CHAPTER – 3

Materials and Methods

3.1. Location of Sampling

For the study the four valley districts of Manipur viz. Imphal East, Imphal West, Thoubal, and Bishnupur were selected as the sampling site. The bacterial samples are isolated from both rhizosphere soil and root nodules of the five legume plants during the month of September-October. The exact spot of collecting samples were done from the mentioned plant sources growing nearby agricultural field of the respective valley districts.

3.2. Selection of Legumes

The selection of leguminous plant sources were solely base on their availability or their economic importance for the state of Manipur, India.

3.2.1. *Sesbania sesban*

Fig. 2: A young *Sesbania sesban* plant.

Sesbania species are perennial plants capable of growing effort lessly in challenging locations and do not need any complex management for maintaining its productivity. They have many characteristics that make them attractive as multipurpose plants and potentially beneficial species for agricultural production systems. These species have had a long history of agricultural use in India, primarily

as green manures and as sources of forage (Anon. 1924, Whyte *et al.* 1953). Most of the early research on the use of perennial *Sesbania* for forage production was conducted in India (Patel 1966, Kareem and Sundararaj 1967). Annual *Sesbania* species e.g., *S. cannabina, S. rostrata*and *S. Bispinosa* are widely used in Asia as green manures in paddy rice cultivation mainly due to their capacity to withstand water logging. In this context, *Sesbania sesban* is used to a lesser extent which may be mainly because of their slow growth rate in comparison to other annual species. Sivaraman, (1951) reported that the use of *S. sesban* leaf as a green manure in southern India, results in the increase in rice yields upto 20-40%.

In the state of Manipur, India, *Sesbania sesban* are found growing in plenty in the wild as well as sometimes in cultivated manner in fields, open grounds and kitchen gardens. The sole reason for the plant to be growned by people is that it serves as a very good source of alternative vegetable. The young leaves and the young pods are consumed in various form of preparations.

3.2.2. Leucaena leucocephala

Fig. 3: Flowering *Leucaena leucocephala* tree.

Leucaena leucocephala has a worldwide reputation as a 'miracle tree' for its wide-ranging variety of uses and multiplicity of roles of the species. Most importantly, the leaves are highly nutritious for ruminants and several outstanding animal production data have been published approving the fodder value of Leucaena (B.W., Norton *et al.,* 1992).*Leucaena leucocephala* was also credited by Guevarra et al.,1978 for its very high nitrogen-fixing prospective, ranging from 600 - 1000 kg N_2 fixed. Just a few decades ago, acidity tolerant genotypes of *L. leucocephala* were identified to acidic Amazonian soils (Hutton 1984), signifying that the trees could be selected and grown in acidic soils, where they could efficiently fix nitrogen in association with acid-tolerant *Rhizobium* strains.

This thornless long-lived shrub or tree which may grow to heights of 7-18 m are also found growing in plenty almost everywhere in Manipur. However, the main importance of this plant in the state is regarding consumption by people of various communities. The young leaves and green pod are consumed either raw or cooked for various preparations. Its value and importance as an alternative vegetable in Manipur has climbed up rapidly in recent times.

3.2.3. *Parkia roxburghii*

Fig. 4: Fruiting *Parkia roxburghii* tree.

Parkia roxburghii (Tree Bean) in Manipur is considered as one of the most costly vegetable where both the flowers and pods are eaten. The price of the vegetable rises as soon as the fruit gets maturity. Being a leguminous plant, the tree bean is rich in protein and amino acids. Flowers, tender pods and seeds of this plant are edible and are a good source of proteins, fats, carbohydrates, vitamins and minerals compared to other legumes (Jekendra et al., 2009).In fruiting season a full grown plant can bear upto 10,000 -15,000 pods. The Manipuri people consume this vegetable as raw in preparation of "Singju" (a typical Manipuri salad), sometimes cooked with fish, in preparation of typical delicious curry the "Iromba" and as several other side dishes. Various other tribes in Northeast India such as Mizos, Garos, Kacharis, Nagas, Mikir setc. also consumed the pods as vegetables.

3.2.4. *Crotalaria junceae*

Fig. 5: Fruiting and flowering *Crotalaria juncea* plant.

Crotalaria juncea (Sunn hemp), a tropical Asian plant is generally considered to have originated in India, where it has been cultivated since prehistoric times (Montgomery 1954). The plant is a member of the legume family (Fabaceae), has great potential as an annually renewable, multi-purpose fiber crop. It is the most

important species of the *Crotalaria* genus, which is comprised of over 350 species located in the tropics and subtropics of both hemispheres. Being one of the most extensively grown green manure crops throughout the tropics, it is often grown in rotation with several different crop species (Kundu 1964; White and Haun 1965; Lai et al. 1967; Purseglove 1968; Srivastava and Pandit 1968; Barros Salgado et al. 1972; Mascarenhas et al. 1980; Rotar and Joy 1983).

In the context of Manipur, its sole utility of *Crotalaria juncea* lies in the consumption of its young leaves and buds as vegetable. Sometimes the plant is also grown by certain section of people as an ornamental plant. Its availability in plenty in different types of soil also makes it a choice for the present work as a source plant.

3.2.5. *Crotalaria pallida*

Fig. 6: Fruiting and flowering *Crotalaria pallida* plant.

Crotalaria pallida, an erect shrub, annual or short-lived perennial herb of 1.5 m or more tall does not have much of an economic importance in the state of Manipur. Its availability in plentiness only makes it a choice for the present work as a source plant.

3.3. Collection of Samples

For isolation of rhizosphere diazotrophs, soil samples were collected from the rhizosphere of the five selected wild legume plants grown nearby or around paddy fields in the valley districts of Manipur. Soil upto the depth of 20 cm are collected in germ-free polybags, then brought to the laboratory and kept at 4°C in the refrigerator before any examination. The soils are then serially diluted and plated on nitrogen free media (Burk's media) for the isolation.

For isolation of root nodules diazotrophs, the roots with nodules are carefully uprooted from wild legume plants and the roots are washed gently with tap water. Then the nodules are separately removed from the root along with little part of the root so as to avoid injuries to the nodules. The nodules are collected and put in 50% glycerol in plastic vials, brought to the laboratory and stored in -80°C in deep freezer. Aliquots of the macerated nodules are then spread plated of N_2 free Yeast Extract Mannitol Agar (YEMA) medium for isolation.

3.4. Bacterial Growth Media

3.4.1. Isolation of Rhizosphere diazotrophs

Burk's N-free medium comprising: 10g dextrose, 0.41g KH_2PO_4, 0.52g K_2HPO_4, 0.05g Na_2SO_4, 0.2g $CaCl_2$, 0.1g $MgSO_4.7H_2O$, 0.005g $FeSO_4.7H_2O$, 0.0025g $Na_2MoO_4.2H_2O$, and 15g agar for solid medium was used throughout the study (Wilson and Knight, 1952). The pH of the medium was adjusted to 7.

3.4.2. Isolation of Root nodule diazotrophs

Yeast extract mannitol agar (YEMA) comprising: 1g yeast extract, 10g mannitol, 0.50g dipotassium phosphate (K_2HPO_4), 0.20g $MgSO_4$, 0.10g NaCl, 1g Calcium Carbonate and 15g Agar for solid medium was used throughout the study (Zhang et al, 1991). The pH of the medium was kept at 7.

3.5. Isolation Procedure:

3.5.1. Rhizosphere Isolates

3.5.1.1. Serial dilution

a) A known amount (10g) of soil is taken and added in a conical flask containing 90ml of sterile blank to make 1:10 dilution (10^{-1}).
b) The flask is vigorously shaken on a magnetic shaker for 20-30 mins to obtain uniform suspension of microorganisms.
c) Six sterile test tubes were taken and labeled them as 10^{-2},10^{-3}….10^{-7}.
d) In each of the tubes 9ml of sterile water blank is poured.
e) 1ml of suspension from the conical flask is transferred into the test tube labeled 10^{-2} with a sterile pipette under aseptic conditions to make 1:100 dilution (10^{-2}).
f) Next dilution of 1:1000 (10^{-3}) is prepared by pipetting 1ml of suspension from 10^{-2} dilution into the test tube labeled 10^{-3}.
g) Further dilutions of 10^{-4} to 10^{-7} are prepared by pipetting 1ml suspension into the remaining sterile water blanks as prepared above.

For the present study only 10^{-4} to 10^{-7} dilutions are taken into consideration as priority is given more on the isolation of free living diazotrophic bacteria.

3.5.1.2. Inoculation

Burk's nitrogen free medium is prepared and let it cooled down to around 45ºC. The medium is poured into sterile petri plates and let to solidify. After solidification, soil suspensions is inoculated into the medium following spread plate method. In this method 0.2ml of the suspensions from each dilution of 10^{-4} to 10^{-7} were pipette into separate plates containing the medium using a sterile pipette and spread thoroughly using a spreader. The whole process is done under aseptic condition under a Laminar Air Flow hood.

3.5.2. Root Nodules Isolates

For the isolation of root nodule diazotrophs from roots nodules of the four selected legume plants (Parkia roxburghii is excluded due to lack of root nodules), the following procedures are carried out-

1. Root nodules with a marginal portion of root is cut off using a scalpel and washed free of soil using tap water.
2. The nodules are surface sterilized for 5 minutes in 0.1% Mercuric chloride in water.
3. Then repeatedly washed with sterile water to get rid of the chemical.
4. The nodules are then washed in 70% Ethyl alcohol for 3 minutes.
5. The step is followed by more washings with sterile water.
6. Few drops of sterile water is pipetted into another sterile petri plate and the portion of the nodules is added using a sterile forcep.
7. The root nodules are then macerated using a sterile glass rod to produce a milky fluid.
8. Serial dilutions of the suspension are then prepared to obtain sparse and distinct colonies when appropriate dilution is plated on YEMA.

3.5.2.1. Inoculation

Inoculation is done on Yeast Extract Mannitol Agar (YEMA) medium either by Spread plate method or Streak plate method-

For Spread plate, 0.2 ml of the root/nodule macerate is pipetted and spread on the surface of YEMA medium using a spreader. The plate is labeled and then incubated at 25-27°C for 3-4 days.

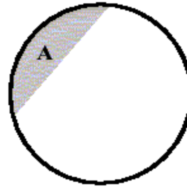
In Streak plate method, a sterile wire loop is used and a loopful of the root/nodule macerate is taken and streak it out on the YEMA medium. The plate is inverted and incubated at 25-27ºC for 3-4 days.

3.6. ISOLATION OF PURE CULTURE

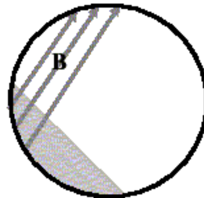
Pure cultures were isolated by streaking on new agar plates. The Streak-plate method is a rapid qualitative method which essentially involves spreading a loopful of culture over the surface of an agar plate. Although many types of procedures can be performed, only Four-way of Quadrant streak is performed in the present study.

1. Mixed culture about 5-7days old is taken and a single colony is touched using a sterile wire loop.

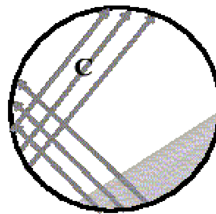
2. Holding the loop parallel with the surface of the new/fresh agar, the inoculums is smeared backwards and forwards across a small area (A) of the medium.



3. After flaming the loop and allowing it to cool, the plate is turned. The loop is streak from (A) across the agar to the area (B) in three straight lines.



4. The plate is turned again and the loop is again streak across the surface of the agar from area (B) to area (C).



5. The plate is turned again and the loop is streak on the agar surface from (C) to (D) as shown in figure below without touching the area (A).

6. The plates are then incubated upside down at 30°C for 72 hrs.

3.7. Preparation of stock culture

Glycerol stocks are prepared to save bacterial culture for a long period so that it could be used later on during the study.

1. 3 ml of Nutrient broth is inoculated with a single bacterial colony.

2. Then it is let to grow for 12-24 hrs at 28-29°C.

3. 1.4 ml of liquid bacterial culture is then pipetted each into 2 screw-cap eppendorf tubes.

4. Then 0.6 ml sterile 50% glycerol is added to each tube and mixed.

5. Finally, it is stored in a deep Freezer at -80°C.

3.8. MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS

Colony characteristics were done in the current study for preliminary characterization of the bacterial isolates by examining the growth of the isolates on solid media in petri plates. The colony characteristics like size, forms, margins, elevations and colony colour were taken into account as per the accepted methods described by Annon, (1957).

3.8.1. Hanging Drop Mount Method

This technique is an important procedure in microbiology by which the motility of the bacterial samples can be determined without any difficulties. The principle of this technique is based on the fact that due to the small size of the bacterium and close refractive index to that of water, do not allow them to be observed readily under unstained contion. By this technique the motility and the binary fission could be observed easily. This technique of motility test by Hanging Drop Mount Method was performed as described by Dubey and Maheshwari, (2002).

3.8.1.1. Requirements: Bacterial culture, Microscope, Cavity slide, Cover slip, Vaseline or petroleum jelly, Inoculating needle and Bunsen burner.

3.8.1.2. Procedure:

(i) First a drop of bacterial sample is placed in the centre of the cover slip.

(ii) It is then inverted over the well of the cavity slide in such a way that the drop does move or contact the side wall of the well.

(iii) Finally the edges of the cover slip were sealed by using Vaseline or petroleum jelly.

3.8.1.3. Observation:

The slide is taken for observation under a microscope, then it is focused under low power objective and a drop of oil immersion is placed on the cover slip. The motility of the bacterial sample can then be observed under oil immersion lens of the compound microscope. The motility or non-motility can be determined from the fact that bacteria that are definitely motile use to progress continuously in a given direction.

3.8.2. Gram Staining

Gram staining is an empirical method of differentiating bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell walls. This method was developed by Christian Gram in 1884. The importance of this determination to correct identification of bacteria cannot be overstated as all phenotypic methods begin with this assay.Gram staining was performed using regular practice as preliminary physiological test following the procedure described by Dubey and Maheshwari, (2002).

3.8.2.1. Procedure

1. First, a loopful of a pure culture is smeared on a slide and allowed to air dry.
2. Fix the cells to the slide by heat or by exposure to methanol. Heat fix the slide by passing it (cell side up) through a flame to warm the glass.
3. Crystal violet (a basic dye) is then added by covering the heat-fixed cells with a prepared solution. Allow to stain for approximately 1 minute.
4. Briefly rinse the slide with water. The heat-fixed cells should look purple at this stage.
5. Add iodine (Gram's iodine) solution (1% iodine, 2% potassium iodide in water) for 1 minute. This acts as a mordant and fixes the dye, making it more difficult to decolorize and reducing some of the variability of the test.
6. Briefly rinse with water.

7. Decolorize the sample by applying 95% ethanol or a mixture of acetone and alcohol. This can be done in a steady stream, or a series of washes.

8. Rinse with water to stop decolorization.

9. Rinse the slide with a counterstain (safranin or carbolfuchsin) which stains all cells red.

10. Blot gently and allow the slide to dry. Do not smear.

3.8.2.2. Observation:

Gram positive bacteria will retain the crystal violet and appear blue in colour. On the other hand, the Gram negative bacteria will not retain crystal violet and appear pink in colour due to Safranine.

3.9. BIOCHEMICAL CHARACTERIZATION:

Biochemical characteristics of the isolates were determined by performing several biochemical tests such as Catalase test, Nitrate reduction test, Citrate test, Urease test, Starch hydrolysis test and Oxidase tests. The tests were performed as per microbiological practical protocols described by Dubeyand Maheshwari, (2002) in Practical Microbiology book.

3.9.1 Catalase Test

Most of aerobes and facultative anaerobes have the characteristic of showing catalase activity. Actually these organisms utilize oxygen to produce hydrogen peroxide. The hydrogen peroxide is toxic to their enzyme system. Hence, these organisms produce an enzyme called catalase, which converts the hydrogen peroxide to water and oxygen. It is also that the reason of not surviving anaerobes in the presence of oxygen is H_2O_2 and absence of enzyme catalase.

$2H_2O_2$ _catalase_ $2H_2O+O_2$

3.9.1.1. Requirements

Bacterial cultures, Inoculation loop, Slide and H_2O_2.

3.9.1.2. Procedure

1. A colony is picked up from a plate and transferred on a microscopic glass slide in a drop of water.
2. Few drops of 3% H_2O_2 is put over the culture.

3.9.1.3. Observation:

Appearance of bubbles shows that the organism shows positive catalase activity. Absence of bubbles shows negative catalase activity.

3.9.2. Starch Hydrolysis test

Starch is an insoluble polymer of glucose which acts as a source of carbon and nitrogen for microorganisms which have an ability to degrade them. Starch degrading microbes transport the degraded form across the cytoplasmic membrane of the cell. Some bacteria possess the ability to produce amylase that breaks starch into maltose. The amylase is an extracellular enzyme which is released from the cell of microorganisms.

3.9.2.1. Requirements

Bacterial culture, inoculation loop, Starch agar medium, petri dish, Iodine.
Starch Iodine Medium composition:
Starch (20g), Beef extract (3g), Peptone (5g), Agar (15g) Iodine solution all diluted to 1 lt distilled water.

3.9.2.2. Procedure

1. Starch agar plates are prepared and streak with suitable culture.
2. The microbes are allowed to grow at 37ºC for 48 hrs.
3. Iodine solution is then pour in the plate.

3.9.2.3. Observation:

The blue-black colour appears due to formation of starch-iodine complex. If the area around streaked culture remain clear it indicates the degradation of starch due to production of amylase.

3.9.3. Nitrate reduction test

Certain bacteria use nitrate (NO3-) in place of oxygen as an external terminal electron acceptor. In the beginning, nitrate can be reduced to nitrite. In case of aerobic bacteria, oxygen is first used to prevent nitrate reduction and then utilize nitrite. The nitrite may further give rise to nitrogen, ammonia; nitrogen oxide (N2O).The enzyme reaction is catalyzed by nitrate reductase. The enzyme reaction is catalyzed by nitrate reductase as given below:

$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O$

3.9.3.1. Requirements-

1. Nitrate Broth comprising: 1g KNO_3, 5g Peptone, 3g Beef extract and 1 litre distilled water.
2. Nitrate test reagent comprising: 20g Zinc chloride, 4g starch, 2g Potassium iodide and 1 litre distilled water.
3. Powdered zinc metal.
4. Dilute Sulphuric acid.

3.9.3.2. Procedure

1. The nitrate broth is inoculated with a suitable culture and incubated at 37ºC for 48 hours.
2. 3 drops of nitrate test reagent is taken in a porcelain plate and 1 drop of sulphuric acid is added. To the mixture, 1 drop of culture is added.

3.9.3.3. Observation:

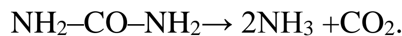
Appearance of blue colour indicates that nitrite is produced. If no blue colour develops, then there is two possible reason-

1. there is no occurrence of nitrate to nitrite reduction or
2. all the nitrite so formed must have been converted further to other products.

In such situation, zinc dust is added to each spot that appears to be negative for blue colour. Still if there is no blue colour, it means bacteria reduce nitrate and nitrite is formed. Another possibility is to get blue colour and it means that the bacteria did not reduce the nitrate.

3.9.4. Urease test

Urea is a waste nitrogenous material and excreted out by animals. Some bacteria degrade the urea into ammonia and CO_2. Due to production of ammonia, the urease production can easily be demonstrated by the following reaction:



$NH_2–CO–NH_2 \rightarrow 2NH_3 + CO_2$.

3.9.4.1. Requirements

Urea Broth Medium comprising: 20g Urea, 0.1g Yeast extract, 9g KH_2PO_4, 9.5g K_2HPO_4, 0.01g Phenol red, 1 litre Distilledwater. The final pH is adjusted to 6.8.

3.9.4.2. Procedure

1. Urea broth medium was taken in test tubes and inoculated with bacterial culture.
2. The cultures were then incubated at 37°C for 48 hrs.

3.9.4.3. Observation:

The phenol red indicator will turn pink due to alkaline nature of medium because of ammonia production. Otherwise, indicator will remain unchanged at acidic range of pH which shows no urease production by the given microorganism.

3.9.5. Oxidase test

Oxidase activity is one of the tests to differentiate certain group of bacteria. Certain bacteria like members of family Enterobacteriaceae are oxidase negative while Pseudomonas shows oxidase positive. A dye p-aminodimethylanilinoxalate or dimethyl-p-phenylenediamine hydrochloride is used which donates electron to cytochrome C, becomes oxidised, and produces a colour. For the present study the customary procedure was not followed and as an alternative oxidase test for bacterial isolates were performed using the **HIMEDIA,** Bacteriological differentiation discs, for oxidase testing, DD018-1VL.

3.9.5.1. Procedure-

1. Some amount of the bacterial colony is taken out carefully with the help of a loop and smeared on the oxidase test disc.

2. Whether the colour of the smeared area on the disc changes to blue or remain unchanged is noted.

3.9.5.2. Observation:

If the colour change to blue, it is Oxidase positive but if it remain unchanged it is negative.

3.9.6. Citrate test

Simmons citrate agar tests is performed to determine the ability of organisms to utilize citrate as a carbon source. Simmons citrate agar contains sodium citrate as the sole carbon source, ammonia as the sole source of nitrogen, other nutrients, and the pH indicator bromthymol blue. Citric acid is an intermediate of the metabolic product of Kreb's cycle which oxidises pyruvate to CO_2. The bacterium must have the ability to transport it across the membrane. Citrate test is part of the IMViC tests and is helpful in differentiating the*Enterobacteriaceae*.

3.9.6.1. Requirements:

(i) Simmon's citrate agar medium comprising: 2.0g Sodium citrate, 0.2g $MgSO_4$, 1.0g $(NH_4)\ H_2PO_4$, 1.0g K_2HPO_4, 5.0g NaCl, 0.08g Bromothymol blue, 15g Agar, 1 litre Distilled water and the final pH adjusted to 7.0.

(ii) Bacterial culture

(iii) Inoculation needle

(iv) Culture tubes

(v) Incubator

3.9.6.2. Procedure:

1. Few slants of Simmon's citrate agar medium are prepared.
2. The slants are inoculated by stabbing to the base of the slant and streak on the surface thereafter.
3. Finally the tubes are incubated at 37°C for 48 hours and the examined for result.

3.9.6.3. Observation:

If the growth of the culture change the colour of the medium to blue due to change of pH becoming alkaline following citrate utilisation, the test is positive. It indicates the culture belongs to Enterobacter. For negative result, the unutilized citrate indicates no colour change to the growth medium.

3.9.7. Antibiotic Sensitivity test

For determining the sensitivity of antibiotics by the bacterial isolates, HIMedia Susceptibility discs were used for the present study. HIMedia Susceptibility discs were placed over the bacterial lawns on Mueller Hinton medium, and after incubation for 48 hours the zone of inhibition is observed and measured to determined the susceptibility of the isolate against the antibiotics (Cappuccino et al., 2005). For the work Six antibiotics were used *viz.* Imipenem (IPM), oxacilin (OX), chloramphenecol (C), novobiocin (NV), ciproflocacin (CIP) and amoxyclav (AMC).

3.9.7.1. Requirements:

Broth cultures of bacteria, Mueller Hinton Media, spreader, micro pipette, HIMedia Susceptibility discs, ruler, forceps, Bunsen burner and safety cabinet chamber.

3.9.7.2. Procedure:

(i) Mueller Hinton Media was pour in sterile petri plates and let to solidify.

(ii) The plates are labeled with the test organisms to be inoculated.

(iii) 100 µl of bacterial broth culture is pipetted on the media, spread with a spreader and then allowed to dry for few minutes.

(iv) The susceptibility discs are then placed over the surface of the media and gently pressed with a sterile forcep in order to ensure that the disc adhere to the media surface.

(v) All the inoculated plates are then incubated at room temperature for 48 hours in inverted positions.

3.9.7.3. Observation:

Zones of inhibition shown by isolates were observe and measured to determine the antibiotic sensitivity.

3.10. Acetylene Reduction Assay

Through the method of ARA (Acetylene Reduction Assay) the assessment for determining the activity of nitrogenase enzyme accountable for Nitrogen fixation was elucidated for the bacteria which were isolated. The process of Acetylene reduction assay was executed at IBSD (Institute of Bioresource and Sustainable Development), Imphal by following the method described by Hardy et al, (1968) and Hardy *et al.* (1973) with some slight modification. The underlying reason for the method is reduction of acetylene into ethylene with the support of enzyme nitrogenase; the process is accessed by using (Thermo scientific CHEMITO CERES 800 plus) Gas chromatography. The method of acetylene reduction assay (Hardy *et al.*, 1973) is an essential practice for determining the ability of the enzyme nitrogenase, the enzyme that is accountable for the fixation of N_2.

Pure culture of all the isolates were inoculated in 10 ml of corresponding broth media prepared both for free living and symbiotic isolates and incubated for 3-5 days. After incubation 5 ml of the broth cultures were transferred in sterile 20 ml cryo tubes provided with rubber stoppers. 1.5 ml (10%) of the air is withdrawned from the tubes using a sterilized syringe. The withdrawned amount of air is replaced with 1.5 ml of Acetylene gas injected with a syringe and then incubated for 2 hrs at 30ºC. 0.5 ml (5%) of Trichloro acetic acid (TCA) is injected after 2 hrs. Then 1 ml of sample gas is removed from the tubes and injected into gas chromatography column and the resulting chromatograph is observed.

3.11. DNA extraction

Genomic DNA was obtained by using the **HIMEDIA, HiPurA^{TM}Bacterial and Yeast Genomic DNA Purification Spin Kit** following the protocol of **Gram Negative Bacterial Preparation.** Bacterial cells were harvested by centifuging 1.5ml of an overnight broth culture for 2 minutes at apprx. 13,000-16,000 rpm. The pellets

are resuspended thoroughly in 180 µl of Lysis Solution (AL) (DS0015). 20 µl of proteinase K solution (20mg/ml) is added to the sample and incubated for 30 minutes at 55ºC. Cells were lysed by adding 200 µl of Lysis Solution (C1) (DS0010) and incubated at 55ºC for 10 minutes. To the lysate 200 µl of ethanol (95-100%) is added and mixed thoroughly by vortexing for few seconds. The lysate thus obtained is transferred onto **HiElute Miniprep Spin Column** (DBCA02) provided with the kit. It is then centrifuged at 10,000 rpm for 1 minute and the flow-through liquid is discarded. 500 µl of Prewash Solution is then added to the column, centrifuged at 10,000 rpm for 1 minute and the flow-through liquid is again discarded. To the column, now 500 µl diluted Wash Solution (WS) (DS0012) is added and centrifuged for 3 minutes at max. speed of 13,000-16,000 rpm. Then the column is transferred to new collection tube and centrifuged again at the same speed for an additional 1 minute to dry the column. The **HiElute Miniprep Spin Column** is transferred to new collection tube and into it 200 µl of Elution Buffer (ET) (DS0040) is pipette directly without spilling to the sides. Finally it is incubated at room temperature for 1 minute and then centrifuged at 10,000 rpm for 1 minute to elute the DNA.

3.12. Identification of Nif genes

For amplification of nif gene, 2 µl of the extracted DNA is used as template for polymerase chain reaction with primers ***zehrf*** **(5'-TGCGACCCAAAAGCAGA-3')** and ***zehrr*** **(5'- AACGCCATCATCTCACC-3')** as in accordance with Zehr and McReynolds, (1989)**.** Polymerase chain reaction was performed using **Applied Biosystem, Veriti 96 Well Thermal Cycler.** The amplification reactions were performed in a 50.0 µl volume by mixing template DNA with 2.0 or 2x Taq Master Mix Kit, **Ampliqon, Bioreagents & PCR Enzymes** having 150 mM Tris-Hcl pH 8.5, 40 mM $(NH_4)_2SO_4$, 3.0 or 4.0 mM $MgCl_2$, 0.2% Tween 20®, 0.4 mMdNTPs, 0.05 units/µl AmpliconTaq DNA polymerase and 0.8 µl each of the primers *zehrf* and *zehrr* (**Xcelris Genomics PrimeX**).The amplification reactions were performed with an initial denaturation for 5 minutes at 95 ºC, then 30 cycles of 30 seconds at 94 ºC, 30 seconds of 50 ºC, 45 seconds at 72 ºC and a final extension for 7 minutes at 72 ºC and 4 ºC for storage.Electrophoresis of nif gene amplification products (3µl) was carried on 1.5% agarose gels in 1XTAE (40mM Tris, pH 8.0, 20mM acetic acid and

1mM EDTA) at 110 V for 50 min. Gels were stained with ethidium bromide prior to image capture.

3.13. 16S rDNA gene amplification

Amplification of 16S rDNA gene was carried out by performing polymerase chain reaction using **Applied Biosystem, Veriti 96 Well Thermal Cycler.** The amplification reactions were performed in a 50.0 µl volume by mixing template DNA with 2.0 or 2x Taq Master Mix Kit, **Ampliqon, Bioreagents & PCR Enzymes** having 150 mM Tris-Hcl pH 8.5, 40 mM $(NH_4)_2SO_4$, 3.0 or 4.0 mM $MgCl_2$, 0.2% Tween 20®, 0.4 mMdNTPs, 0.05 units/µl AmpliconTaq DNA polymerase and 0.8 µl each of the primers 27f and 1492r (**Xcelris Genomics PrimeX**). The primers sequences used for PCR amplification of the 16S ribosomal DNA were: 27f (5’-AGAGTTTGATCCTGGCTCAG -3’) and 1492r (5’-TACGGYTACCTTGTTACGACTT-3’) in accordance with the work done by Weisburg et al, (1991).

The thermocycling conditions consisted of an initial denaturation at 95 ºC for 5 minutes, 35 amplification cycles of 94 ºC for 45 seconds (denaturation), 55 ºC for 45 seconds (annealing), and 72 ºC for 1.30 minute (extension) and final polymerization at 72 ºC for 7 minutes. Electrophoresis of 16S rDNA gene amplification products (3µl) was carried on 1.5% agarose gels in 1XTAE (40mM Tris, pH 8.0, 20mM acetic acid and 1mM EDTA) at 110 V for 50 min. Gels were stained with ethidium bromide prior to image capture.

3.14. Agarose Gel Electrophoresis

Agarose Gel Electrophoresis is a highly efficient tool used for separation, identification and purification of DNA and other metabolites. All the DNA and PCR products from the previous molecular works are separated and identified by using this technique. The methods and procedure of this technique was carried out as described byDubeyand Maheshwari, (2002) in Practical Microbiology book.

3.14.1. Preparation of gel:

A desired amount of agarose gel (0.8 – 1.5%) is added in TAE (tris/acetate) buffer. The gel is mixed homogenously by using a microwave oven. The gel is then let to cool down for a while and before the gel solidifies, Ethidium bromide is added which helps in visualisation of DNA under UV source. Gel comb is inserted in the casting plateform to make wells and the still liquid gel is poured in the tray. The gel comb is then removed carefully after the gel sets and the plateform is placed inside an electrophoresis chamber or tank, which is filled with TAE buffer upto a depth where it allows the gel to sink properly. An appropriate quantity of DNA sample is then loaded onto the wells in the gel. One well is also used separately for loading the appropriate standard DNA molecular weight marker. The voltage is then set on power supply (1 to 10 V/cm to gel) to start the electrophoresis. After a preset time the power supply is swith off and the gel can be visualised in a UV hood.

3.15. 16S rDNA Gene Sequencing

The amplified products were purified and sequenced using automated DNA Sequencer (ABI 3500 Genetic Analyzer; Applied Biosystem, Inc. USA) in the Molecular Medicine Laboratory in the Department of Biotechnology, Assam University. The sequencing reaction was performed using BDTv3.1, 5x sequencing buffer (Applied Biosystems, USA); a final concentration of 3.2 pmol of each of the primers was maintained in separated reaction. The Chain termination reaction was carried out at Thermal cycler and fragments were then purified by Sodium Acetate/EDTA/Ethanol method prior to run in the 3500 Genetic Analyzer. The details of the sequencing reaction is summarized below

Reagent (Concentration)	**Volume**
BDT v3.1 Ready reaction Mix (2.5x):	4 ul
5x Sequencing buffer:	2 ul
Primer (3.2 pmol):	1 ul
Template (20-40 ng):	1 ul
Nuclease free Water:	12 ul
Total	**20 ul**

The thermo-cycling was done for initial denuration at 96°C for 1 minute followed by 25 cycles of denaturation at 96°C for 10 secs, annealing at 50°C for 5 secs; and extension at 60°C for 4 minutes as per the manufacturer's protocol.

3.16. Phylogenetic Tree Construction

Molecular Evolutionary Genetic Analysis or MEGA is used for the construction of phylogenetic tree. MEGA 4.1 version is used for the construction and analysis of the nucleotide sequences (Tamura et al., 2007). The sequence were first aligned by using Clustal IW and the output of Clustal IW was used as an input for Mega 4.1. Analysis is conducted using "Kimura-2-parameter" model for nucleotide sequences. The phylogenetic tree was drawn using Neighbour-Joining (NJ) method (Saitou and Nei, 1987).