

## ISOLATION AND CHARACTERIZATION OF *RHIZOBIUM* FROM ROOT NODULES OF *ARACHIS HYPOGAEA* L. (GROUNDNUT)

May Thazin Aung<sup>1</sup>, Phyu Phyu Oo<sup>2</sup>

### Abstract

The present study was to isolate the beneficial nitrogen fixing bacteria *Rhizobium* from root nodules of *Arachis hypogaea* L. (Groundnut). Plant sample and root nodules sample were collected from cultivated field of Munkhrain Quarter, Myitkyina Township in Kachin State. This study was done at Research Center, University of Myitkyina from January to May 2019. The five selected *Rhizobium* strains ( MTZA 1, MTZA 2 , MTZA 3, MTZA 4, MTZA 5) were subjected to culture on Yeast Mannitol agar ( YMA ) and Yeast Mannitol Broth (YMB). The method of Vincent (1970) was used for isolation of *Rhizobium* from root nodules. *Rhizobium* strains were medium sized, rod shaped, Gram negative and no spore. Colonies were sticky appearance, circular, varying from flat to raise shaped, margin entire and milky white colour. The *Rhizobium* strains were aerobic, motile, turbidity and the positive chemical reaction was observed in Catalase, Lactase, Glucose Peptone agar (GPA), Triple Sugar Iron Agar and Antibiotic test. The negative chemical reaction was observed in Gelatin Hydrolysis and Methylene blue test.

**Keywords:** *Rhizobium*, *Arachis hypogaea* L. isolation, characterization, biochemical tests.

### Introduction

The groundnut, *Arachis hypogaea* L., a highly nutritious food, is rich in protein, minerals and vitamins. The groundnut contain fat 34 - 54% and very important in crop rotation system as they help in biological nitrogen fixation. Groundnut, *Arachis hypogaea* L., a member of family Fabaceae is usually nodulated by rhizobia of genus *Bradyrhizobium* demonstrated by Van Rossum *et al.* (1995).

Plants in Fabaceae through their symbiotic relationship with certain gram-negative soil bacteria, collectively known as rhizobia help to fix atmospheric nitrogen to obtain nutrients from the plant and producing nitrogen in process called nitrogen fixation (or) Biological Nitrogen Fixing (BNF) (Herridge *et al.* 2008).

Rhizobia is the group of soil bacteria that infect the roots of legumes to form root nodules. Rhizobia are found in the soil and after infection, produce nodules in the legumes where they fix nitrogen gas (N<sub>2</sub>) from the atmosphere turning it into a more readily useful form of nitrogen. From here, the nitrogen was exported from the nodules and used for growth in the legume. Once the legume dies, the nodule breaks down and releases the rhizobia back into the soil where they live individually or rein fact a new legume host (Herridge *et al.* 2008).

Nitrogen fixation used biological agents is mostly associated with legumes and contributes to the sustainable agricultural development (Marta *et al.* (2014). This is a very good example of a symbiotic relationship accounting to the beneficial plant microbe interaction (Francisco *et al.* 2016).

Biological nitrogen fixation (BNF) is the cheapest and environmental friendly procedure in which nitrogen fixing microorganisms interacting with leguminous plants, fix aerobic nitrogen into soil (Md. Jakaria *et al.* 2013). Atmospheric nitrogen fixation is carried out by microorganisms to fix forms to nitrogen, such as ammonia and nitrate to be used by the plants.

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Restoring, maintaining and increasing soil fertility are major agricultural priorities in many parts of the developing countries where soils are inherently poor in plant nutrients and the demand for grain food and raw materials is increasing rapidly. Sustainable production of crops cannot be maintained by using chemical fertilizers only. Nutrients used to be added from other sources such as organic manure and biofertilizer for providing soil fertility (Masharof *et al.* 2012).

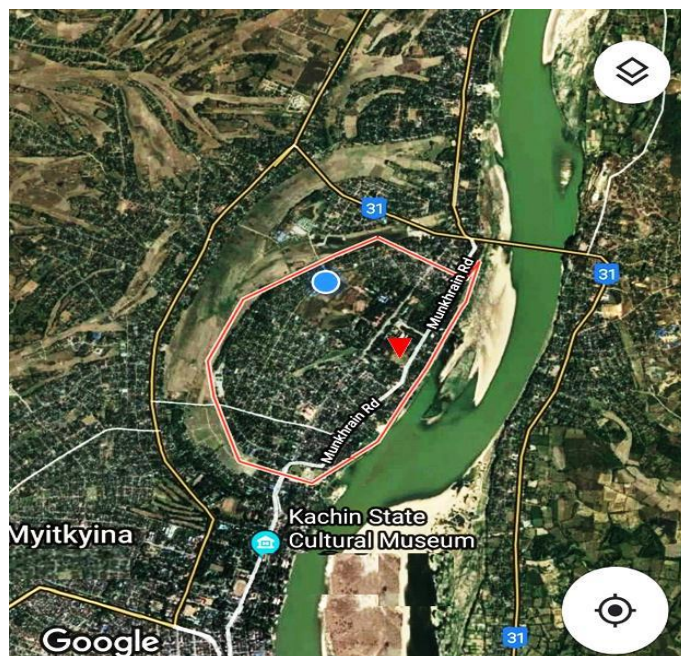
In recent years, there is increasing in fertilizers cost, so increases crop cost. In addition, chemical fertilizers have harmful effect on the environment. Therefore a process known as inoculation or biofertilization instead developed. Rhizobial inoculants are known as an alternative to the use of industrial nitrogen fertilizers and mean to maintain or improve soil fertility (Peoples *et al.* 1995, Alver *et al.* 2003 and Chalk *et al.* 2006).

The aims and objectives of this research was to isolate the *Rhizobium* from root nodules of *Arachis hypogaea* L. from Munkhrain Quarter, Myitkyina Township, Kachin State and to identify the bacteria by using morphological characters and some biochemical tests.

## Materials and Methods

### Study area and collection of root nodules of *Arachis hypogaea* L.

The sample of plant, soil and root nodules of *Arachis hypogaea* L. were collected from cultivated field of Munkhrain quarter, Myitkyina Township, Kachin State. (Figure 1)



**Figure 1** Location map of the study area

Source from Internet

### Identification of plant sample and soil sample

The collected plants were subjected to identify with the help of literatures (Hooker 1879; Backer 1964; Dassanayake 1981). The pH value of Yeast Mannitol Broth (YMB) medium was also detected with the help of portable pH meter. The experiments of isolation and biochemical

tests were carried out at the Department of Botany, Research Center, University of Myitkyina, from January to May 2019.

### Isolation of pure culture of *Rhizobium* strain isolation media, Vincent (1970)

In the isolation procedure, Yeast Mannitol Broth (YMB) and Yeast Mannitol Agar (YMA) were used as basal isolation and culture media. The composition of YMB & YMA was as follows.

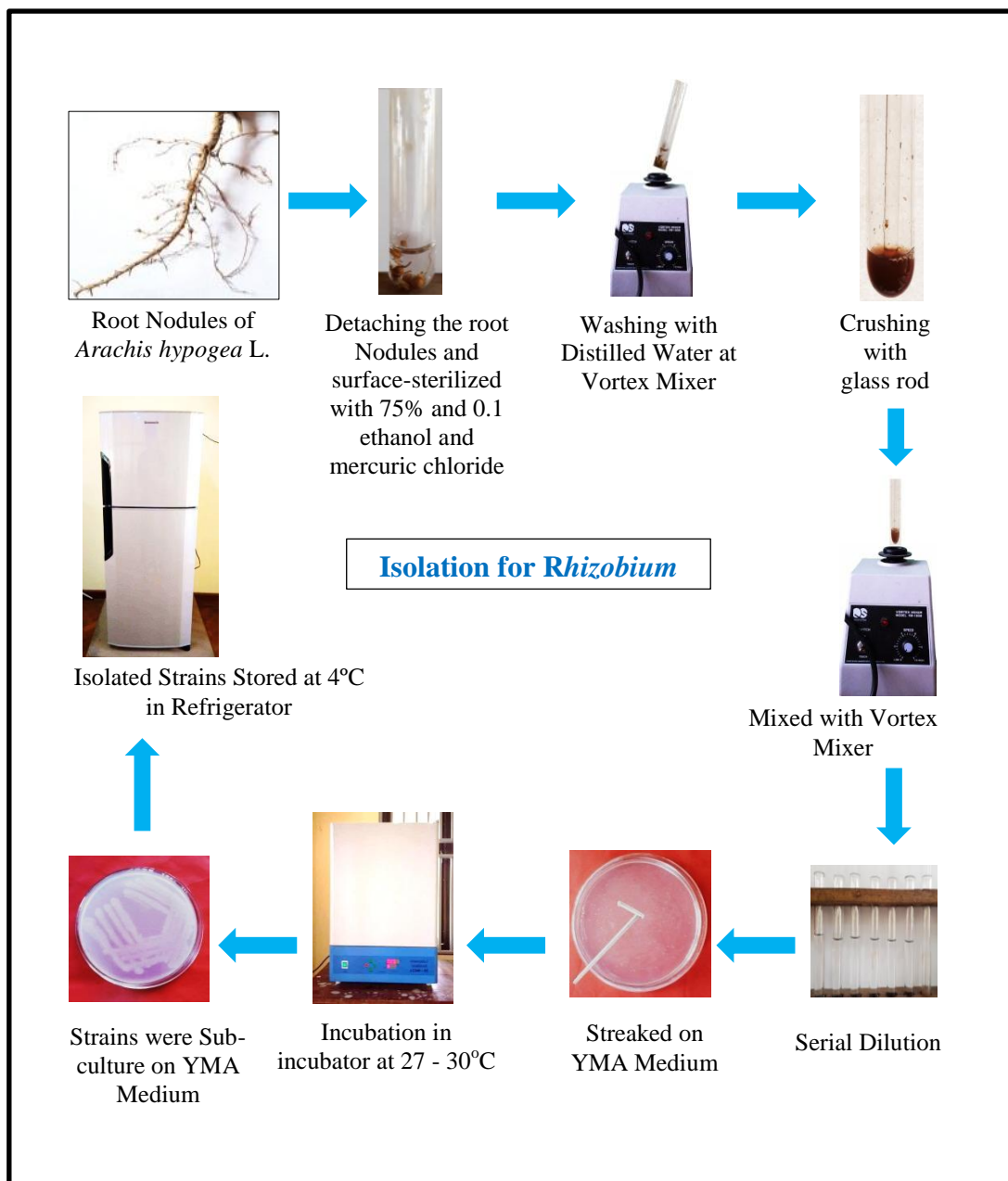
**Table 1 The composition of Yeast Mannitol Broth (YMB)**

Mannitol	10 g
Yeast Extract	500 mg
K <sub>2</sub> HPO <sub>4</sub>	500 mg
MgSO <sub>4</sub> .7H <sub>2</sub> O	200 mg
NaCl	100 mg
Distilled water	1 liter
pH	6.8

**Table 2 The composition of Yeast Mannitol Agar (YMA)**

Mannitol	10 g
Yeast Extract	500 mg
K <sub>2</sub> HPO <sub>4</sub>	500 mg
MgSO <sub>4</sub> .7H <sub>2</sub> O	200 mg
NaCl	100 mg
Agar	17 g
Distilled water	1 liter
pH	6.8

In the case of Yeast Extract Agar (YMA) preparation, 17 g/l of agar was dissolved into YMB and sterilization was done by autoclaving 15 lb/sq at 121°C for 15 minutes. 5 µL of nodule-bacterial suspension was dropped on to the culture plate with YMA medium and then were spread and incubated at 27 – 30° C in incubator single colony on YMA medium plates, selected from old cultures after 3 - 5 days. All strains were sub-cultured onto the YMA media to obtain pure culture for all experiments. Finally culture plates containing isolated bacterial strain were stored at 4°C in refrigerator for further experimental works (Figure 2).



**Figure 2** Isolation procedure of *Rhizobium* from root nodules

**Gram staining methods of *Rhizobium* strains**

The staining procedure was carried out according to the methods described by Santra *et al.* (1998).

Place a loopful of sample on a clean glass slide at angel on it. Prepare a smear/ thin film on a clean glass slide by dragging the slide over it. Air dry and heat fix the smear by passing through flame. Immerse the smear in crystal violet for one and a half minutes. Wash the slide with water and then immerse in Gram's iodine for one to one and a half minutes. At this time all cells appear violet. Wash with water and decolorize by shaking the slide gently for 10 - 15 seconds in acetone / alcohol fill the violet color comes off the slide. Immediately wash with

water and subsequently counter stain with safranin for 30 seconds. Finally, once wash the slide with water, blot dry and examine under the oil immersion lens of a microscope.

### **Biochemical and physiological characters of isolated five *Rhizobium* strains**

The biochemical tests were carried at 28°C for 48 hours old culture. All the tests were carried out with three replicates.

#### **Aerobic Test**

Different test tube of broth cultures are prepared and then one is inoculated at room temperature. Different culture of agar test tube is prepared as usual and isolated strains are made as sub-culture and incubated at room temperature. The patterns of growth are checked daily and the oxygen requirement of the isolated strain were recorded (Prescott *et al.* 2002).

#### **Motility Test**

Motility test was tested according to the procedure described by Prescott *et al.* 2002. The composition of medium contains Gelatin 10g, sodium chloride 5.0 g, beef extract 3g, 4.0g and distilled water 1000 ml. This medium was sterilized by autoclaving at 15 psi pressure 121°C for 15 minutes and the isolated strains were inoculated into stabbing medium  $\frac{3}{4}$  of the way to the bottom of the tube. During growth, motile bacteria will migrate from the line of inoculation to form a dense turbidity in the surrounding medium and non motile bacteria will grow only along the line of the inoculation.

#### **Turbidity Test**

YMB cultures medium and *Rhizobium* strain were prepared. This medium was sterilized by autoclaving at 15 lb pressure and 121°C for 15 minutes and the isolated strain were inoculated into the tube of YMB medium. Incubate at the 30°C for the pattern of growth is checked after two days and examine (MacFaddin 2000).

#### **Catalase Test**

Place a few drops 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) solution onto each slide culture and watch for immediate signs of bubbling, which represented positive test; absence of bubbles indicated a negative test (MacFaddin 2000).

#### **Glucose Peptone Agar GPA Test**

GPA test was performed to determine the capability of the *Rhizobium* strains to utilize glucose as the sole carbon for its growth medium. Glucose peptone agar (40g/L glucose, 5 g/L peptone, 15 g/L agar, distilled water 1000 ml) was also used (Deora & Singal 2010).

#### **Lactase Test**

Lactase test was performed to determine capability of *Rhizobium* to utilize lactose present in medium (10 g/L lactose, 5 g/L peptone, 3 g/L beef extract, 15 g/L agar, distilled water 1000 ml) as the sole carbon source for its growth (Kucuk *et al.* 2006).

#### **Methylene Blue Test**

Methylene Blue Test was performed to check the growth of the isolates. In this test, methylene blue dye (1 mL) was added to the YMB (10 mL) and inoculated with *Rhizobium*. Inoculation was given at 30°C for 24 – 48 hours and observations were made (Gao *et al.* 1994).

### Gelatin Hydrolysis Test (Liquefaction Test)

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. The actively grown cultures were inoculated in nutrient gelatin medium (5 g/L peptone, 3 g/L beef extract, 12 g/L gelatin, distilled water 1000ml) and grown for 48 hours. On subjecting the growing culture to low temperature at 4°C for 30 minutes - 60 minutes. The cultures which produce gelatinase remains liquefied while others due to the presence of gelatin become solid (Aneja 2003).

### Starch Hydrolysis Test

The test was performed to determine capability of *Rhizobium* strains to use starch as carbon source. Starch agar medium (5g/1 peptone, 2g/1 beef extract, 3g/1 potato starch, 15g/1 agar, distilled water 1000ml, pH 7.0) were inoculated with *Rhizobium* cultures and incubated at 30°C temperature for 48 hours. 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 (De Oliveira *et al.* 2007).

### Triple Sugar Iron Agar Test

The test was performed to determine the capability of isolates to use various carbohydrates source (sucrose, glucose, lactose) as media for growth. Triple sugar medium consists of beef extract 3g/L, yeast extract 3g/L, peptone 15g/L, NaCl 5g/L, lactose 10g/L, sucrose 10g/L, dextrose 1g/L, ferrous sulfate 0.2g/L, sodium thiosulfate 0.3g/L, phenol red 0.24g/L, agar 15g/L, distilled water 1000 ml). After inoculation and incubation color was observed on the butt and the slant. On the basis of capability of organisms for 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 (Kligler 1918).

### Antibiotic Resistance Test

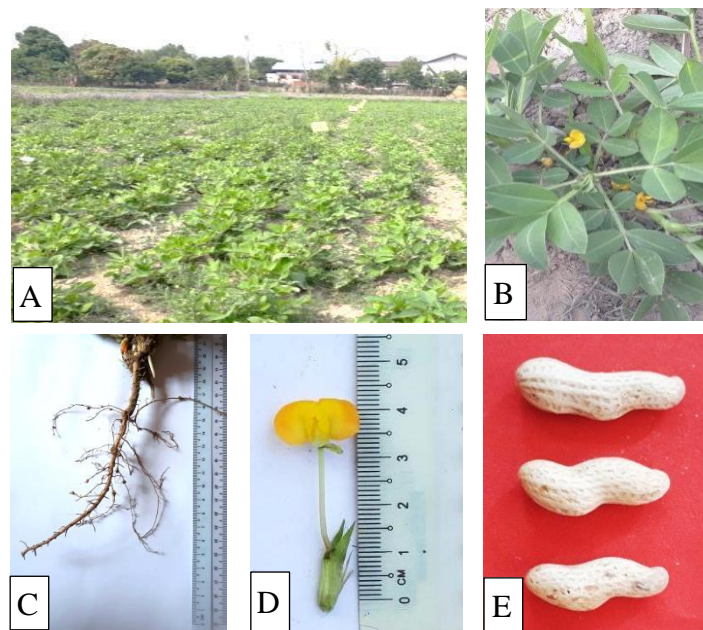
Antibiotic resistance of the *Rhizobium* isolates was tested against to, Streptomycin and Penicillin using paper disc diffusion method (NCCLS, 1999). Cultures were inoculated by swabbing with standard inoculums according to 0.5 McFarland tube over the entire agar surface. The agar surface was allowed to dry for 3-5 minutes before applying the antibiotic discs. Antibiotic disc were placed equidistantly on 90 mm petri plate using sterile forceps. The plates were incubated aerobically at 30°C for 48 hrs. Resistance to an antibiotic was detected by the inhibition zone formed around the discs. The antibiotic was used by Streptomycin (10 µg) and Penicillin (10 µg) (NCCLS 1999).

## Results

### Identification of *Arachis hypogaea* L.

Scientific name	-	<i>Arachis hypogaea</i> L.
Family	-	Fabaceae
Sub - Family	-	Papilionoideae
Myanmar name	-	Myae pe
English name	-	groundnut; peanut

Annual herbs; stems and branches angular, glabrous; leaves pinnately-compound, alternate, paripinnate, 4-foliotate; stipules lanceolate, acuminate at the apex; petiole terete, leaflets elliptic, opposite, 2-6cm by 1.0-3.5cm, green and glabrous on both surfaces, obtuse at the base, entire along the margin, rounded at the apex; petioles minute, pilose, caducous, flowers usually solitary, 1.5-1.8cm in diameter at the anthesis, yellow, bisexual, zygomorphic, pentamerous, hypogynous, bracteate, pedicellate, ebracteolate; bracts narrowly lanceolate, caducous; pedicels terete, short, pilose, calyx campanulate, 5-lobed, pale green, glabrous; tubes cylindric, 4.0-45cm long; lobes lanceolate, 0.8-1.0cm long; corolla papilionaceous; exserted; standard obovate, 0.6-1.2 cm long, yellowish with reddish lines; wings oblong, 4.8 m long, pale yellow; keel boatshaped, 6.8 mm long, pale yellow, acute at the apex; stamens 8 or 10, monadelphous, included; filaments filiform, alternately long and short, white, glabrous; anthers ditheous, oblong, versatile, pale brown, dehiscing by longitudinal slit, ovary superior, subsessile, oblong and unilocular, with 1 to 6 ovate ovules, the marginal placentae; styles filiform, 1.0-1.2 cm long, white glabrous; stigma simple; fruits indehiscent nut, torulose, developing underground, moniliform, commonly 1 to 3 seeded, pale brown; seeds oblongoid, with pale reddish papery coat, glabrous. (Figure 3 A, B, C, D and E)



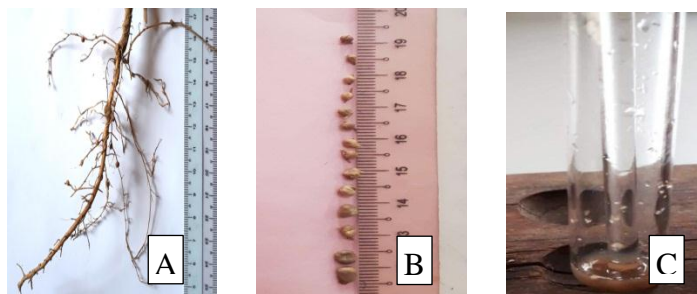
**Figure 3** Identification of *Arachis hypogaea* L.

- A. Cultivation field
- B. Habit
- C. Root nodules
- D. Flower
- E. Pod

### Characteristics of nodule sample

Nodule types and distribution on secondary roots from the plant, as nodules may be found on the lateral roots as well as the taproot. The shape and size of the nodules recovered from the collected plants were noted. Nodules size and shape vary with the *Rhizobia* and host plant

species. Effective nodules may be found on groundnut *Arachis hypogaea* L. about 1-3 mm in diameter, most formed on tap roots and lateral roots. An active N-fixing nodule contains a protein called leghemoglobin. Its presence in the nodule can be noted by the characteristic pink to red coloration. (Figure 4. A, B, C).



**Figure 4** Morphology of root nodules

- A. Roots nodules
- B. Detached root nodules
- C. Crushed root nodules

### Morphological characters of isolated bacterial strains

#### Cell morphology

*Rhizobium* sp. or root nodule bacteria were medium sized, rod-shaped cells, 0.5-0.9 micron meter in width and 1.0-3.0 micron meter in length and they usually aerobic metabolism. Optimal growth of most strains occurs at a temperature range of 25-30°C and a pH of 6.8-7.0 (Table 3, Figure 5. C).

#### Colony morphology

Colonies of *Rhizobium* were obtained on YMA agar medium after incubation at 30°C for 2-3 days. The colonies were having sticky appearance showing the production of mucous though at lower levels. Analysis of colony morphology indicated round colonies, a smooth margin on agar surface. Colonies may be white colored till 2-3 days of growth. Typical colonies had a diameter of 2-5 mm (Table 4, Figure 5. A,B).

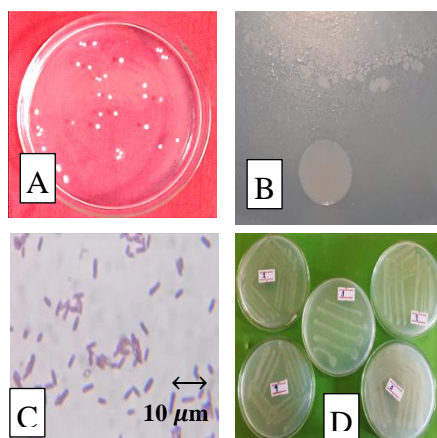
**Table 3** Cell morphological characters of isolated *Rhizobium* strains

Characters	Result
Bacterium shape	Rod
Oxygen demand	Aerobic
Gram's nature	Gram negative
Consistency	Turbid
Width	0.5 - 0.9 $\mu\text{m}$
Length	1.0-3.0 $\mu\text{m}$



**Table 4 Colony morphological characters of isolated *Rhizobium* strains**

Characters	Result
Growth on media	YMA Media
Temperature	30°C
Apperance	Sticky Mucous
Shape	Circular
Color	Milky White
Optical density	Opaque
Margin	Entire
Growth obtained	2-3 days
Diameter	2-5 mm

**Figure 5** Colonies forming *Rhizobium* on YMA medium

- A. Colonies
- B. Single colony
- C. Gram staining (cells)
- D. Sub-culture

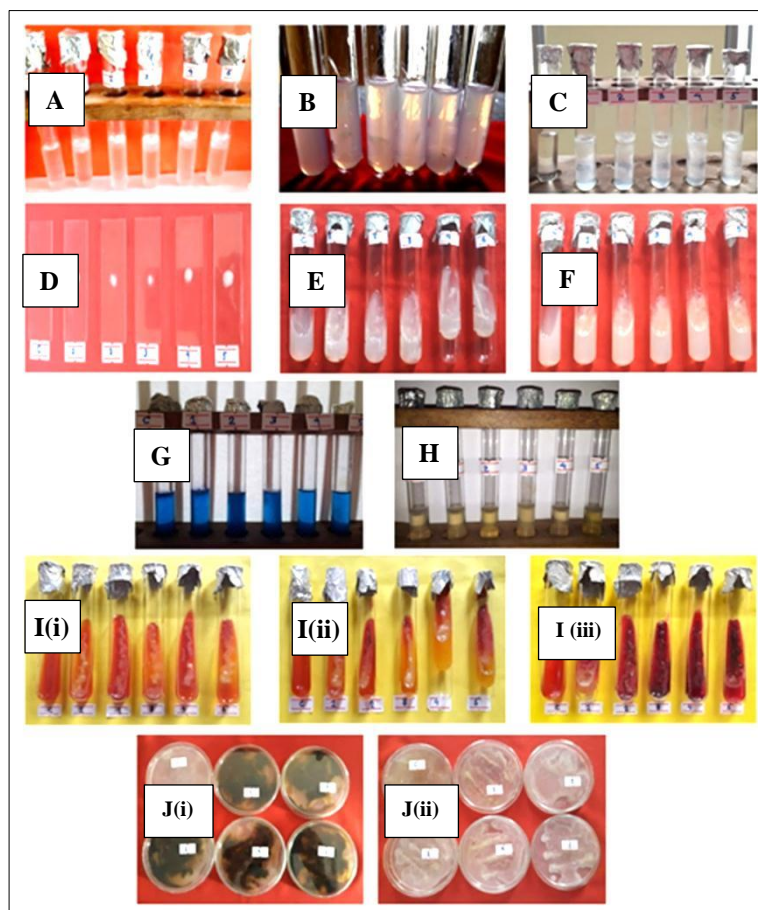
### Biochemical test and physiology characters from five isolated *Rhizobium* Strains

According to these results, the isolated five strains were Gram negative, mucous and aerobic respiration highly positive activities in catalase, turbidity, starch hydrolysis, triple sugar iron agar, lactose and antibiotic test. Negative activities in methylene blue and gelatin hydrolysis test. (Table 5, Figure 6, 7)

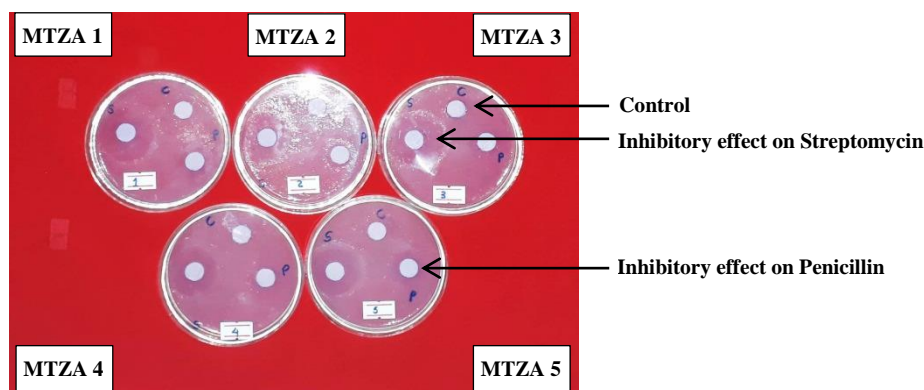
**Table 5 Biochemical characterization of isolated five selected *Rhizobium* Strains**

Sr No.	<i>Rhizobium</i> isolates	AT	MT	TT	CT	GPAT	LT	MBT	GHT	SHT	TSIAT	AT	
												S	P
1.	MTZA 1	+	+	+	+	+	+	-	-	+	+	+	+
2.	MTZA 2	+	+	+	+	+	+	-	-	+	+	+	+
3.	MTZA 3	+	+	+	+	+	+	-	-	+	+	+	+
4.	MTZA 4	+	+	+	+	+	+	-	-	+	+	+	+
5.	MTZA 5	+	+	+	+	+	+	-	-	+	+	+	+

(+) Positive, (-) Negative reaction, AT (Aerobic test), MT (Motility test), TT (Turbidity test), CT (Catalase test), GPAT (Glucose peptone agar test), LT (Lactase test), MBT (Methylene Blue test), GHT (Gelatin hydrolysis test), SHT (Starch hydrolysis test), TSIAT (Triple sugar iron agar test), AT (Antibiotic resistance test).

**Figure 6** Biochemical tests of five *Rhizobium* strains

- |                              |  |
|------------------------------|--|
| A. Aerobic test              | H. Gelatin hydrolysis test test        |
| B. Motility test             | I(i)TSIA test (24 hour)                |
| C. Turbidity test            | I(ii)TSIA test (72 hours)              |
| D. Catalase test             | I(iii)TSIA test(48 hours)              |
| E. Glucose peptone agar test | J.(i) Starch hydrolysis                |
| F. Lactase test              | J.(ii)Starch hydrolysis (after iodine) |
| G. Methylene blue test       |  |



**Figure 7** Antibiotic resistance test of isolated five *Rhizobium* strains Against Streptomycin and Penicillin

### Discussion and Conclusion

In the present study, *Rhizobium* bacteria was isolated from root nodules of *Arachis hypogaea* L. collected from Munkhrain quarter, Myitkyina Township, Kachin State.

*Arachis hypogaea* L. a member of the legume family (Fabaceae) is an annual herb. The leaves are alternate and pinnate with four leaflets (two opposite pairs each leaflet is 1-7 cm long, 1-3 broad. Peanut flowers are borne in axillary clusters above the ground. The pods usually containing from one to three seeds. Each seed is covered with a thin papery seed coat. In the present work, the *Arachis hypogaea* L. were similar to those describe by Katarzyna *et al.* (2011).

According to the Sarah *et al.* (2015) the colonies were Gram negative, milky white, glisterintening and circular in shape. In the present study, five isolates showed the same colony characteristics, after 48 hours of incubation, but the colonies were not translucent. This did not agreed with Sarah *et al.* (2015).

YMA medium was used for the growth of rhizobia isolates. Similar findings were made by Kumari *et al.* (2010) for the characterization of *Rhizobium* isolates from *Indigofera* species. The morphological and cultural characteristics of isolated bacteria from nodules of *Arachis hypogaea* L. incitated that the isolated strains were grown on YMA agar plates, the colonies of isolates appeared in 2-3 days at 30°C as mentioned in the results. This agreed with Burton (1967).

According to Swe Wut Hmone (2014), The bacterium was rod shaped, Gram negative, aerobic, non-spore forming and motile. It showed positive chemical reaction in aerobic, motility, catalase, glucose peptone agar (GPA), methylene blue and triple sugar iron test. In gelatin hydrolysis test, showed the negative chemical reaction. This agreed with Burton (1967). All the strains were shown positive result.

Tittsler and Sandholzer (1936) stated that the motility is observed visually by diffusing growth spreading from the line of inoculation. Certain strains of motile bacteria will show diffuse growth throughout the entire medium, while others may show diffusion from one or two points only, appearing as nodular growths along the stab line. Non-motile organisms grow only along the line of inoculation. In the present work of the motility test result indicated that individual isolated bacteria moving in random direction showing motility. This agreed with Tittsler and Sandholzer (1936). MacFaddin (2000) stated that the cell mass is directly proportional to cell

number. Cells mass and number are also obtained by optical density method. Turbidity is developed in the liquid medium due to the presence of cells which make cloudy appearance to the eyes. In Turbidity test of the present study, all five strains were showed positive result. In the present study of turbidity test was in agreement with MacFaddin (2000).

MacFaddin (2000) stated that the organisms containing the catalase enzyme will form oxygen bubbles when hydrogen peroxide ( $H_2O_2$ ) exposed to it. In the present work, bubbles were appeared to show the positive catalase. This agreed with MacFaddin (1967). All five strains were showed positive result.

In glucose peptone agar test, all five strains were showed negative result. Wei *et al.* (2003) suggested that methylene blue was used as an agent against the growth of the microorganism. Rhizobial cells were unable to grow on medium containing 0.1% methylene blue were also suggested that one of the strain showed growth on medium containing 0.1% methylene blue. Therefore, the present research agreed with those finding by Gao *et al.* 1994 and Singh *et al.* (2008). In methylene Blue test, all five strains were shown negative result.

Kucuk *et al.* (2006) stated that the lactose is a confirmatory test for *Rhizobium* and these are able to utilize lactose as carbon source. In the present work, pure bacteria isolate was able to grow on lactose. In lactase test, five strains were shown positive result.

Hunter *et al.* (2007) stated that the gelatin hydrolysis test was observed that rhizobial cells do not produce gelatinase enzymes as medium containing gelatin solidified when kept at 4°C for 60 minutes. Negative gelatinase activity is also a feature of *Rhizobium*. Therefore, the present research was agreed with Hunter (2007) and isolated strain may be genus *Rhizobium*. In gelatin hydrolysis test, all five strains were showed negative result. In starch hydrolysis test, all five strains were showed positive result after iodine.

According to Hajnaa (1945), yellow slants and red butt by using the utilization of glucose, lactose and sucrose in the triple sugar iron agar medium. In the present study of triple sugar iron agar test, showed yellow slants and red butt. Therefore, the present research agreed with Hajnaa (1945). In Triple Sugar Iron Agar test, of five strains positive result in 24 hours, 72 hours and 48 hours.

Hangaria *et al.* (2000) stated that the *Rhizobium* isolates were sensitive to tetracycline, kanamycin and streptomycin antibiotics. Detection of antibiotic resistance of the *Rhizobium* isolates was tested against Penicillin and Streptomycin using disc diffusion method NCCLS (1999). In the present study, all five strains were showed that antibiotic activities of Streptomycin and Penicillin were used to showed clear zone. Therefore, the present study was agreed with Hungaria *et al.* (2000); Sharma (2009); NCCLS (1999) and Bauer *et al.* (1966).

The present study, the five selected *Rhizobium* strains were isolated from root nodules of *Arachis hypogaea* L. collected from cultivated field of Munkhrain quarter, Myitkyina Township, Kachin State. *Rhizobium* sp- or root nodules bacteria were medium sized, rod-shaped cells. Colonies were obtained on YMA agar medium after incubation at 30°C for 2-3 days. Colonies may be milky white colored till 2-3 days of growth. The isolated five strains were Gram negative, mucous and aerobic respiration, highly positive activities in catalase, turbidity, starch hydrolysis, triple sugar iron agar, lactase and antibiotic test. Negative activities in methylene blue and gelatin hydrolysis tests.

According to cell and colony morphology as well as some biochemical test, the isolated strain of bacteria were confirmed as genus *Rhizobium*. This organism is believed to increase better agricultural practices when inoculated in plant legumes. The *Rhizobium*, nitrogen fixing bacterium is the essential feature of leguminous plants. Increased cultivation of legumes is essential for the regeneration of nutrient-deficient soils and providing nutrients to human and animals.

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### References

- Alvers BJR, Boddey R.M, Urquiaga S. (2003). The success of BNF in Soybean in Brazil. *Plant Soil.*, 252:1-9.
- Aneja, K.R. (2003). *Experiments in Microbiology Plant Pathology and Biotechnology*, 4<sup>th</sup> edition, New Age International Publishers, Dew Delhi, India.
- Backer, C.A. and R.C Bakhuizen Van Den Brink. (1964). *Flora of Java*, Vol. 1. Rijksherbarium, Leyden, N.V.P. Norrdhoff.
- Burton, J.C. (1967). *Rhizobium culture and use*. In: Pepler, H.J. (E.d.), *Microbial Technology*, Van Nostrand Reinhold, New York, pp. 1-33
- Chalk. PM, Souza R. Urquiaga S, Alves BJR and Boddey RM. (2006). The role of arbuscular mycorrhiza in legume symbiotic performance soil *Biology, Biochem*: 38.2944-2951.
- Dassanayake, M.D. (1981). *A Revised Handbook to the Flora of Ceylon*, Vol.7. University of Peradeniya, Department of Agriculture Peradeniya, Sri-lanka.
- De Oliveira A. N., De Oliveira L. A, Andrade.J.S., Chargas J. A. F. (2007). *Rhizobia amylase production using various starchy substances as carbon substrates*. *Braz.J.Microbial*. 38; 208-216.
- Deora, G.S and Singal K. (2010). *Isolation, biochemical characterisization and preparation of biofertilizers using rhizobium strained for commercial use*, *Biosci, Biotech. Res. Comms* (2) 132-136.
- Francisco X.N., Clarisse B., Bernard R.G., and Marcio J.R. (2016). *Review Article the Role of Rhizobial ACC Deaminase in the Nodulation Process of leguminous Plants*.
- Gao, J.L., J.G. Sun, Y. Li, Wang E.T. and Chen W.X. 1994. *Numerical taxonomy and DNA relatedness of tropical rhizobia isolated from Hainan Province, Chin. Int. J. Syst. Bacterial*. 44:151-158.
- Hajnaa, A. (1945). *Triple Sugar Iron Medium for the identification of the intestinal group of bacteria*. *J. Bacterial*. 44:151-158.
- Herridge, D.F.M.B.Peoples and R.M. (2008). *Boddey Global inputs of biological nitrogen fixation to agricultural systems*. *Plant Soil* 2008, 311.1-18.
- Hooker, J. D. (1879). *The Flora of British India*, Vol. 2, L. Reeve & Co, 5 Hemrietta street, Convent Garden,, London
- Hungaria M, D.S. Andrade, L. M., Chueria. (2000). *Isolation and characterization of new efficient and complete bean (Phaseolus vulgaris L.) rhizobia in Brazil*. *Soil Biol. Biochem*. 32:1515-1528
- Hunter, W.J., Kuykendall, L.D and Manter, D.K. (2007). *Rhizobium selenireducens sp. Nov: A Selenite-Reducing, Proteobacteria Jsolated from a Bioreactor*. *Curr. Microbial*. 55. 455-460.

- Katarzyna Suchoszek – Lukaniuk, Arkadiusz Kozubek. (2011). Nuts and Seeds in Health and Disease Prevention. 2011.
- Kligler, I.J. (1918). Modification of culture media used in the isolation and differentiation of typhoid, dysentery and allied bacilli. *J. Exp. Med.* 28:319-322.
- Kumari B.S., Ram M.R., and Mallaiah K.V., (2010). Studies on nodulation, biochemical and analysis and protein of *Rhizobium* isolated from *Indigofera* sp Mal. *J. Microbiol.* 6(2); 133-139.
- Kucuk, C., Kivanc M, and Kinaci E. (2006). Characterization of *Rhizobium* Sp. Isolated from Bean. *Truk. J. Bial.* 30:127-132.
- Macfaddin, J.F. (2000). Biological test for the Identification of Medical Bacteria, 3<sup>rd</sup> ed., Lippincott, William & Wilkins. Co., Philadelphia, P.A.
- Marta L., Ana A. and Solange O. (2014). Legume growth-promoting rhizobia: An overview on the Mesorhizobium genus. *Microbiological Research.* 169: 2-17.
- Masharof HAKM, Musharrat HM, Hakim MA, Islam MM, Jannatul F. (2012). Isolation and selection of *Bradyrhizobium* from the root nodules of indigo plants (*Indigofera tinctoria* L.) *African Journal of Biotechnology*, 11(58)12183
- Md. Jakaria Al-Mujahidy, Md. Mchedi Hassan, Md. Mizanur Rahman, and A.N.M. Ma mun-or Rashid. (2013). Isolation and characterization of *Rhizobium* spp. and determination of their potency for growth factor production.
- National Committee for clinical laboratory Standards. (1999). NCCLS documents M-100-59, Performance Standards for Antimicrobial susceptibility testing 9th information supplement. NCCL: Wayne Pa.
- Peoples MB, Herridge D F. Ladha JK. (1995). Biological nitrogen fixation: an efficient source of nitrogen for sustainable agricultural production *Plant Soil*, 174:3-28.
- Presscott, L.M, Harley, J.P and Klein, D.A. (1996). *Microbiology*, 3<sup>rd</sup> ed. Wiliam C, Brown Publishers Dubuque, Iowa, 935p.
- Santra, S.C., T.P. Chatterjee, and Das, A.P. (1998). *Collage Botany Practical (Vol.II)*. New Central Book Agency (P) Ltd.
- Singh, B., Kaurand R. and Singhk. (2008). Characterization of *Rhizobium* strain isolated from the roots of *Trigonella foenumgraecum* (Fenugreek) *African Journal of Biotechnology.*, 7(20):3671-3676.
- Sharma, K. (2009). *Manual of Microbiology tools and 2<sup>nd</sup> edition*. Department of Botany, M.L. Sukhadia University. Udaipur, Rajasthan.
- Swe Wut Hmone. 2014. Isolation and characterization of *Bradyrhizobium* from Root Nodules of *Arachis Hypogea* L. (Groundnut). MSc Thesis, Department of Botany, Yadanabon University.
- Sarah Sobti, Hamdi Aissa Belhadj, Afaf DJAGHOUBI. (2015). Isolation and Characterization of Native Rhizobia Under Hyper-Salt Edaphic Conditions in Quargla. (Southeast Algeria)
- Tittsler, R.P. and L.A. Sandholzer. (1936). The use of semi-solid agar for the detection of bacterial motility. *J. Bacteriol.* 31:575-580.
- Vincent, J.M. (1970). *A Manual for the Practical Study of the Root Nodule Bacteria* IBP Handbook, Oxford; Blackwell Scientific Publications, Vol-15
- Van Rossum, D, F.P. Schuumans, M.Gills, A. Muyotcha, H.W. Van. Verseveld A.H. Stouthamer and F.C. Boogerd. (1995). Genetic and phenetic analysis of *Bradyrhizobium* strains nodulating peanuts (*Arachis hypogea* L.) roots. *Applied and Environmental Microbiol.* 61:1599-609.
- Wei, G.H., Z.Y. Tan, M.E. Zhu, E.T. Wang and W.X. Chen (2003). Characterization of rhizobia isolated from legume species within the genera *Astragalus* and *Lespedeza* grown in the Loess Plateau of China and description of *Rhizobia* Loessense sp. Nov. *Int. J. Syst. Evol. Microbiol.* 53: 1575-1583.