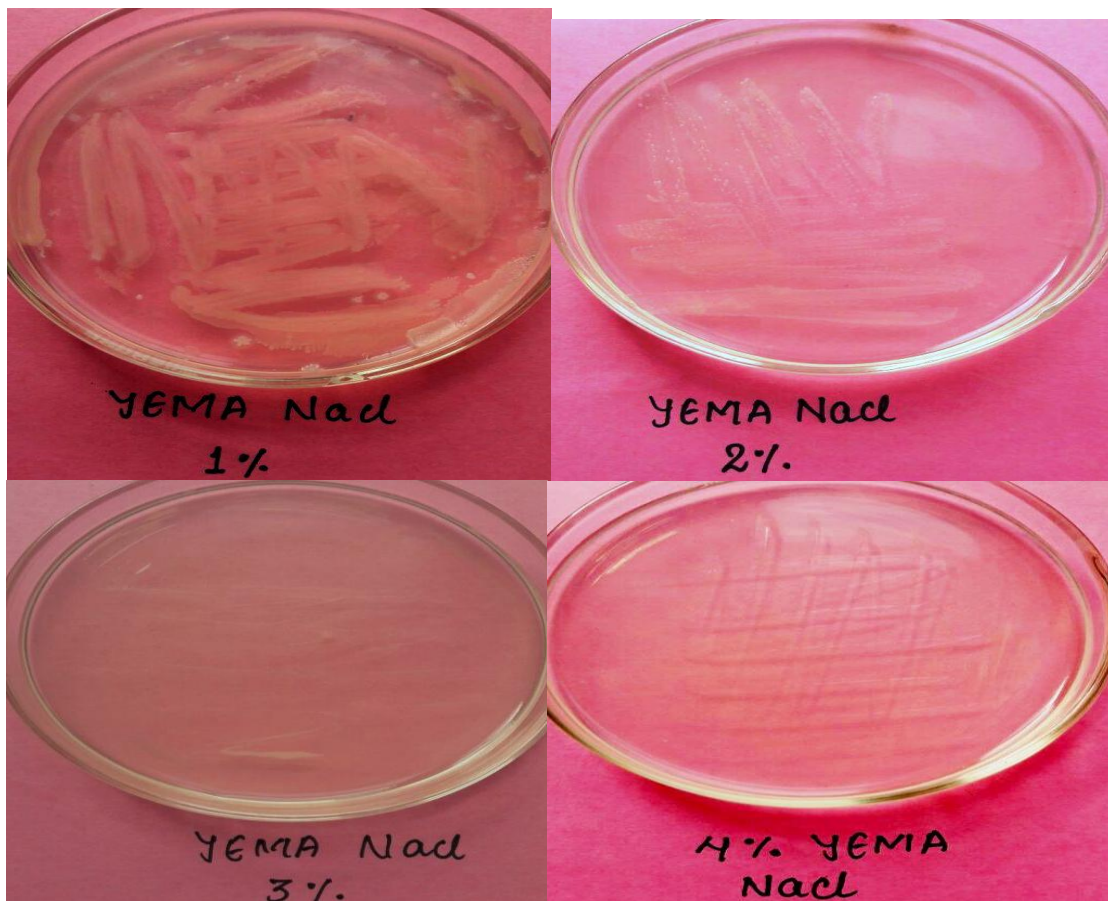




Fig.9 Effect of NaCl on growth of rhizobium



Effect of metal ions on growth of *Rhizobia* strain

Bacteria have developed controlled system to differentiate and cope up with harmful metal ions. Assessing the effect of metal salts on *Rhizobia* strains showed that all the strains were sensitive to mercuric chloride (HgCl_2).

On the other hand, ZnSO_4 had least antibacterial effect at 0.1% (w/v) concentration. Most metal ions have to enter bacterial cells in order to produce physiological and toxic effect. Table 2, represents growth pattern of *rhizobia* strains towards effect of these metal ions i.e. HgCl_2 and ZnSO_4 (Fig. 6). Datta *et al.*, 2015

investigated the effect of metal ions on *Rhizobium* strains observed that all the strains were sensitive to mercuric chloride (HgCl₂). On the other hand, ZnSO₄ and CuSO₄ had minimum antibacterial effect at 0.1% (w/v) and 1% (w/v) concentration.

Intrinsic Antibiotic Resistance (IAR) spectra

An IAR spectrum is used for the identification of nodulating strains in reports intended to assess the ecological competitiveness. IAR test was carried out to identify isolates of *Mesorhizobium* sp. for sensitivity or resistance to various antibiotics. Test isolates were assessed against four antibiotics namely vancomycin, chloramphenicol, kanamycin and gentamycin. This estimation of intrinsic resistance to antibiotics revealed that the test isolates displayed high resistance to kanamycin and least resistance to chloramphenicol. The antibiotic resists the growth of *Rhizobium*. Antibiotic resistance pattern observed from tests was as follows kanamycin>gentamycin>vancomycin>chloramphenicol (Fig. 7). Sharma *et al.*, (2010) reported that most of the *Rhizobium* isolates were sensitive to chloramphenicol. Maatallah *et al.*, 2002; Kucuk and Kivanc 2008 found great variation among chickpea rhizobia with respect to their IAR pattern. Gauri *et al.*, 2012; Berrada *et al.*, (2012) showed that the isolated strains of *Rhizobium* were highly resistant to kanamycin.

Biochemical characterization

Biochemical characterization of selected isolates was carried out on the basis of different biochemical tests *viz.*, Oxidase, Catalase, Citrate utilization, Bromothymol blue, Lysine decarboxylase, Lipase and Caesinase test. In the present study, isolates were positive for oxidase and catalase, whereas negative for lipase, urease, caseinase

test (Table 3; Fig. 8). 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.*, 2012, 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.

In the present study, when test isolates were streaked on Bromothymol blue supplemented YEMA media for further confirmation. It was observed that growth occurs after 2 days of inoculation and turned YEM media from blue to yellow, confirming their nature as acid producers (Fig. 8; Table 3). Similarly, Datta *et al.*, (2015) also observed that when all isolates were streaked on Bromothymol blue added YEMA medium, found after 2 days that growth turned YEM media from blue to yellow confirming their nature of being fast growers and acid producers. As no clear zone around the isolates was observed under starch hydrolysis assay which was performed to determine the production of reducing sugar from starch in bacteria. Similarly, Datta *et al.*, (2015) also found that no clear zones around colonies were observed in *Rhizobium leguminosarum* (Fig. 8; Table 3). Lysine decarboxylase test was performed using bromocresol purple falkow media and no such color change in the medium. Datta *et al.*, 2015

showed change in the color of medium inoculated with *Rhizobium phaseoli*, *Rhizobium trifolii* and *Bradyrhizobium japonicum* but no such color change was found in the medium inoculated with *Rhizobium leguminosarum*.

In our findings use of citrate as a carbon source showed no color change, exhibiting negative citrate test (Fig. 8; Table 3). Datta *et al.*, 2015 found that citrate utilization as a carbon source was positive in *Rhizobium phaseoli* and *Rhizobium trifolii* (the fast growing *Rhizobia*). However, slow growing *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* showed no color change, exhibiting negative citrate test. From all the above findings and results confirms the presence of *Rhizobium leguminosarum* in our study.

Rhizobium is an important microorganism for the environment because of its nitrogen-fixing ability when in symbiotic relationship with plants (mainly legumes). This study confirmed that the root nodules of chickpea plants harbour the nitrogen-fixing bacterium-*Rhizobium*. The present investigation seems to be promising approach to consider the optimum method for the isolation of rhizobia from chickpea plant that act as a potential candidate to be used in nitrogen fixation and lab based experiments. It also showed that these plants, when inoculated with *Rhizobium* isolates, perform better. This organism will greatly enhance agricultural production, if they are often used to inoculate legume plants, thereby reducing the environmental threat of synthetic nitrogen fertilizers. These findings allow us a new scope for extensive research in Agricultural Biotechnology.

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