# Isolation & Characterization of *Rhizobium* Species and its Effect on Growth on Monocot Plant used as Biofertilizer

**Chitra Bhattacharya[1] & Bhawana Pandey**

Department of Microbiology and Biotechnology
Bhilai Mahila Mahavidyalaya, Hospital Sector, Bhilai, Chhattisgarh
always.smilechitrabhattacharya@gmail.com
bhawanapandey15@gmail.com

**Abstract**

*Rhizobium plays an important role in agriculture by including nitrogen-fixing nodules on the roots of legume plants. The present study describes the physiological and biochemical characterization of Rhizobium species isolated from root nodules of Pea plant. Microscopic examination of Rhizhobium sp. was done it is rod shaped, gram negative acid and mucous producing. It utilizes starch as sole carbon source. Yellow slants and red butt was obtained showing the utilization of glucose and sucrose in the triple sugar ion agar medium. Rhizobial cells were able to grow on the urease media showing the utilization of urea as source by the Rhizobium sp. It was unable to hydrolysis the gelatinase activity. The effect of Rhizobium inoculums as biofertilizer with manure, chemical fertilizer and charcoal fertilizers were analyzed for yield and quality of wheat seeds. It was found that the seed yield was higher in the Rhizobium inoculums treatment of coated manure than in the chemical fertilizer treatment, the effect on monocot plant growth were identified after 18 days of experiment. The use of plant growth-promoting bacteria to increase the soil fertility and improve growth and yield of agronomical important crops is a significant alternative to chemical fertilizers in sustainable agriculture. Strains of the Rhizobium genera are the most well-known nitrogen fixing bacteria, which when co-inoculated in soil with manure can improve growth of different legumes, as well as monocot plants including wheat.*

**Key Words:**

*Rhizobium* sp.; Pea Plant; Characterization; Wheat Seeds; Biofertilizer

## Introduction

*Rhizobium* plays an important role in agriculture by including Nitrogen-fixing nodules on the roots of legume plants. The *Rhizhobium* sp. were rod shaped, gram negative acid and mucous producing. Developed a rapid, inexpensive method for isolating and identifying *Rhizobium leguminosarum* by phaseoli strains from bean root nodules. (Gauri *et al*. 2009, Gwyn *et al* 1989) *Rhizobium* is special bacteria that can live in the soil or in nodules formed on the roots of legumes. In root nodules, they form a symbiotic association with the legume, obtaining nutrients from the plant and producing nitrogen in a process called biological nitrogen fixation, or BNF. Microorganisms producing root and stem nodules in legume plants are devided into five genera (*Rhizobium, Azorhizobium, Mesorhizobium, Sinorhizobium and Bradyrhizobium*), with a separate sixth genus Allorhizobium. Not all legumes bear nodules; 90% of Mimosoideae and 97% of Papilionoideae are nodulated, however, only 3% of Caesalpiniodeae are nodulated (Graham P. H., 2004). The rhizobium-legume symbiosis and its different aspects were studied time to time. Soil bacteria

associated with plant roots that can exert beneficial effects on their hosts are designated as plant growth promoting rhizobacteria (PGPR) (Olivera *et al*., 2012). In the present study was performed to isolate non-rhizobial endophytes from the surface sterilized root nodules of pea (*Pisum sativum*) and assess their effects on growth of wheat (*Triticum aestivum*) plant.

**Materials and Methods:**

**Isolation of Microorganism:** The traditional method for isolation and identification of an infecting *Rhizobium* strain are laborious and time-consuming. Isolation typically involves handling each nodule individually while both sterilizing the nodule surface and disrupting the nodule to release bacteria. Nodule disruption is accomplished by either cutting (El Hassan *et al*., 1986) stabbing(Brewin *et al*., 1983) or crushing(Bromfield *et al*.,1984), the nodule. Rhizobia culture was gram-negative rod shaped and it was maintained on yeast extract mannitol agar after periodic sub-culturing at 15-30 days intervals.

**Biochemical Analysis:**

**Starch Hydrolysis:** Amylase is an exoenzyme that hydrolyses (cleaves) starch, a polysaccharide into maltose, a disaccharide and some monosaccharides such as glucose. Culture was inoculated in starch agar medium plate and incubated in 48 hours at 37ºC (Aneja K. R., 2003), in the laboratory it is tested by performing the starch test to determine the absence or presence of starch in the medium by using iodine solution as an indicator.

**Triple Sugar Ion Agar Test:** TSI contains three carbohydrates: glucose (0.1%), sucrose (1%), and lactose (1%). TSI is similar to Kligler's iron agar, except that Kligler's iron agar contains only two carbohydrates: glucose (0.1%) and lactose (1%). Besides the carbohydrates mentioned, the medium also contains beef extract, yeast extract, and peptones which are the sources of nitrogen, vitamins and minerals. Phenol red is the pH indicator, and agar was used to solidify the medium and culture to be inoculated kept at 48 hrs for 37ºC. When any of the carbohydrates are fermented, the drop in pH will cause the medium to change from reddish-orange (the original color) to yellow.

**Gelatin Hydrolysis:** Proteins are organic molecules composed of amino acids, in other words proteins contain carbon, hydrogen, oxygen and nitrogen, though some proteins contain sulphur too. Prepared gelatin-agar medium and culture was inoculated at 37ºC for 4 to 7 days. After incubation, placed the tubes into a refrigerator at 4ºC for 15 minutes. Flood the incubated agar plates with mercuric chloride solution and allow the plates to stand for 5 to 10 minutes (Dubey R.C. ,2002).

**Urease Activity:** Some microorganisms have the ability to produce the enzyme urease. The urease is a hydrolytic enzyme which attacks the carbon and nitrogen bond amide compounds (e.g. urea) with the liberation of ammonia. Urease test was performed by growing the test organisms on urea broth or agar medium containing the pH indicator phenol red (pH 6.8). Inoculate the culture in slants and incubated for 24-48 hours at 37ºC and observed result.

**Effect of Selected Chemical and Biofertilizer with *Rhizobium* Culture on Growth of Wheat Plant (*Triticum aestivum*):** This experiment involved following steps:

**1. Preparation of *Rhizobium* inoculum:** *Rhizobial* isolate were inoculated in loop full amount in 250 ml. of 4 different sterile conical flask using Yeast Extract Mannitol broth medium and incubated at 25°C for 4-5

days to get heavy growth. Then this was used as *Rhizobium* inoculam.

**2. Preparation of Pots:** Sieved fertile soil was first washed 3-4 times to remove clay particles & taken in a disinfected tray and sterilized in hot air over for 2 successive days. Then it was filled in round bottom plastic bags of 13 cm length & 17.5 cm width under aseptic conditions. These plastic bags had four holes created initially at the bottom. The experiment was run in a set, each containing seven pots labeled as A (Simple soil as control), B, C, D, E, F, G. seven pot was labeled. Manure, NPK, *Rhizobium* culture inoculum and charchol were mixed with equal amount of ratio (250 gm and 250 ml).

**Pot A (Simple soil as control)** = Pot with wheat seeds.
**Pot B (Soil + *Rhizobium* culture inoculums)** = Pot with wheat seeds, respective only *Rhizobium* culture inoculum.
**Pot C (Soil + Manure)** = Pot with wheat seeds, respective manure.
**Pot D (Soil + Manure + *Rhizobium* culture inoculum)** = Pot with wheat seeds, respective
*rhizobial* isolate & manure.
**Pot E (Soil + NPK)** = Pot with wheat seeds respective NPK (chemical fertilizer).
**Pot F (Soil + NPK + *Rhizobium* culture inoculum)** = Pot with wheat seeds respective *rhizobial*
isolate and NPK (chemical fertilizer).
**Pot G (Soil + Charcoal + *Rhizobium* culture inoculum)** = Pot with wheat seeds respective
*rhizobial* isolate & charchol powder.

**3. Surface-sterilizing the seeds:** Checked the germination (percentage viability) of the wheat seeds and surface sterilized a sufficient number of uniform, undamaged seeds to give about 200 germinated seeds. Sterilized by immersing seeds in 3% sodium hypochlorite solution for 3-5 temperature (25-30°C) until the radicals were 0.5 - 1.0 cm long. Avoided overcrowding agar plates with the seeds. (Contact between seeds in an overcrowded plate increases the risk of cross-contamination from a partially sterilized seed to neighboring seeds. Uncrowded plates (approximately 25-30 seeds) produce more uniform and better germination due to better availability of moisture.)

**4. Planting and inoculating of seeds:** Followed the above method for planting and inoculating the seeds in each pot. Label the pots and indicate assignment. Group the treatments according to assignment and keep them separated. It is mandatory to observed that the growth of the wheat seeds (leaves length and number of leaves) at a regular interval of 3 days.

**Result and Discussion**
To the present investigation colonies of *Rhizobium* sp. were obtained on YEMA medium after incubation at 30ºC for two days. General microscopic view of the isolates showed them to be rod cells and germ negative in nature (Fig.2). It was observed that rhizobial cells do not produce gelatinase enzymes as medium containing gelatin solidified when kept at 4ºC for 30 as well as 60 min. (Fig.6). Nagative gelatinase activity is also a feature of *Rhizobium* (Hunter *et al.,*2007). Positive results were obtained from the starch hydrolysis assay. On subjecting inoculate plates to iodine test, clear zones around the colonies were seen and the colonies turned yellow in appearance, whereas, blue colour appears on no growth areas (Fig.4). This indicates that the isolates have the potential to hydrolyze starch present in the medium. It was observed that *Rhizobium* strains can utilize starch obtained from different sources (De Oliveria *et al.,* 2007). Yellow slants and red butt (Fig.5) was obtained showing the utilization of glucose and sucrose in the

triple sugar ion agar medium(Hajnaa A.,1945). No such studies have been conducted on *Rhizobium* strains. Rhizobial cells were able to grow on the urease media showing the utilization of urea as source by the *Rhizobium* (Fig.7). The use of plant growth-promoting bacteria to increase the soil fertility and improve growth and yield of agronomical important crops is a significant alternative to chemical fertilizers in sustainable agriculture (Yadegari *et al.,* 2010). In pot experiment with non-sterile soil showed a significant effect of some fertilizers when co-inoculated with *Rhizobium* on nodulation and growth parameters of wheat in respect to inoculation with *Rhizobium* alone and compared with simple soil as control, soil and manure, soil and chemical fertilizer etc. Co-inoculation of wheat with *Rhizobium* was found to positively influence nodule number (Table-2, 3 and Graphs shown), and in the case of plant growth number of leaves and length of leaves were increased.

**Table 1: Morphology and biochemical characterization of the bacterial isolates-**

| **Biochemical Characters** | **Bac-1** |
|---|---|
| Gram's reaction | - |
| Shape | Rod |
| Starch hydrolysis | + |
| Urease activity | + |
| Gelatin hydrolysis | - |
| Triple Sugar Ion Agar test | + |
| **Identified Bacteria** | ***Rhizobium* sp.** |

Key: (+) = Positive, (-) = Negative, sp.= Species, A= Acid, Bac. = Bacteria

**Table 2: Effect of selected chemical and biofertilizer with *Rhizobium* culture inoculums on growth of wheat plant (*Triticum aestivum*) of Day third, sixth, ninth –**

**Day Zero-** It was a seeds planting day, so no growth was obtained.

| **Source** | **Day Third** | | **Day Sixth** | | **Day Ninth** | |
|---|---|---|---|---|---|---|
| | **Leaf Length (cm.)** | **No. of Leaves (cm.)** | **Leaf Length (cm.)** | **No. of Leaves (cm.)** | **Leaf Length (cm.)** | **No. of Leaves (cm.)** |
| Pot A (Simple Soil as Control) | 5-7 | 5 | 3-4 | 13 | 18-20 | 16 |
| Pot B (Soil+*Rhizobium* sp. inoculums) | 3-7 | 20 | 7-8 | 27 | 16-17 | 32 |
| Pot C (Soil + Manure) | 2-5 | 11 | 8-10 | 14 | 16-18 | 14 |
| Pot D (Soil + Manure + *Rhizobium* sp. incls.) | 3-8 | 37 | 12-15 | 46 | 23-25 | 70 |
| Pot E (Soil + NPK) | Nil | Nil | 1-2 | 02 | 3-4 | 09 |
| Pot F (Soil + NPK + *Rhizobium* sp. incls. ) | Nil | Nil | 2-3 | 08 | 6-7 | 25 |
| Pot G (Soil + Charcoal + *Rhizobium* sp. incls.) | 3-7 | 27 | 7-8 | 35 | 18-19 | 50 |

**Table 3: Effect of selected chemical and biofertilizer with *Rhizobium* culture inoculums on growth of wheat plant (*Triticum aestivum*) of Day twelveth, fifteenth, eighteenth –**

| Source | Day Twelveth | | Day Fifteenth | | Day Eighteenth | |
|---|---|---|---|---|---|---|
| | Leaf Length (cm.) | No. of Leaves (cm.) | Leaf Length (cm.) | No. of Leaves (cm.) | Leaf Length (cm.) | No. of Leaves (cm.) |
| Pot A (Simple Soil as Control) | 22-26 | 18 | 26-27 | 39 | 26-28 | 41 |
| Pot B (Soil+*Rhizobium* sp. incls.) | 19-20 | 40 | 20-22 | 40 | 19-23 | 45 |
| Pot C (Soil + Manure) | 18-19 | 16 | 23-25 | 20 | 25-27 | 22 |
| Pot D (Soil +Manure + *Rhizobium* sp. incls.) | 25-27 | 110 | 27-30 | 115 | 27-30 | 120 |
| Pot E (Soil + NPK) | 7-9 | 10 | 12-15 | 15 | 13-16 | 15 |
| Pot F (Soil + NPK + *Rhizobium* sp. incls.) | 11-13 | 28 | 16-18 | 29 | 16-18 | 29 |
| Pot G (Soil + Charcoal + *Rhizobium* sp. incls.) | 22-25 | 70 | 25-27 | 100 | 25-28 | 112 |

**Biochemical Analysis:**

Fig 1. Root nodules of *Pisum sativam*

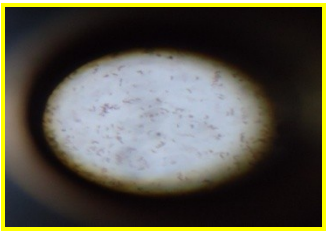


Fig 2. Gram Negative Bac. (*Rhizobium* sp.)

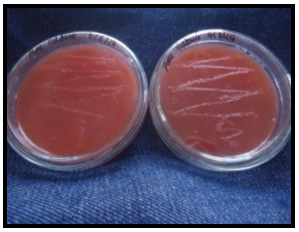


Fig 3. Pure Culture of *Rhizobium* sp.

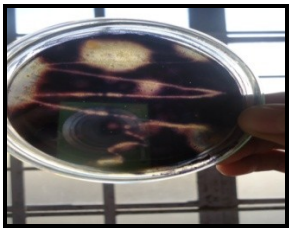


Fig 4. Starch Hydrolysis (Positive)

Fig 5. TSI Test (Positive)

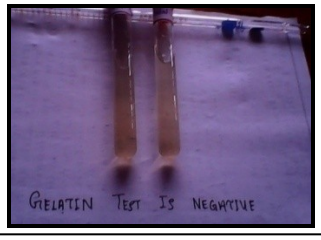



Fig 6. Gelatin Hydrolysis (Negative)

Fig 7. Urease Test (Positive)

**Pot Culture Method:**

**Zero Days growth of Wheat (*Triticum aestivum)* Planting:**


Fig 8. Plant growth on Wheat (***Triticum aestivum) of Zero Day***

**Eighteenth Days growth of Wheat (*Triticum aestivum)* Plant:**

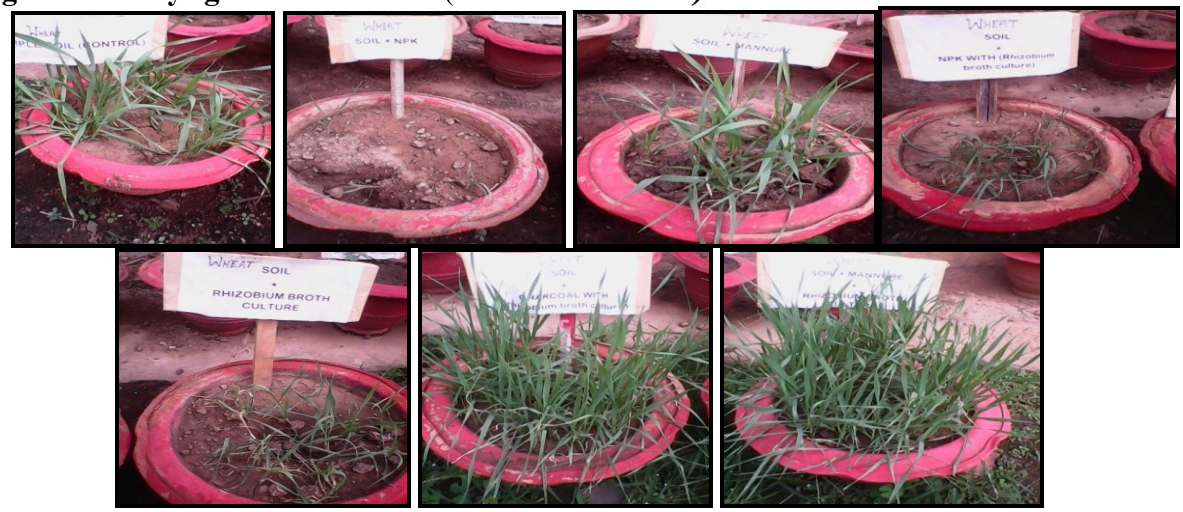
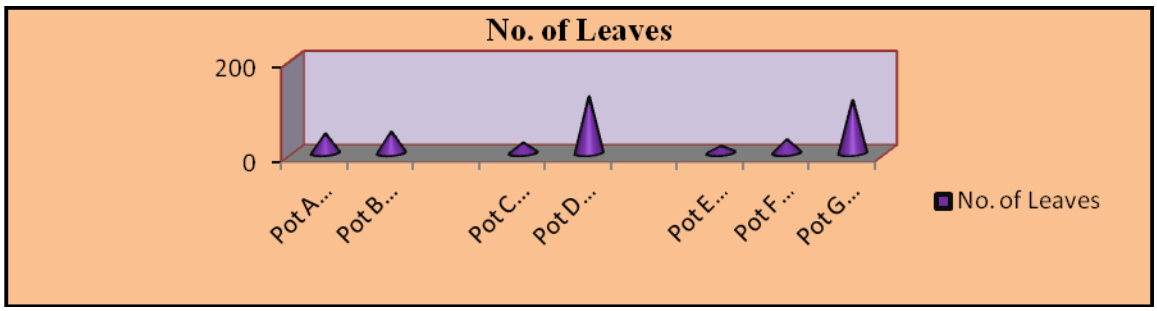
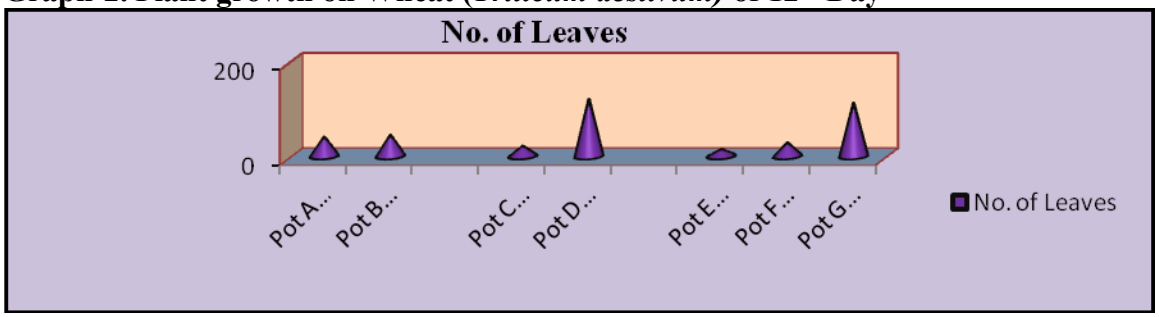



Fig. 9 Plant growth on Wheat (***Triticum aestivum)*** of **Eighteenth Day**

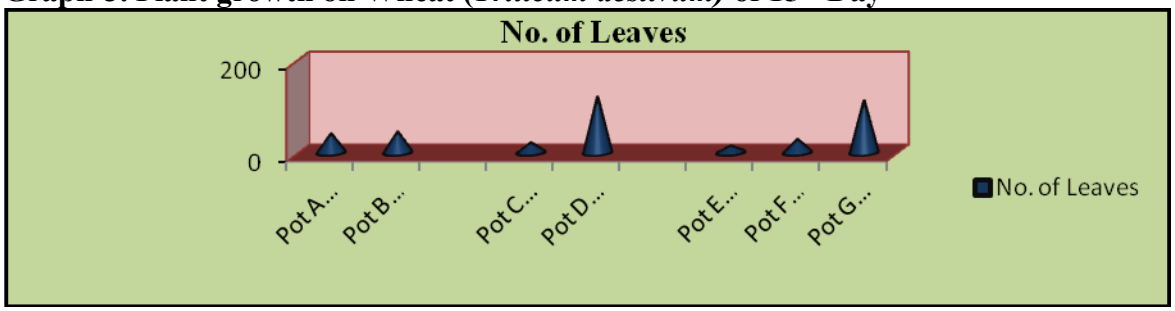
**Graph-1. Plant growth on Wheat (*Triticum aestivum)* of 9th Day**



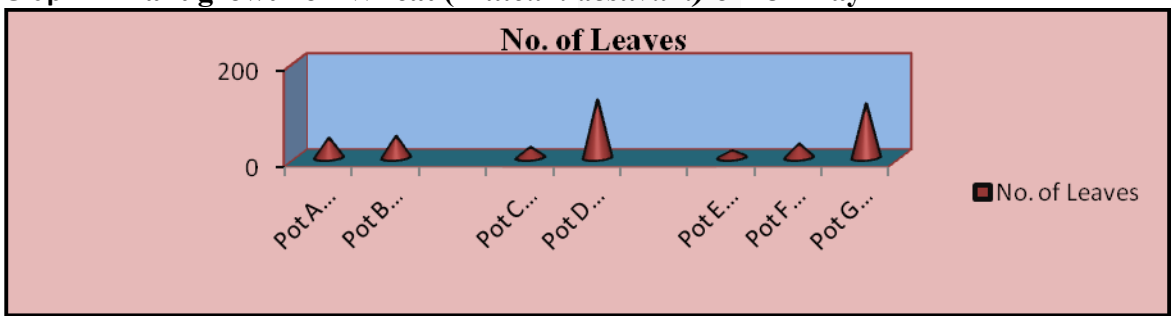
**Graph-2. Plant growth on Wheat (*Triticum aestivum)* of 12th Day**




**Graph-3. Plant growth on Wheat (*Triticum aestivum)* of 15th Day**




**Graph-4. Plant growth on Wheat (*Triticum aestivum)* of 18th Day**




## Conclusion

The present study demonstrated a significant positive effect of co-inoculation with *Rhizobium* sp. on growth, N and P contents of common wheat plants compared to inoculation with *Rhizobium* added with manure in soil. These data suggest that *Rhizobium* sp. can be used in further investigations as a potential agent of new biofertilizer for improved monocot plant production to expressing plant growth-promoting ability in this study should be tested further with the aim of confirming the good results under field condition.

## References:

1. Aneja, K. R. (2003). "Experiments in Microbiology Plant Pathology and Biotechnology." 4th edition, *New Age International Publishers,* New Delhi, India.
2. Brewin, N. J., Wood, E. A. and Young, J. P. W. (1983). "Contribution of the symbiotic plasmid to the competitiveness of *Rhizobium leguminosarurn." J. Gen. Microbiol.* 129, 2973-2977.

3. Bromfield, E. S. P. (1984). "Variation in preference for *Rhizobium meliloti* within and between *Medicago sativa* cultivars grown in soil." *Appl. Environ. Microbiol.* 48: 1231-1236.
4. De Oliveria, A. N., de Oliveria, L. A., Andrade, J. S. and Chagas, J.A.F. (2007). "*Rhizobia* amylase production using various starchy substances as carbon substrates." *Braz. J. Microbiol*. 38: 208-218.
5. Dubey, R.C. (2002). "Practical Microbiology." first edition, *S. Chand publication* New Delhi.
6. El Hassan, G. A., Hernandez, B. S. and Focht, D. D. (1986). "Comparison of Hup trait and intrinsic antibiotic resistance for assessing rhizobial competitiveness axenically and in soil." *Appl. Environ. Microbiol.* 51: 546-551.
7. Gauri, Singh, A. K,. Shailja, P., Manjinder, K. B., Alemayehu, W. (2009). "The effect of indigenous Root Nodulating Bacteria on Nodulation and Growth of faba bean (*Vicia faba*) in low input agricultural systems of Tigray Highlands, Northern Ethopia." *MEJS* (Mekelle University) 1(2): 30-43.
8. Graham, P., H., (2004). "Nodule formation in legumes." Microorganisms in The *encyclopedia of microbiology,* edited by M Schaechter, Consulting editor J Lederberg (Acadmic Press, New York): 715-724.
9. Gwyn, A., Beattie and Handelsman, Jo. (1989). "Inexpensive method for isolating and identifying *Rhizobium leguminosarum* by.*phaseoli* strains from bean root nodules."
10. Hajnaa, A., (1945). "Triple- Sugaar Ion Medium for the identification of the intestinal group of bacteria." *J. Bacteriol*. 49: 516-517
11. Hunter, W., J., Kuykendall, L., D. and Manter, D., K., (2007). "*Rhizobium* sp. Isolated from a Bioreactor." *Curr. Microbiol.* 55: 455-460.
12. Olivera, Stajković, Bogić, Miličić, Dušica, Delić, Bai, Y., Aoust, D. F., Smith D.L. and Driscoll, B.T. (2002). "Isolation of plant growth promoting *Bacillus* strains from soybean root nodules." *Can. J. Microbiol*. 48: 230-238.
13. Yadegari, M., Asadi Rahmani, H., Noormohammadi, G., Ayneband, A. (2010) "Plant growth promoting rhizobacteria increase growth, yield and nitrogen fixation in Phaseolus vulgaris." *J. Plant Nutr.,* 33 (12): 1733-1743.