ABSTRACT

A study was undertaken for the isolation and characterization of nitrogen fixing bacteria from five selected wild legume plants growing in Manipur, India. The study deals with the isolation of bacteria from both the rhizosphere and the root nodules of the selected legume plants. Rhizospheric bacteria were isolated from the rhizosphere of five legume plants viz., *Sesbania sesban, Leucaena leucocephala, Crotalaria junceae*, *Crotalaria pallida* and *Parkia roxburghii* growing in the four valley districts (Imphal East, Imphal West, Thoubal and Bishnupur) of Manipur. Root nodulating bacteria and endophytic bacteria were also isolated and characterized from the root nodules of four leguminous plants viz., *Sesbania sesban, Leucaena leucocephala, Crotalaria junceae* and *Crotalaria pallida* growing in the four valley districts. Isolation of bacteria from root nodules of *Parkia roxburghii* was an exception and omitted from the study as the plant does not bear nodules on its root system. 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.

Selection of the leguminous plant sources is a vital part of the present study and the selection is solely base on their availability or their economic importance for the state of Manipur, India. 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. *Leucaena leucocephala*, a 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. *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, Mikirs etc. also consumed the pods as vegetables. *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 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. *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.

For isolation of rhizospheric 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 symbiotic 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.

Several bacterial strains were isolated from the rhizosphere of the five (5) selected legume plants. However, a total of twenty-six isolates (26) were selected for further study in the current work. The selected isolates viz., *Crotalaria juncea*: CJS.T-1, CJS.IE-1A, CJS.IE-

2, CJS.B-1 and CJS.IW-1, *Crotalaria pallida*: CPS.B-1, CPS.B-2, CPS.T-1, CPS.IW-3, CPS.IE-2A and CPS.IE-3, *Sesbania sesban*: SS.T-1, SS.IE-1, SS.B-1, SS.IW-1 and SS.IW-3, *Leucaena leucocephala*: LLS.IE(B)-1, LLS.IE(B)-2, LLS.B-1, LLS.T-1 and LLS.IW-2 and *Parkia roxburghii*: PS.T(A)-2, PS.T(B)-2, PS.B-3, PS.IW-1 and PS.IE-2 were isolated by using the nitrogen free Burk's media as described by Wilson and Knight (1952). Pure colonies of the isolates were obtained through repeated streaking of the isolates on the same medium.

Isolation of bacterial isolates was also done from the root nodules of four legumes viz., *Crotalaria juncea, Crotalaria pallida, Sesbania sesban* and *Leucaena leucocephala.* Several numbers of bacterial strains were isolated from the root nodules of the four (4) selected legume plants. Here, isolation of bacteria from *Parkia roxburghii* was omitted as there are no nodules present in the root. However, a total of twenty (20) bacterial samples were selected for further study. The selected isolates viz., *Crotalaria juncea*: CJN.IE-1, CJN.IE-2 CJN.IW-1, CJN.B-1 and CJN.T-1, *Crotalaria pallida*: CPN.IE, CPN.B, CPN.IW-1, CPN.IW-2, CPN.T-1 and CPN.T-4, *Sesbania sesban*: SN.IE, SN.IW, SN.B-1 and SN.T and *Leucaena leucocephala*: LLN.T-1, LLN.IE-1, LLN.IW-1, LLN.B-1 and LLN.B-2 were isolated by using the nitrogen free YEMA media. Pure colonies of the isolates were again obtained through repeated streaking of the isolates on the same medium.

After the isolation of bacteria from both the sources 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). 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 condition. 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).

After the preliminary colony characteristics and motility test Gram staining was performed as the next step which 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).

For observation of colony characteristics of rhizospheric samples, bacterial isolates are streak plated in Nutrient Agar medium and the examination was done after 48 hours of incubation. The largest single colony was observed to be upto 8mm in size, however majority of the single colony of the isolates range in size between 2-3mm. Motility test of the isolates were also done by Hanging Drop Mount Method**.** A total of seventeen (17) isolates were found to motile, six (6) were non-motile and 3 (three) were variable meaning they could not be ascertained. Out of the twenty-six bacterial strains, fourteen strains were found to be gram negative rod shaped and five were found out to be gram positive rod shaped bacteria. The remaining seven bacterial are found to be gram negative spherical shaped bacteria.

Observation for colony characteristics of bacterial isolates obtained from root nodules was also done by streaking on Nutrient Agar medium and the examination was done after 48 hours of incubation. The largest single colony was observed to be upto 6mm in size, however majority of the single colony of the isolates range in size between 2-5mm. After performing Motility test, seventeen isolates out of twenty were found to be motile and the remaining three isolates were non-motile**.** The details of Colony characteristics as well as motility of the bacterial isolates are given in Table no. 2. Out of the twenty bacterial isolates fifteen were detected to be gram negative rod, three were gram negative sphere and two isolates were gram positive rod.

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 Dubey and Maheshwari, (2002) in Practical Microbiology book. 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).

All the twenty-six bacterial samples isolated from the rhizosphere soil of legume plants showed positive result for Catalase test. All the samples isolated from the rhizosphere soil of *Crotalaria pallida* are found to show positive result for Starch hydrolysis test whereas those isolated from *Crotalaria juncea* were found to be negative for the same test. All the isolates from both the plant source as well as those from *Sesbania sesban* are found to be positive for Nitrate reduction test. Only the two isolates, LLS.IE(B)-2 and PS.IE-2 shows negative result for Nitrate reduction test. Majority of the samples shows negative result for Urease test and only five samples shows positive result. Every bacterial sample isolated from the rhizosphere soil of *Parkia roxburghii* tested positive for Oxidase test. Seventeen out of twenty-six samples turns out to be Citrate test positive and the remaining nine isolates shows negative result.

All the twenty bacterial samples isolated from the root nodules of the four legume plants showed positive result for Catalase test. Four isolates out of five from the root nodules of *Crotalaria juncea* are found to be positive for Starch hydrolysis test. Again two isolates from the same plant tested positive for oxidase test. All the isolates from *Sesbania sesban* are found to be positive for Nitrate reduction test as well as oxidase test. The isolate SN.B-1 from the same plant was detected to be negative for Urease test. Only one isolate from Leucaena leucocephala gives negative result for Oxidase test whereas only one isolate gives positive result for Citrate Test.

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 said by 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.

ARA was performed for all the twenty-six bacterial isolates obtained from the Rhizosphere and chosen for the study. Nineteen isolates were found to show some ability for fixing Nitrogen (nitrogenase activity), whereas the remaining seven isolates showed negligible or no nitrogenase activity at all. The bacterial samples isolated from the rhizosphere of *Crotalaria juncea* particularly failed to show any significant amount of reducing Acetylene to Ethylene, thus it could be considered that it may not have the capacity of fixing nitrogen. However the bacterial isolate CJS.IE-1 isolated from the rhizosphere of *Crotalaria juncea* and isolate SS.IW-3 from the rhizosphere of *Sesbania sesban* were selected for 16S rDNA sequencing considering their morphology, biochemical characteristics and nifH gene amplification along with other isolates.

ARA was also performed for all the twenty bacterial isolates obtained from the Root Nodules of the selected legume plants. Seventeen isolates were found to show some ability for fixing Nitrogen (nitrogenase activity), whereas the remaining three isolates showed negligible or no nitrogenase activity at all. Bacterial isolate SN.IW obtained from the nodules of *Sesbania sesban,* and two isolates obtained from the *Leucaena leucocephala viz.*, LLN.T-1 and LLN.IW-1 failed to show any significant amount of reducing Acetylene to Ethylene, indicating that they may not have the capacity of fixing nitrogen. However, the isolates LLN.T-1 isolated from the rhizosphere of *Crotalaria juncea* and isolate SS.IW-3 from the rhizosphere of *Leucaena leucocephala* was selected for 16S rDNA sequencing considering their morphology, biochemical characteristics and nifH gene amplification along with other isolates.

Extraction of Genomic DNA from the bacterial isolates constitutes the initial step for molecular characterisation of the isolates. 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.** Amplification of NifH gene and 16S rDNA followed as the primary steps after DNA extraction. Amplification of NifH gene was performed to determined the nitrogen fixing capability of isolated bacterial isolates. The step was also performed in order to check the accuracy of the result given after performing Acetylene Reduction Assay (ARA). It is a necessary step for the confirmation of nitrogenase activity of the bacterial isolates as the result given by ARA tends to be inaccurate sometimes due to contamination or culture conditions of isolates. NifH gene amplification was carried out by performing polymerase chain reaction using a **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 mM dNTPs, 0.05 units/µl Amplicon Taq DNA polymerase and 0.8 µl each of the primers *zehrf* and *zehrr* (**Xcelris Genomics PrimeX**).

All the selected twenty-six isolates from Rhizosphere and the twenty isolates obtained from Root Nodules were subjected to nifH gene amplification through polymerase chain reaction after ARA. Identification of replicon with a molecular weight of approximately 300 bp indicates the presence of nif gene in the isolates. In some isolates multiple replicons were formed which may be due to minute variations in the concentration and purity of template DNA as observed by Berg *et al.* (1994).

After considering both the results of ARA and NifH amplification, 16S rDNA gene amplification was performed. In few cases either of the two results was taken into consideration. Amplification of 16S rDNA gene was carried out by performing polymerase chain reaction using a **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').

Amplification of 16S rDNA was carried out for twelve selected isolates from rhizosphere and fourteen selected isolates from amongst the Root Nodule isolates. Selection of the isolates were done after observing and comparing both the results from ARA and NifH gene amplification. Both the ARA results and NifH amplification were taken into consideration and in few cases either of the two results was taken into consideration. Amplification of the 16S rDNA of all the selected samples through PCR gives band between 1400bp to 1600bp as shown in the gel image below. Amplification between 1400bp to 1600bp indicates the amplification of the targeted 16S rDNA. The PCR product was then sent for sequencing to perform further sequence analysis and phylogenetic studies.

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 by Dubey and Maheshwari, (2002) in Practical Microbiology book.

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.

After ARA and NifH gene amplification, the following twelve stains isolated from the rhizosphere *viz.* SS.T-1, SS.IW-3, CPS.B-1, CPS.B-2, CPS.IW-3, CPS.T-1, CJS.IE-1, LLS.T-1, LLS.IE(B)-1, PS.IE-2, PS.IW-1 and PS.T(B)-2 were subjected to 16S rDNA phylogenetic analysis. These isolates were subjected to 16S rDNA gene sequencing in accordance with their result regarding the previous two confirmatory steps. However, some isolates are still selected for 16S rDNA gene sequencing even if the result from either of the previous test were not convincing. This is mainly due to the fact that both the ARA and NifH gene amplification cannot give perfect and accurate result everytime as many factors including culture condition and the state of the extracted DNA sometimes dictate the outcome of the test. The sequences of all the above 12 strains were deposited in GenBank under accession numbers: SS.T-1 (KM456220), SS.IW-3 (KM925076), CPS.B-1 (KM382276), CPS.B-2 (KM456219), CPS.IW-3 (KM925077), CPS.T-1 (KM925078), CJS.IE-1 (KM598638), LLS.T-1 (KM925075), LLS.IE(B)-1 (KM396262), PS.IE-2 (KM396263), PS.IW-1 (KM269071) and PS.T(B)-2 (KM598639) respectively. The bacterial isolates obtained from rhizosphere conforms to different organisms *viz. Bacillus spp., Enterobacter spp., Beijerinckia spp., Cedecea spp., Klebsiella spp.* and *Pseudomonas spp.*

From the total of twenty bacterial samples isolated from the root nodules of the source plants and selected for ARA and NifH gene amplification, 16S rDNA amplification and

sequencing have been performed for fourteen bacterial sample in accordance with their results from ARA and NifH gene amplification. However, some isolates are still selected for 16S rDNA gene sequencing even if the result from either of the previous test were not convincing. Again this is mainly due to the same reason as mentioned before. The sequences of the said bacterial sequences deposited in GenBank along with their accession numbers are as follows: SN.T (KP331546), SN.IE-1 (KU355544), SN.B-1 (KX281718), CJN.T-1 (KP331547), CJN.B-1 (KU355542), CJN.IW-1 (KU355543), CJN.IE-2 (KX434625), CPN.B (KU935450), CPN.IW-1 (KU935451), CPN.T-1 (KU935452), CPN.T-4 (KU935453), LLN.B-1 (KU955582), LLN.T-1 (KU955583) and LLN.IE-1 (KX281719). The bacterial isolates obtained from rhizosphere conforms to different organisms *viz. Mesorhizobium spp., Pseudomonas spp*., *Rhizobium spp., Pantoea spp., Bacillus spp., Bradyrhizobium spp., Enterobacter spp., Stenotrophomonas spp.* and *Cedecea spp.*

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).

Four bacterial isolates obtained from *Crotalaria pallida viz.*,CPS.B-1, CPS.B-2, CPS.T-1 and CPS.IW-3 put these strains into three different family of organism after 16S rDNA analysis. The stains CPS.IW-3 positioned itself as *Bacillus subtilis,*CPS.B-1 as *Beijerinckia fluminensis* and both CPS.B-2 and CPS.T-1 positioned themselves as *Enterobacter asburiae*.16S rDNA analysis of two bacterial isolates form *Sesbania sesban* conformed to two different organisms. Isolate SS.T conforms to *Enterobacter cloacae* and the isolate SS.IW-3 has been established as the organism *Cedecea davisae* in accordance with the result suggested after 16S rDNA analysis and NCBI Blast.Analysis of 16S rDNA sequences of two isolates from *Leucaena leucocephala i.e.,* LLS.T-1 and LLS.IE(B)-1 positioned themselves as the organisms *Enterobacter kobei* and *Bacillus altitudinis* respectively. Three isolates from the source plant *Parkia roxburghii viz.,* PS.IW-1, PS.IE-2 and PS.T(B)-2 which indicated some nitrogenase activity and also presence of NifH gene were also further determined through 16S rDNA analysis. The isolate PS.IW-1 got positioned as *Bacillus subtilis,* PS.IE-2 as *Klebsiella oxytoca* and the isolate PS.T(B)-2 conformed itself

to the organism *Pseudomonas cedrina.* Only one isolate from *Crotalaria junceae,* CJS.IE-1 was found conforming to *Cedecea davisae* after 16S rDNA analysis. The isolate was selected for the same analysis even as it does not indicate any nitrogenase activity, however it produced a good band on NifH amplification.

Four bacterial isolates obtained from the root nodules of *Crotalaria pallida viz.*,CPN.B, CPN.IW-1, CPN.T-1 and CPN.T-4 put these strains into four different family of organism after 16S rDNA analysis. The isolate CPN.B conformed to the organism *Enterobacter hormaechei,* CPN.IW-1 as *Stenotrophomonas maltophilia,* CPN.T-1 as *Pantoea agglomerans* and isolate CPN.T-4 positioned itself as *Cedecea davisae*. 16S rDNA analysis of three bacterial isolates form the nodules of *Sesbania sesban* conformed to three different organisms. Isolate SN.T conforms to *Mesorhizobium huakuii,* the isolate SN.IE-1 as *Pseudomonas azotoformans* and isolate SN.B-1 has been established as the organism *Neorhizobium huatlense* in accordance with the result suggested after 16S rDNA analysis and NCBI Blast. Analysis of 16S rDNA sequences of three nodules isolates from *Leucaena leucocephala i.e.,* LLN.B-1, LLN.T-1 and LLN.IE-1 positioned themselves as the organisms *Bacillus toyonensis, Pseudomonas hibiscicola* and *Mesorhizobium plurifarium* respectively. Four isolates from the nodules of *Crotalaria juncea* were chosen for 16S rDNA analysis which produced three different organisms. Isolates CJN.T-1 and CJN.B-1 were found to conformed with the organism *Pantoea agglomerans,* isolate CJN.IW-1 with *Bacillus subtilis* and the isolate CJN.IE-2 got positioned as the organism *Bradyrhizobium japonicum.*

Isolation and characterization of nitrogen fixing bacteria from wild legume haves never been reported from this region under study and the present study is the first from the state of Manipur, India. Studies regarding both legumes and rhizobia as well as legume-rhizobia interactions have not been fully documented from the region. Besides the studies about both free living as well as associative nitrogen fixer are not yet documented. As such the determination and categorisation of symbiotic, endophytic and rhizospheric nitrogen fixing bacteria from the region has a very prospective and worthwhile undertaking. In the present study more emphasis was given to the determination of the bacteria on their species and genus level through molecular characterization. Legume crops at least have been documented well by many worker all over the world but documentation of wild legumes and their interactions with bacteria still have a huge area to be explored. In the present study, fresh strain of symbiotic, endophytic as well as rhizospheric (associative) nitrogen fixing bacteria are isolated from the root nodules and rhizosphere soil of the source plants

respectively. The isolated bacteria after preliminary examination through morphological, physiological as well as biochemical characteristics were screen for their nitrogen fixing ability through Acetylene Reduction Assay (ARA). The isolates showing positive readings in ARA were then subjected to NifH gene amplification to ascertain their nitrogenase activity. Finally incorporation of 16S rDNA gene amplification and sequencing in the study provided the information of the bacterial isolates upto their species level. During the study, isolation of *Enterobacter sp.* were more common of which many of the species were known nitrogen fixers. Isolation of *Beijerinckia, Pseudomonas* and *Bacillus sp.* further confirmed that the rhizosphere of wild legumes harbours a wide diversity of bacteria that are capable of nitrogen fixation. Isolation and identification very less known nitrogen fixing organism such as *Enterobacter hormaechei* and *Bacillus altitudinis* gives a very exciting prospect for future studies. Moreover, isolation of well known nitrogen fixing bacteria of the different *Rhizobium* species in the present study such as *Mesorhizobium huakuii, Neorhizobium huautlense, Bradyrhizobium japonicum* and *Mesorhizobium plurifarium* provided a very positive aspect for future studies and researches. Although there are significant reports regarding the studies about the nitrogen fixing organism from around the world by many workers, both about free living and symbiotic, there is insufficient report of such research from the region. With the wide diversity of leguminous plants available in the region, huge scope is available for the such research to find out several bacteria associated with them regarding Biological Nitrogen Fixation. The process of biological nitrogen fixation being an eco-friendly approach towards increase crop productivity and soil enrichment is proving a good alternative to chemical fertilizer due to its safer and comparatively cleaner ways. Covering a broader area of sampling site and increasing the number of plant sources promises the possibility of finding out even more numbers of organisms capable of Biological Nitrogen Fixation.

The present study was carried out for the first time both for this region as well as for the concerned leguminous plants for the determination and categorization of rhizospheric, endophytic and symbiotic bacteria isolated from the rhizosphere and roots nodules of the selected legume plants growing in the four valley districts of Manipur, India. Considering the importance of legume plants economically in the form of food crops, their effect on soil fertility as well as a source of alternative vegetable in few cases, the present study provide a glimpse of the potential of the legume, plants particularly their interactions with nitrogen fixing bacteria. Besides, the current study may prove worthwhile to recognise and determine the diversity of bacteria which perhaps potentially enhance the growth and development of

the wild legume plant whose potentials are still not properly exploited. In the present study fresh strains of rhizospheric, endophytic and symbiotic bacteria are isolated and monitored for the presence of nitrogenase activity through Acetylene Reduction Assay and NifH gene amplification. Finally through 16S rDNA sequencing and phylogenetic analysis the species level of the isolated bacterial isolates are determined and recognised. The present study provided the chance for identification of fourteen nitrogen fixing bacteria associating with the selected five legume plants. Besides, unaccounted and unexploited wild legume plants are available in every possible environment even in harsh conditions. Consequently, covering a broader area of sampling site and increasing the number of wild legume plant sources for study promises the possibility of finding out even more numbers of organisms capable of Biological Nitrogen Fixation. Works as such being never done before from the state of Manipur gives the possible chances of finding out undocumented noble species whereby the ecological importance and the diversity of the nitrogen fixing microbes found associated with the wild legumes plants could be ascertained and described.