# CHAPTER – 1

# Introduction

## 1.1 INTRODUCTION

Approximately 80% of the atmosphere is occupied by nitrogen gas ($N_2$) which unfortunately is unusable by most living organisms. All form of organisms whether plants, animals or micro-organisms can die of nitrogen deficiency, even when they are surrounded by $N_2$which they cannot use. All organisms use the ammonia ($NH_3$) form of nitrogen to manufacture amino acids, proteins, nucleic acids, and other nitrogen-containing components necessary for life.

Nitrogen (N) is the nutrient element most frequently found limiting to the growth of green plants. This results from the continual loss of nitrogen from the reserve of combined or fixed nitrogen, which is present in soil and available for use by plants. Processes such as microbial denitrification, soil erosion, leaching, chemical volatilization, and perhaps most important, removal of nitrogen-containing crop residues from the land are the causes for the continual depletion of $N_2$ from the soil. The nitrogen reserve of the soils must therefore be replenished periodically in order to maintain an adequate level for crop production. This replacement of soil nitrogen is generally accomplished by the activity of biological nitrogen fixation (BNF) systems or by addition of chemically fixed nitrogen in the form of commercial inorganic fertilizers. Biological nitrogen fixation is carried out through symbiotic and non-symbiotic means. Symbiotic fixation occurs through the association of leguminous plant roots with nitrogen fixing micro-organisms called Rhizobium while the symbionts of a few non-leguminous species belong to a genus of *Actinomycetes, Frankia*. Besides, free living soil organisms affected the Non symbiotic fixation which are a significant factor in natural ecosystem and have relatively modest nitrogen requirements from outside system.

Biological nitrogen fixation is the process that changes inert atmospheric $N_2$ to biologically useful nitrogenous substances. Only bacteria can mediate this process in nature. Other plants benefit from nitrogen-fixing bacteria when the bacteria die and release nitrogen to the environment or when the bacteria live in close association with the plant. In legumes and some of non-leguminous plants, the bacteria live in small out-growths on the roots called nodules and fix nitrogen. Nitrogen fixation is done by

the bacteria, and the $NH_3$ produced is absorbed by the plant within these nodules. Nitrogen fixation by legumes is a partnership between a bacterium and a plant.

Nitrogen fixation by legumes and non-legumes is a strong symbiotic relationship between a bacterium and a plant. In most natural ecosystems, nitrogen (N) is the primary nutrient that limits crop productivity (Vitousek et al., 1997). Biological nitrogen fixation can take many forms in nature, including blue-green algae, lichens and free-living soil bacteria. Rhizobium-legume plant symbiosis is the best example for the symbiotic relationship which is considered the most proficient type of association between nitrogen fixing micro-organisms and plants, and is of major importance for agricultural practices, such as soybean crops in Brazil (Dobereiner, 1997).

The nitrogen-fixing capabilities of these so called nitrogen fixing bacterial strains can be utilized, after thorough analysis and studies to more appropriate application, like in agriculture and other plantation works for nitrogen source. The genetic potential of the gene for the nitrogen fixation could be analyzed through accurate molecular characterization of several unknown nitrogen-fixing organisms by sequencing of the *nif*H gene (Zehr *et al.*, 1995). *Nif*H genes can be used as markers for the revealing and studying genetic multiplicity of diazotrophic organisms in the community of microbial society in the roots of plants like rice (Ueda *et al.*, 1995) or can be acquired from the forest soil (Widmer*et al.*, 1999).

Human-induced activities and disturbance are responsible for increase contributions to the N cycle, through energy and fertilizer production, and mobilization of N from long-term storage pools (Galloway 1998; Goulding et al., 1998). This extra reactive N can affect ecosystem processes such as N deposition and soil acidification, and microbiologically mediated soil processes such as mineralization, immobilization, nitrification, denitrification and emission of nitrous oxide and methane (Goulding et al., 1998; Fageria and Stone, 2006). Of all sources, biological nitrogen fixation is the most 'environmentally friendly' process of supplying nitrogen to ecosystems (Jensen and Hauggaard-Nielsen 2003). Leguminous and actinorhizal plants forms nodules with their respective host plants and fix nitrogen

in association with rhizobia or Frankia (Franche et al. 2009). Rhizobium–legume symbiosis represents the major mechanism of biological nitrogen fixation compared with the nitrogen fixing heterotrophs and associative bacteria and actinorhizal plants (Zahran, 2001). Through symbiotic fixation, these nitrogen fixing plants provide high inputs of nitrogen to the ecosystem (Watt et al. 2003; Augusto et al., 2005). Such nitrogen supply increases soil fertility and builds up the soil nitrogen pool through the decomposition of nitrogen rich litter, and releasing of nitrogen from roots and nodules (Ehrenfeld, 2003; Goldstein et al. 2010) can contribute significantly to the global nitrogen cycle (Colebatch et al., 2002). The quantity of symbiotic nitrogen fixation remains problematic to assess as it differs from species to species and depends on environmental conditions ( Fageria and Stone, 2006; Jackson et al., 2008).

The legume plants could be attributed for the major conversion of nitrogen from atmospheric $N_2$ to ammonia ($NH_3$) for which they are considered very significant not only ecologically but also agriculturally. Therefore, the isolation of microorganisms with the ability to fix nitrogen and to further study them has become a main practice of importance in the field of agriculture and science. As a consequence, extending application of biological nitrogen fixation by any means is of huge importance.

### 1.1.2. Role of nitrogen in the biosphere

As an essential component of proteins, nucleic acids and other cellular constituents, nitrogen of all the mineral nutrients available, is the most important for the promotion of growth of all organisms and which is required in large amounts. The abundant supply of nitrogen (nearly 79%) in the earth's atmosphere is in the form $N_2$ gas, which is inert owing to the presence of triple bond between the two nitrogen atoms, consequently making them unavailable for use by most organisms. Hence, in order for nitrogen to be used for growth of organisms it must be "fixed" (combined) in the form of ammonium ($NH_4$) or nitrate ($NO_3$) ions. The weathering of rocks as a natural process discharges these ions, however due to its slow process it has a negligible effect on the availability of fixed nitrogen.

Microorganisms have a significant role in almost all aspects of nitrogen availability and thus for life support on earth. Several bacteria have the capacity to convert $N_2$ into ammonia by the process termed Nitrogen Fixation; these bacteria are either free-living, associative or form symbiotic associations with plants or other organisms (e.g. termites, protozoa). Some other bacteria on the other hand can also bring about transformations of ammonia to nitrate, and of nitrate to $N_2$ or other nitrogen gases. Many bacteria and fungi are responsible for degradation of organic matter, by which fixed nitrogen are released for reuse by other organisms. All these processes contribute to the Nitrogen Cycle.

### 1.1.3. Nitrogen Fixation

Lightning as a natural process produced comparatively a small amount of ammonia (Slosson, 1919; Hill et al., 1979). Industrially also by the Haber-Bosch process, using an iron-based catalyst, very high pressures and fairly high temperature some amount of ammonia is also produced. However the major conversion of $N_2$ into ammonia, and then into proteins, is accomplished by microorganisms in the process known as Nitrogen fixation (or dinitrogen fixation).In the atmosphere, nitrogen is found as $N_2$ which is not usable by most plants and animals. The nitrogen must be converted into ammonia ($NH_3$), in order for plants and humans to acquire the nitrogen they need. This conversion of atmospheric nitrogen to the usable form of ammonia is known as nitrogen fixation (Postgate, 1998). It can also be defined as the process of producing ammonia from atmospheric nitrogen. Without the process of nitrogen fixation, most plants and animals would not have the nitrogen necessary their life processes. Nitrogen fixation, involving the chemical reduction of $N_2$ to $NH_3$ or $NH_4$, requires a source of electrons. Sources of electrons for the nitrogenase activity can vary from organism to organism. The sources for electrons such as flavodoxin, ferredoxin, nicotinamide, or ademine dinucleotide (phosphate) are all small proteins and highly reductive molecules. It is usually accepted that $N_2$ fixing systems require more Phosphorus (P) than non-$N_2$-fixing systems. Processes vital for nitrogen fixation such as for plant growth, nodule formation and development, and ATP synthesis, all of them requires the availability of Phosphorus.

### 1.1.4. Biological Nitrogen Fixation

Biological nitrogen fixation (BNF) can be defined as the process whereby atmospheric nitrogen (N≡N) is reduced to ammonia in the presence of the enzyme Nitrogenase (Postgate, 1998). It is a biological catalyst found naturally only in certain microorganisms such as the symbiotic *Rhizobium* and *Frankia,* free-living *Azospirillum* and *Azotobacter* or other associative organisms like *Enterobacter, Pseudomonas* etc.

Biological nitrogen fixation is carried out by free-living or associative soil microorganisms and by symbiotic associations of microorganisms with higher plants. Leguminous plants fix atmospheric nitrogen by functioning symbiotically with special bacteria, rhizobia, which thrives in the root nodules. The nodules are formed when Rhizobia infect root hairs of the leguminous plants. The nodules act as the home for bacteria obtaining energy from the host plant and take free nitrogen from the soil air and then processing it into combined nitrogen. The plant in return, receives the fixed N from nodules and produces food and forage protein.

Biological nitrogen fixation (BNF) by multiplicity of symbiotic, associative, and free living microorganisms has remarkable importance to the environment and to the world as a whole. It represents as one of the fundamental steps of the nitrogen cycle by replenishing the overall nitrogen content of the biosphere and reimbursing for the losses that are suffered due to the process of denitrification. The fixed $N_2$that is delivered through BNF is less prone to leaching and volatilization as it gets consumed in situ. Consequently, this biological process contributes as an important and sustainable input into agriculture (Dixon and Kahn 2004).Symbiosis in the root nodule with $N_2$-fixing bacteria delivers legumes with greater capability to obtain fixed $N_2$ (Quispel1974).The ultimate question of whether such a relationship is possible for non-legume plants came to the fore due to the discovery of symbiosis between $N_2$-fixing bacteria and legumes (Mia et al., 2010).Based on our understanding of nitrogen fixation biology in legumes, molecular mechanisms of BNF in non-legume crop species also needs to be ascertain through more research (Godfray et al. 2010).

The capability to fix atmospheric nitrogen is limited to prokaryotes in the bacterial and archaeal domains, collectively so-called diazotrophs that convert atmospheric nitrogen into ammonia (Canfield et al. 2010).Interestingly, diazotrophs are available in an extensive range of habitats including free-living in soils and water, associative symbioses with grasses, actinorhizal associations with woody plants, cyanobacterial symbiosis with various plants, and root–nodule symbioses with legumes (Dixon and Kahn 2004; Kneip et al. 2007).Several bacterial species belonging to genera *Azospirillum*, *Alcaligenes*, *Arthrobacter*, *Acinetobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Rhizobium* and *Serratia*al so formed associations with the plant rhizosphere and are capable of exerting beneficial effects on plant growth (Tilak*et al*., 2005, Egamberdiyeva, 2005). There is a variety of bacterial existence in soil rhizosphere, rhizoplane and internal of the plant tissues (Hallmann et al., 1997).The contribution of Biological nitrogen fixation to fixed nitrogen globally reached180 X $10^6$ metric tons/year (Postgate, 1998), out of which symbiotic associations' produces 80% and the rest comes from free-living or associative systems (Graham 1988).The capability to reduce and derive such considerable amounts of nitrogen from the atmospheric reservoir and supplement the soil is confined to bacteria and Archaea (Young 1992). These include symbiotic nitrogen fixing ($N_2$-fixing) forms, viz. *Rhizobium,* the obligate symbionts in leguminous plants and *Frankia* in non-leguminous trees, and non-symbiotic (free-living, associative or endophytic) $N_2$-fixing forms such as cyanobacteria, *Azospirillum*, *Alcaligenes*, *Arthrobacter*, *Acinetobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Rhizobium,Serratia, Azotobacter*, *Acetobacter diazotrophicus* and *Azoarcus*etc.

### 1.1.5. Mechanism of Biological Nitrogen Fixation:

The biochemical mechanism of $N_2$ fixation can be written in simplified form as the production two moles of ammonia from one mole of nitrogen gas, at the expense of 16 moles of ATP and a supply of electrons and protons (hydrogen ions) in the presence of enzyme Nitrogenase (Chung et al., 2014).

$$\mathbf{N_2 + 8H+ + 8e^- + 16\ ATP = 2NH_3 + H_2 + 16ADP + 16\ Pi}$$

Prokaryotes (the bacteria and related organisms) have the capacity to perform this reaction exclusively by using an enzyme complex termed nitrogenase**.** This enzyme consists of two proteins - an iron protein and a molybdenum-iron protein, as shown below(Fig.1).

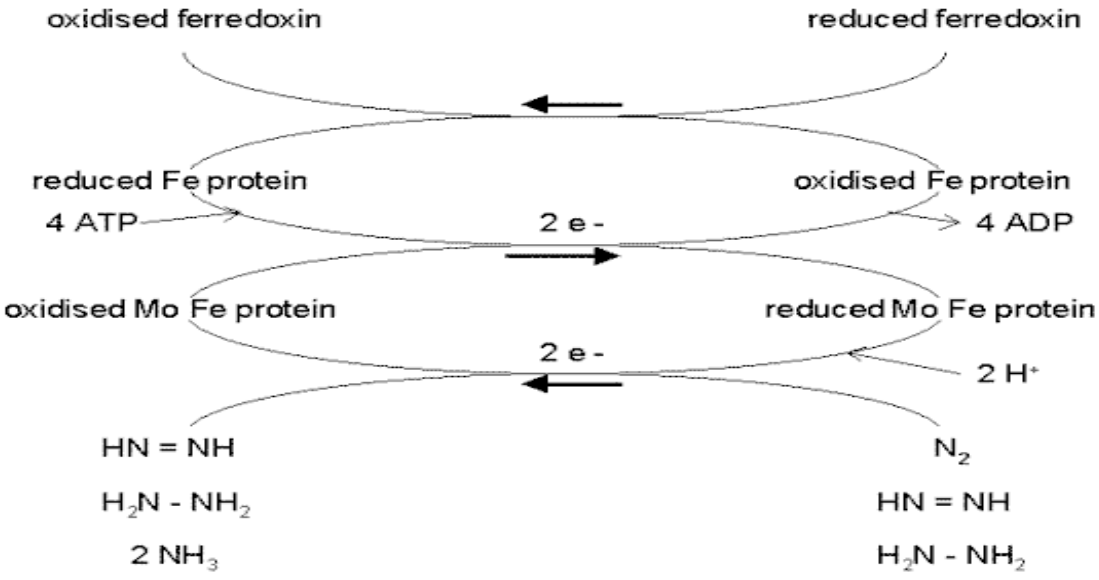


**Fig.1: Working of enzyme Nitrogenase**

The above reactions takes place when $N_2$ is bound to the nitrogenase enzyme complex. First, the electrons donated by ferredoxin reduced the Fe protein. Then the reduced Fe protein binds ATP and reduces the molybdenum-iron protein, which donates electrons to $N_2$, producing HN=NH. In two additional cycles of this process (each requiring electrons donated by ferredoxin) HN=NH is reduced to $H_2N$-$NH_2$, and this in turn is reduced to $2NH_3$. Depending on the type of microorganism, the reduced ferredoxin that supplies electrons for this process is produced by photosynthesis, respiration or fermentation.

The above mechanism according to Peoples et al., (1989) and Takishima et al., (1989),specifies that $N_2$-fixing systems can flourish in soils poor in N, that they are a source of proteins, and also provide N for soil fertility. Adenosine triphosphate (ATP) is the source of energy essential for the cleavage and reduction of $N_2$ into ammonia.

For instance, in rhizobia, ATP is produced as a consequence from the oxidative degradation of sugars and related molecules. The host-plant manufactured these sugars during photosynthesis and consequently get transferred to the nodules. In general, for each gram of $N_2$ fixed by *Rhizobium,* the plant fixes 1-20 grams carbon (C) through photosynthesis. This is an indication that symbiotic $N_2$ fixation requires additional energy which, in nitrate-fed plants, can be used to produce more photosynthates (products of photosynthesis). The extra energy cost of $N_2$ fixation can, however safely be carried by most field-grown legumes with little or no loss of production.

Nitrogenase is an oxygen sensitive enzyme. The low oxygen tension condition is realized through compartmentation in cyanobacteria (heterokcysts in *Anabaena azollae*), active respiration (in *Azotobacter*), synthesis of leghemoglobin (in *Rhizobium* legume). Leghemoglobin is a macromolecule synthesized by both symbiotic partners, the rhizobia and the host plant. *Rhizobium* synthesizes the heme portion, and the plant synthesis the globine. Like human hemoglobin, leghemoglobin fixes $O_2$. It is responsible for the red or brown color of active (i.e., $N_2$-fixing) nodules. Non-$N_2$-fixing nodules have white nodule content or a green content when the globine has degenerated (Peoples et al., 1989; Takishima et al., 1989).

### 1.1.6. Discovery of Nitrogen Fixation

Even though people observed “bumps” on legume roots as early as the 17$^{th}$ century as evidenced by a drawing published in 1679 by Malpighi (who thought they were insect galls), the mechanism whereby legumes accumulated nitrogen was unknown. It took a German scientist, Hermann Hellriegel, in collaboration with Hermann Wilfarth, to recognize that the legume root nodules themselves were responsible for the conversion of atmospheric nitrogen to ammonia (Hellriegel et al., 1888). The organisms inside the nodule were thought by some to be vibrio like or bacteria-like organisms, but others were of the opinion that they were fungi. The microorganisms were first isolated and cultured from nodules of a number of different legume species in 1888 by Martinus Beijerinck of Holland. This isolate happened to be a Rhizobium leguminosarum strain. Over time, modifications to the culture media

were made to ensure easy isolation and growth of the nodule bacteria, which were placed in the genus Rhizobium(rhiza= root; bios= life).

Beijerinck in 1901 and Lipmanin 1903 were responsible for isolation of Azotobacter spp., while Winodgradsky in 1901 isolated the first strain of Clostridium pasteurianum. Discovery of nitrogen fixation in blue-green algae (now classified as cyanobacteria) was established much later (Stewart, 1969). By 1960, the nitrogen fixation capacities of free-living soil bacteria had been established for only a dozen genera. This was a long way from our present knowledge of the distribution of nitrogen fixation ability in most phyla of the Bacteria domain (Postgate 1981; Balandreau 1983; Young 1992; Henson et al. 2004;Lindstrom and Martínez-Romero 2007; Schmid and Hartmann 2007). Indeed, identification of new nitrogen-fixing genera and species has long been hampered by technical limitations due to both the unavailability of proper tools for taxonomy and phylogeny and the difficulty in proving nitrogen fixing capacity.

Meyen first observed nodules on alder roots in 1829, which was confirmed by Woronin in 1866. In contrast to legume nodules, which are small and ephemeral, alder nodules are large, woody, and perennial. Also, their anatomy is like a root in that there is a central vascular bundle. Considerable controversy existed for a while as to the identity of the microbes inside the nodules. They were filamentous, so some believed that they were filamentous fungi, e.g., Brunchorst, who named the microbe Frankia subtilis. Hiltner (1898) recognized the nodule inhabitant as an actinomycete, Gram-positive bacteria closely related to Streptomyces. Pommer (1959) was probably the first person to obtain an isolate, but it did not reinfect its host plant. Other actinomycetes were also being isolated from the nodules of diverse actinorhizal plants, but not much attention was being paid to them. In the late 1980's, several actinomycetes had been isolated from nodules of Casuarina trees (indigenous to Australia) growing in Mexico. These trees, normally nodulated by Frankia strain, were introduced to Mexico to serve as windbreaks in farmers' fields. Maria Valdés and her colleagues in Mexico City were planning to isolate Frankia from the nodules to see whether the strains differed from the ones that typically nodulate Casuarina.

Instead, what they found were actinomycetes that were smaller in diameter than Frankia, but like Frankia, fixed atmospheric nitrogen to ammonia. This was determined by: 1) their ability to grow in N-free medium; 2) acetylene reduction assay, a test diagnostic for nitrogenase activity, the bacteria were revealed to reduce acetylene to ethylene; and 3) a 15N isotopic dilution assay, which definitively showed that these novel actinomycetes fixed nitrogen. Since this discovery, several other actinomycetes have been found to have nif genes, and a number of them have been shown to be Micromonospora. It is likely that many more will be characterized in the next 10-15 years. Exactly how they fix nitrogen in association with plants is still a mystery but will also be an important topic of study.

### 1.1.7. Symbiotic Nitrogen Fixers

*Rhizobia* and *Frankia*are the two groups of nitrogen fixing bacteria have been studied extensively so far. Even if the symbiotic relationship about *Frankia*is not as well understood, it is however an established fact that *Frankia*forms root nodules on more than 280 species of woody plants from 8 different families (Schwintzer and Tjepkema 1990). *Frankia* has been documented to form effective symbiosis with the species of *Alnus*and *Casuarina* (Wheeler and Miller 1990, Huss-Danell 1990, Werner 1992, Dommergues and Marco-Bosco 1998). There are also several individual species which may improve plant nutrition by liberating plant growth regulators, siderophores and hydrogen cyanide as well as by increasing phosphate availability (Antoun*et al*., 1998). Yanni*et al*., 1997, reported about an increase in rhizosphere populations after crop rotation with non-legumes consequently the abundance benefiting subsequent crops (Barriuso and Solano 2008).Considerable changes in taxonomic status has been observed during the last few years. Based on the polyphasic taxonomic approach, Sahgal and Johri (2003) defined the status of rhizobial taxonomy and registered 36 species dispersed among seven genera i.e., *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Methylobacterium*, *Rhizobium* and *Sinorhizobium*. With the diversity of legumes of economic importance growing under different agro-climatic conditions in India, there is huge anticipation for the presence of native rhizobia associated in a symbiotic relationship with the legumes.

### 1.1.8. Free-living Bacteria

Free-living diazotrophic bacteria are referred as those that shows no association with plants and are found thriving in soils that are free from the direct influence of plant roots. These microorganisms are physiologically very diverse and are abundant in both terrestrial and aquatic environments (Reed et al., 2011). Since the amount of $N_2$being fixed by these organisms in soil is restricted by access to energy sources, *i.e.,* substrates to generate adenosine triphosphate (ATP) and micronutrients essential for the synthesis and functioning of nitrogenase, as a result generally most soils are limited in Carbon and Nitrogen content (Reed et al., 2011).The problem of severe oxygen sensitivity of nitrogenase makes the BNF by free-living diazotrophs very limited (Postgate, 1998), however this problem has been at least partially solved in different ways by diazotrophs contributing in symbiotic nitrogen-fixation. Another problem is the further reduction of the amount of $N_2$ fixed by these organisms byantagonistic microbial interactions such as parasitism and competition for nutrients (Cacciari et al., 1986; Bashan and Holguin, 1997; Bashan et al., 2004). The general belief about freeliving diazotrophs is that their contribution of fixed N to most terrestrial ecosystems is low, perhaps 3-5 kg/ha/yr (Postgate, 1998; Newton, 2007), however their collective N contributions are assumed to be important in some tropical and temperate forest ecosystems (Cleveland et al., 1999; Gehring et al., 2005; Reed et al., 2007; 2008).

### 1.1.9. Associative Nitrogen Fixation

Associative nitrogen fixation are carried out exclusively by the organism which are generally available in the rhizosphere of plants. The rhizosphere is defined as soil that surrounds plant roots and is under their direct metabolic influence (Curl and Truelove, 1987). Rhizosphericdiazotrophs can have a reasonable advantage due to their ability to fix $N_2$, over non-$N_2$ fixing bacteria in the rhizosphere and prevail in it mostly when soil N is limited (Dobereiner and Pedrosa, 1987).Little attention was given up to the 1970's to the probability of nitrogen-fixing bacteria associated with non-legume crops, particularly the cereal grasses, and additionally, that these bacteria helped plant growth by supplying fixed nitrogen to their hosts. During her early work,

Döbereiner found a number of bacteria in Brazilian soils, including *Azotobacter paspali* from the cereal grass *Paspalumnotatum* (Dobereiner 1966, 1970) and *Beijerinckia fluminenesis*, from the rhizoplane of sugarcane (Dobereiner and Ruschel 1958). A number of Brazilian species of Azospirillum, the only other genus other than the rhizobia used as inoculants for crops was also investigated during her work. Döbereiner and her colleaguesin the 1980's, also found a number of nitrogen-fixing bacteria colonizing the inner tissues of plants (diazotrophic endophytes). In addition, rhizosphere diazotrophs signifyingnumerous genera such as *Acetobacter,Azoarcus, Azospirillum, Azotobacter, Beijerinckia, Burkholderia, Enterobacter, Herbaspirillum, Klebsiella, Paenibacillus*and *Pseudomonas* etc. have been revealed to improve the growth of the plants that generate a suitable rhizosphere. These comprises agriculturally-important plant species such as rice, wheat, barley, potato and several vegetable crops (Dobelaere et al., 2003). Diazotrophic rhizosphere bacteria in addition to the assumption that they improveplant growth through BNF, these bacteria are also found often with capability to produce plant growth-enhancing phytohormones and pathogen-suppressing antibiotics (Chanway, 2002). Many of these bacteria are also able to improve the availability of N, P and S in the rhizosphere enzymatically, consequently making it difficult to determine with certainty the mechanism by which plant growth is stimulated. Christine Kennedy and her co-workers later confirmed the capability of Nitrogen-fixation by these bacteria using $15N_2$ incorporation and Nifmutants. Thus, associative nitrogen fixation was resolutely recognized as a means of providing fixed nitrogen to plants, and shows the prospective partnerships between bio-fuel crops and nitrogen-fixing bacteria.

Research efforts are now focusing heavily on the associative nitrogen-fixers as well as the symbiotic species because these bacteria have considerable potential for generating alternative energy sources. For example, in addition to the enzyme nitrogenase, which reduces nitrogen gas to ammonia, many bacteria possess hydrogenase, a reaction coupled to nitrogen fixation whereby hydrogen gas is oxidized. However, if no nitrogen is present, nitrogenase produces hydrogen as long as sufficient chemical energy is supplied as ATP. Hydrogen is considered to be a

powerful alternative fuel source, but much more research is needed to get nitrogen-fixing microbes to produce hydrogen efficiently and rapidly.

### 1.1.10. Endophytic Rhizobia

Endophytes are defined as those microorganisms that live within plant tissues for all or part of their life cycles and cause no apparent infections or symptoms of disease (Wilson 1995; Azevedo et al. 2000; Bacon and White 2000; Saikkonenet al. 2004). Endophytes are also described by Hallmann et al. (1997) as those organisms that can be isolated from surface-sterilized plant parts or extracted from inner tissues and that are no source for any damaging effect to the host plant. Instead in many cases, they may stimulate host growth through several mechanisms such as biological control, stimulation of systemic resistance to pathogens, nitrogen fixation, production of growth regulators, and improvement of mineral nutrients or water uptake (Ryan et al. 2008).

Classical microbiological procedures including cultivation of bacteria establish soil bacteria of several genera such as *Azospirillum, Azotobacter, Alcaligenes, Bacillus, Beijerinckia, Campylobacter, Derxia,* several members of *Enterobacteriaceae (Klebsiella, Pantoae)* and *Pseudomonas* as belonging to endophytes ( Rennie 1980; Balandreau 1983; Elmerich et al., 1992; Yan et al. 2008). Majority of these strains were isolated from surface-sterilized root samples indicating colonization of root tissues by these bacteria. Moreover, many additional isolates, such as *Azoarcus* (Hurek and Reinhold-Hurek 2003), *Burkholderia* (Caballero-Mellado et al. 2004), *Herbaspirillum* and *Gluconacetobacter* (Baldani and Baldani 2005), or *Klebsiella pneumoniae* strain 342 (Chelius and Triplett 2000), were also found to belong to endophytes.

### 1.1.11. Biological Nitrogen Fixation by Endophytic Bacteria

The ability to enhance plant growth and nutritional improvement of plants through nitrogen fixation and other mechanisms by Endophytic bacteria made its study a huge agronomic interest (Boddey et al. 2003; Sevilla et al. 2001). More than rhizospheric bacteria, diazotrophic endophytic bacteria were found to deliver more of

fixed nitrogen since the interior of plants is a more appropriate niche for nitrogen fixation due to the low partial oxygen pressure (pO2) and direct availability of the fixed nitrogen to the plants (James and Olivares 1998). Hence the application of $N_2$-fixing endophytic bacteria as biofertilizer has become one of the most effective and environmentally viable methods for increasing the growth and yield of crop plants (Singh et al. 2011).

The isolation of endophytic *Gluconacetobacter diazotrophicus* from Brazilian sugarcane resulted in the widespread research on endophytic bacteria and its valuable effects on plant growth (James and Olivares 1998) and later on several $N_2$-fixing bacteria are found associated with sugarcane (Boddey et al. 2003). Studies during the 1980s, gave many accounts on endophytic bacteria having nitrogen-fixing activity in gramineous plants (Olivares et al. 1996; Reinhold-Hurek and Hurek 1998; Mano and Morisaki 2008). The concept of BNF by endophytes (Dobereiner 1992) has resulted to studies on the potential uses of endophytic nitrogen-fixing bacteria colonizing graminaceous plants. Promotion of growth in rice plants by some endophytic bacteria have also been reported. During a study for the isolation and characterization of bacteria associated with soybean, Kuklinsky-Sobral et al. (2004) evaluated 75 endophytic isolates for their ability to fix atmospheric nitrogen by considering bacterial growth in a nitrogen-free medium (NFb medium) and then PCR specific for the nifH gene (encodes nitrogenase protein component II). Govindarajan et al. (2007) suggested that such crop-associated native nitrogen fixers may be agronomically significant as they could possibly supply part of the nitrogen that the crop requires.

### 1.1.12. Significance of Legume Plants

Legume plants are the group of plants that belongs in the family Fabaceae (or Leguminosae),which are primarily grown agriculturally for their grain seed called pulse, soil enhancing green manure as well as for livestock forage and silage. Legumes, generally defined with their unusual structure of flower, podded fruit, and 88% of the species examined to date with the capacity to form nodules with rhizobia (de Faria et al., 1989), are second only to the Graminiae in their significance to humans. Legumes are concurrently one of the largest families of crop plants and a

corner stone in biological nitrogen cycle (Choi et al, 2004). The 670 to 750 genera and 18,000 to 19,000 species of legumes (Polhill et al., 1981) include important grain, pasture, and agroforestry species. Legumes are distinguished from the reason that most of them harbours symbiotic nitrogen-fixing bacteria in structures so-called root nodules, due to which they play a vital role in crop rotation.

Several legumes have symbiotic bacteria called *Rhizobia* thriving within the root nodules and these bacteria have the special capability of fixing nitrogen from atmospheric molecular nitrogen $N_2$ into ammonia $NH_3$ (Deacon, 1997).This indicates that the root nodules are nitrogen sources for legumes, making them reasonably rich in plant proteins and all proteins contain nitrogenous amino acids. When a legume plant dies, all of its remaining nitrogen which are incorporated into amino acids inside the plant parts, is released back into the soil. In the soil, the amino acids are converted to nitrate ($NO_3^-$), making the nitrogen available to other plants, thereby serving as fertilizer for future crops (Postgate 1998, Smil 2000).

Legumes are as much important to the natural environment as it is to different agriculture environment. Sprent and Parson (2000), suggested that growing of grain and forage covers 2-15% of the Earth's arable surface, accounting for 27 % of the world's primary production, with grain legumes alone contributing 33 % of the dietary protein nitrogen needs of humans. For centuries, the foundation for dairy and meat production have been laid possible with the use of forage legumes (Russelle, 2001). When appropriately managed, legume plants provides a rich sources of protein, fiber, and energy.

In addition to its role as a source of protein N in the diet, the importance of symbiotic $N_2$ fixation in legumes lies due to the fact that, the fixed N is essentially "free" N for use by the host plant or by associated or subsequent crops. Moreover, fertilizer N is often unavailable to subsistence farmers, which madethem dependent upon $N_2$fixation by legumes or other $N_2$-fixing organisms. Interest in BNF has been focusing on the legume-rhizobia symbiotic systems as these associations have the highest quantitative impact on the nitrogen cycle. Deficiency in mineral N is often a limiting factor for plant growth which ultimately gives rise to the evolution of the so-

called symbiotic relationships between plants and a range of $N_2$-fixing organisms (Freiberg et al., 1997). The $N_2$, fixed symbiotically by the association between *Rhizobium* species and the legumes, represents a renewable source of N for agriculture. Estimated values for numerous legume crops and pasture species are often remarkable, usually falling in the range 200 –300 kg N ha − 1 per year (Peoples et al., 1995).

Apart from the crop legumes, the nodulated wild herb and tree legumes also have the potential for nitrogen fixation, reforestation and control of soil erosion (Ahmad et al., 1984). It has been reported by Jha et al., 1995, that a novel, suitable associations of wild legume-*Rhizobium* are beneficial in providing a vegetational cover in degraded lands. Sprent and Parsons (2000) discuss the significance of woody tree legumes in forestry, which includes important genera such as *Acacia*, *Anadenathera*, *Calliandra*, *Dalbergia*, *Erythrina*, *Gliricidia*, *Melanoxylon*, *Parkia*, *Prosopis*, *Pterocarpus*, and *Samanea*. One of the wide-host range legume tree is*Leucaena leucocephala*, which is nodulated by some strains of bean-nodulating rhizobia (Mhamdi et al., 2000), thus emphasizing the capability of wild legume trees to harbour bacterial strains belonging to an entirely different groups in their root nodules. The diversity of rhizobial populations nodulating wild legumes, nevertheless, is a common character which is also seen in other legumes, e.g. common bean (*Phaseolus ulgaris*), which harbour a diversity rhizobial strains belonging to different species of genus *Rhizobium*: *R. leguminosarum*, *R. etli*and *R. tropici*(Van Berkum et al., 1996).

Prevailing situations like food security issues, pressure on the land, and increasing soil degradation (Franzluebbers et al., 1998; Cassman, 1999; Sanchez, 2002) have led to increase in interest for research in tree-fallow and alley cropping systems for subsistence farmers in Africa and Asia. In tree fallows, legume species like *Sesbania* spp., *Leucaena* spp., *Tephrosia*spp., *Crotalaria*spp., *Glyricidia*spp., or *Cajanus*spp. are interplanted into corn, and allowed to grow as dry-season or longer-term fallows. In Costa Rica, Henriksen et al., 2002, reported about *Phaseolus* spp. grown between *Erythrina poeppigiana* rows and supplied prunings from these trees yielding 15% to 50% more than the crop grown in monoculture. *Sesbania* sp. has been

used similarly for alley cropping in rice. Crop rotation concerning legumes is common practice in many traditional and organic farming practices. By alteration of growing between legumes and non-legumes, the field regularly receives an abundant amount of nitrogenous compounds thereby producing a good result, even when the crop is non-leguminous. Legumes are sometimes referred to as "green manure".

### 1.1.13. Morphological and Biochemical analysis

Preliminary identification of microbes according to their morphological traits *viz.* shape, size, colour, margin etc. as well as in accordance with their biochemical characteristics by performing numerous biochemical tests available is of utmost importance. Preliminary identification as such helps in singling out and screening the microbes of interest from the bulk population before further more precise methods are used. Preliminary tests are done to identify a microbe upto the genus level. Preliminary identification were then followed up with further confirmatory tests and later more improve molecular tools for characterizations are incorporated for more precise identification of the microbes upto the species level. For the assessment of microbes isolated from the rhizosphere soil and root nodules of wild legumes of Manipur, India, the methods carried out were on the basis as described by Cappuccino *et al* (2005) and Dubey and Maheshwari (2002).

### 1.1.14. Acetylene Reduction Assay

Determination of the nitrogen fixing capability of microbes through Acetylene Reduction Assay is solely based on the reducing capability of the enzyme nitrogenase. In addition to reducing $N_2$ to $NH_3$, the enzyme nitrogenase can also reduce certain compounds like Acetylene (Subba Rao 2008). An important achievement for the rapid determination of nitrogenase activity was the introduction of an analysis called the Acetylene Reduction Assay or ARA (Hardy et al. 1973), that could be applied also to excised roots, soil cores and greenhouse experiments in addition to its application to pure cultures and cellular extracts (Bergersen 1980).This assay is based on the nitrogenase-catalyzed reduction of $C_2H_2$ (Acetylene) to $C_2H_4$ (Ethylene), which can be determined by using gas chromatography. Through this assay, quantification of the amount of nitrogen fixed can be established by the extent of reduction of $C_2H_2$ to

$C_2H_4$. In the present study, bacterial samples with the most prominent and healthy growth from the respective nitrogen free media were selected and their ability to fix atmospheric nitrogen were evaluated through Acetylene Reduction Assay (ARA).

### 1.1.15. Amplification by Polymerase Chain Reaction:

Polymerase Chain Reaction (PCR), atechnique developed by Kary Mullis (1993),provides amplification in vitro specific DNA sequences. The invention of this machine enable the amplification of large fragments of DNA without the need of cloning the fragments, with the use of a thermo stable DNA polymerase enzyme and specific primers. Primers are oligonucleotides consisting of 15-20 bases whose sequence is complementary to the target DNA sequence which is supposed to be amplified. PCR technique is generally handy to employed when the biological sample is available in very small quantity (Dubey 2004). Separating the double stranded DNA (dsDNA) to a single stranded (ssDNA) by heating which results in denaturing the DNA comprises the first step of the reaction procedure. The next step is the annealing step where the primers after addition gets attached to the single stranded DNA. The third step where the polymerase enzyme initiates the synthesis of the complementary nucleotide chain starting at the known primer sequence represents the extension step. This process is automated in a thermo cycler where products of distinct length are formed exponentially to a million-fold or more, which are detected even with poorly sensitive probes or sometimes without any probe. It could also be detected only by staining the electrophoretic gel of the PCR products prepared by Southern bolt method.

### 1.1.16. Differentiation through 16S r-DNA sequence analysis

16S ribosomal RNA constitute a part of the 30S small subunit of prokaryotic ribosome. The genes coding for it are referred to as 16S rDNA and are used in reconstructing phylogenies because of their slow rates of evolution of this region of the gene (Woese and Fox, 1977). The sole reason for the use of 16S rRNA gene for phylogenetic studies (Weisburg et al., 1991)lies in fact that it is highly conserved amongst different species of bacteria and archaea (Coenye and Vandamme, 2003). Moreover, it is present in most microbes and shows proper changes and consequently

16S rRNA gene is used as the standard for classification and identification of microbes.

Apart from the presence of highly conserved primer binding sites, 16S rRNA gene sequences contain hyper variable regions that provides species-specific signature sequences useful for bacterial identification (Pereira et al., 2010; Kolbert and Persing, 1999). Consequently for this reason, sequencing of 16S rRNA gene has emerged as a prevalent technique in microbiology proving to be a speedy and inexpensive alternative to phenotypic methods of bacterial identification (Clarridge III, 2004). Even though it was initially used for identification of bacteria, 16S rRNA sequencing was also consequently found to be capable of reclassifying bacteria into completely new species (Lu et al., 2009), or even genera (Weisburg et al., 1991; Brett et al., 1998). Moreover the technique has also been used for describing new species that have never been successfully cultured (Schmidt and Relman, 1994; Gray and Herwig, 1996).

### 1.1.17. Phylogenetic Analysis

Every organism shares a common ancestry (Steel and Penny, 2010). However, during the course of evolution due to inheritance, variation, mutation, recombination or selection (Darwin, 1989), some phenotypic and genotypic variations had taken place which eventually led to diversification of different families, genera and species. Consequently, in surge to study the evolutionary relationship amongst organisms to determine their evolutionary position, a new branch is evolved called Phylogenetics.

Phylogetic analysis or evolutionary sequence analysis is one of the major areas of sequence analysis which deals with the evolutionary connection between sequences of biological species. By determining relationships among different organisms, the evolutionary history of organisms during the course of evolution could be determined. A phylogenetic tree or evolutionary tree is a branching diagram or tree displaying the inferred evolutionary relationships among various biological species or other entities based upon similarities or dissimilarities data that has been used to build a hierarchical relationship among organisms (Hodge and Cope, 2000). A phylogenetic tree consists of a set of nodes linked together by branches, therefore representing

relationship among separate sequences or taxa. The nodes represented the ancestral sequences, and the branches represents the topological relationships between the nodes (Saitou and Nei, 1987). The length of each branch represents the evolutionary distance that separates the nodes (Maher, 2002).

The amount of variance between homologous DNA, RNA or protein sequences in different organisms is used as a measure of how much the organisms have diverged from one another evolutionarily (Schmidt and Relman, 1994). rRNA sequences differ between species due to mutation. Through these variations in rRNA sequences the determination of organisms upto the species level and trace evolutionary relationships could be achieve.

### 1.1.18. Current Work:

The present study account with the isolation of several nitrogen fixing bacteria from five selected wild legume plants viz. *Crotalaria pallida*, *Crotalaria juncea, Leucaena leucocephala, Sesbania sesban* and *Parkia roxburghii.* Isolation of the bacteria are done both from the rhizosphere soil as well as from the root nodules of the above mentioned legume plants. The current study give emphasis to isolate, categorize and to determine their biochemical as well as molecular characteristics. The present work is really a significant approach as so far no work in this area has been attempted from the region. Through morphological, biochemical and molecular characterization, the position of the isolated samples are determined upto their species level.

### 1.2. MAIN OBJECTIVE

Identification of potential nitrogen fixing bacteria through 16Sr DNA analysis of isolates from the selected regumes

#### 1.2.1. SPECIFIC OBJECTIVES

1. To isolate several bacterial strains from different wild plant sources.

2. Characterization of isolated species by using phenotypic and biochemical methods, in order to assess their taxonomic and genetic diversity.

3. To investigate their ability to fix atmospheric nitrogen and the occurrence of *nif*H-like genes.

4. Molecular characterization of isolated species by performing 16s rDNA sequencing.

5. Suitable tools of bioinformatics may be used for phylogenetic analysis.