Chapter 1
INTRODUCTION

Dutch microbiologist and botanist Martinus Willem Beijerinck (1851-1931) first discovered the process of biological nitrogen fixation. While working in Wageningen University and Polytechnische Hogeschool Delft (Delft University of Technology), he thoroughly studied the various nitrogen fixing organisms and found that certain bacteria can convert atmospheric nitrogen into ammonia. He had isolated *Bacillus rudicicola* and proved that it forms nodules on the roots of members of the family Fabaceae. Later he isolated *Rhizobium* species, studied nitrogen fixation, and demonstrated nitrogen fixation by free-living microorganisms, particularly *Azotobacter chroococcum* (http:// en.wikipedia.org/wiki/ Martinus_Beijerinck). Some of his students also did excellent work in this field. Prof. A.H. Van Delden, who was a student of Prof. Beijerinck, worked in this field of microbiology. He worked on *Bacillus oligocarbophilus*, which is a nitrogen fixer. At the same time Sergei Nikolaievich Winogradsky (1856 – 1953) in his short working period in St. Petersburg (1891-1905) as chief of the division of general microbiology of the Institute of Experimental Medicine, first identified the ability of obligate anaerobe *Clostridium pastorianu* in fixing atmospheric nitrogen. Basically, in biological nitrogen fixation, the prokaryotic organisms convert the atmospheric nitrogen into ammonium ion, which is absorbed by the plants. *Frankia* is one of those prokaryotic organisms which have this unique property.

The bacterium *Frankia* is a microaerophilic, nitrogen fixing, gram positive to gram variable, sporulating actinomycetes belong to the Darmatophilous group. This bacterium has a high G+C content of 68-72% (An *et al*, 1983). It produces characteristic sporangia in submerged liquid culture and also form vesicles. This

morphology differentiates *Frankia* from other actinomycetes (Myrold, 1994). This bacterium produces three characteristic cell types, namely vegetative hyphae, sporangium and vesicle in the culture medium as well as *in planta* and it is assumed that these structures are also produced in the soil. In solid agar medium *Frankia* produce submerged colonies without aerobic hyphae. In liquid culture *Frankia* produce submerged growth with out any floating or aerial growth (Benson and Silvester, 1993). It is an obligate heterotrophic, microaerophilic, slow growing bacterium with a doubling time of about 15hr or more in culture; however their growth in symbiotic condition seems to be unrestricted, because timing of root infection, nodule development, and host cell infection are similar to that of rhizobia-legume system (Wall, 2000).

Vesicle is the site of the nitrogenase activity of *Frankia*. The lipid coating layers of the vesicle provide the necessary oxygen protection towards the nitrogenase. This property of the vesicle is revealed by Freeze fracturing technique of electron microscopy (Parsons *et al*, 1987). The actinorhizal nodules show an optimal nitrogenase activity at around 20K Pa of oxygen and significant inhibition above 25K Pa of oxygen is noted (Rosendahl and Huss.Danell, 1988; Silvester *et al*, 1990).

An (1985) was the first to use the DNA homology technique in classification of *Frankia* (An *et al.*, 1983; An *et al.*, 1985; An *et al.*, 1987). With the advent of Field emission scanning electron microscopy, Polymerase Chin Reaction (PCR) technology, Sequencing technology, DNA hybridization and various bioinformatics software like CodonW, CAI calculator etc gave a significant boost in the *Frankia* research. Various *Frankia* specific PCR primers targeting to 16s rRNA, 16s-23s rRNA spacer and *nif*D-*nifK* intergenic region have been developed. Workers used these techniques to study both pure cultures as well as uncultured *Frankia* directly from nodules (Benson *et al.*, 1996; Normand *et al.*, 1996; Murry *et al.*, 1997; Jamman *et al.*, 1993; Jeong *et al.*, 1999; Sur *et al.*, 2006, 2007, Bose *et al.*,2007; Ventura *et al.*,2007; Sen *et al.*, 2008, 2010). Thus, it has become easy to study the evolution, systematic and diversity of *Frankia* with the help of these molecular approaches. Recently the whole genome of six *Frankia* strains have been sequenced (e.g. *Frankia*

EAN1pec NC 009921; *Frankia* Eul1c NC 014666, *Frankia* NC 015657; *Frankia* ACN14a NC 008278; *Frankia* CcI3, NC 007777; and *Frankia* EUN1f, NZ ADGX00000000) (http:// www.ncbi.nlm.nih.gov/genome).

In addition to these molecular techniques, traditional culture based approaches are also on to elucidate the mechanism of nitrogen fixation by *Frankia*-actinorhizal system. Heavy metal resistance pattern (Richard *et al.*,2002 Bose and Sen.,2006, 2007) and antibiotic resistance pattern (Tisa *et al.*,1999) of *Frankia* have been established in the determine the effective genetic marker in *Frankia*.

Unlike *Rhizobium*, *Frankia* make symbiotic association with a number of woody dicotyledonous plants collectively known as actinorhizal plants (*action* from actinomycetes, *rhiza* the plant root) (Tjepkema and Torrey, 1979). To be precise *Frankia* infects about 194 species of dicotyledonous plants belonging to 25 genera, 8 families and 7 orders. Surprisingly there are representatives of actirhizal plants in almost every orders cutting across the evolutionary lineage (Benson and Silvester, 1993, Normand and Fernandez, 2006). The nitrogen fixing capability of those plants under the influence of *Frankia* helps them becoming key representative of different ecosystem and various agro forestry programme. These plants fixed sustainable amount of molecular nitrogen, which ranges from 240-350 kg N_2 $ha^{-1}y^{-1}$ and comparable with those of leguminous plants (Dawson, 1990; Hibbs and Cromack, 1990; Wheeler and Miller, 1990, Wall, 2000). This nitrogen improves the nitrogen condition of the soil and this nitrogen fixing capability is advantageous to the yield of associated plant group and reduces the use of chemical nitrogenous fertilizers. They increase the yields of associated crops by way of soil nitrogen with adding litters. They also increase the concentration of nutrients to the surface of the soil by extracting them from the deeper layer. Y. R. Dommergues (1987) postulated six important concepts for optimal utilization of biological nitrogen fixation in agro forestry which are as follows:

a. Plant species with high nitrogen fixing ability with good adaptability should be used.

b. Identify and multiplication of best host.

c. The best should show high nitrogen

fixing potential in field condition.

d. The host should provide with required nutrients for expression of full nitrogen fixing potential.

e. Secondary effects should not be overlooked

f. The host should be analyzed through short term and long term method.

Among the actinorhizal species *Alnus* and *Casuarina.* (Akkermans and Houvers, 1983; Gauthier *et al.,* 1984; Bond, 1983) are important for agro forestry.

Casuarina, a member of Casuarinaceae, consists of 82 species, mostly from Australia, South-East Asia and the Pacific islands. Johnson (1982) recognizes four genera under this family: *Casuarina* (e.g *C. equisetifolia, C. cunninghamiana, C. obesa, C. junghuhniana,* syn. *C. montana, C. Oligodon, C. glauca,*); *Allocasuarina* (e.g., *A. torulosa, A. littoralis, A. fraseriana, A. stricta, A. decaisneana*); *Gymnostoma* (e.g., *G. rumphiana, G. deplancheana, G. papuana*), and *Ceuthostoma. Casuarina* is suitable for agroforestry systems. One classical example is found in Papua New Guinea, where *C. oligodon* and *G. papuana* are used in the highlands and *C. equisetifolia* used in the lowlands, as intercrop with food crops, and are used as shade trees (Thiagalingam, 1983; Bourke, 1985). In India, *C. equisetifolia* plantations are associated with crops such as peanuts, sesame and various pulses (Kondas, 1981). *Casuarina* is planted as windbreaks. *C glauca* is used in this purpose in Tunisia and *C. equisetifolia* is used in Senegal. *Casuarina* has profuse nodulation property and this property of *Casuarina* explains its high nitrogen -fixing potential. A selected clone of *Casuarina equisetifolia* was reported to fix 42.5 g of N_2 per tree during the first nine months following plantation. *Casuarina* can tolerate high degree of salt concentrations. *C. obesa* can tolerate 15,000 ppm of chloride. So, they are considered as good candidates for the reclamation of salt-affected soils (Bajwa, 2004).

On the other hand, genus *Alnus* belongs to the family Betulaceae. Important *Alnus* species are *A nepalensis, A. viridis, A. glutinosa* etc. They are also good candidates for agro forestry. *Alnus* prefer cool and moist climate with mean annual temperature of 13-26°C and mature trees are frost tolerant. They can grow up to 3000m with annual rainfall of 500-2500mm. They can also grow on non fertile hill soil. *Alnus* is introduced in several

countries for species trial in agro forestry which include Bolivia, Uganda, Burundi and Java (Bajwa, 2004).

The improvement of soil texture and environmental condition by actinorhizal nitrogen fixation depend upon the improvement of host and symbiont, that is *Frankia.* A well characterized *Frankia* with good host can change the scenario of nitrogen deficient soil in the countries like India.

Therefore The Research Needs-Difficulties in *Frankia* Research

In comparison to other field, modern *Frankia* research has not grown up satisfactorily. The slow growth rate of *Frankia* and presence of contaminations are responsible behind this phenomenon (Benson and Schultz, 1990). The growth rate is the characteristic of an organism and a very little is left in the hand of workers. However researcher can reduce or eliminate the contamination. Sodium hypochlorite and osmium tetra-oxide are the potent candidates for this purpose (Benson and Silvester, 1993). Corrosive nature of osmium tetra-oxide creates problem in the isolation of *Frankia.*

Another difficulty in *Frankia* research is the absence of a universal media for isolation and maintenance of pure culture. *Frankia* is a poor competitor for carbon sources in comparison to its contaminants. The cortical layers of actinorhizal root nodules are heavily contaminated with soil microorganisms. The growth rate of which is generally faster than the *Frankia.* So, the prerequisite for successful isolation of *Frankia* was to eliminate these contaminants without damaging the endophyte in the cortical tissue. Lechevalier and Lechevalier (1990) suggested simple tap water agar, define propionate minimal medium to complex Qmod (Lalonde and Calvert, 1979) medium for isolation and maintenance. Bose and Sen (2006) pointed out the problems in using the two types of media. The minimal medium suppressed the contaminations, which may grow upon transfer into richer medium and on the other hand richer medium invite contaminations from environment. *Frankia* also need various nutritional supplements for growth (Lechevalier *et al.,* 1982). The isolation problem also inhibits the sequencing based diversity work. Limited work is done in recent years and six strains of *Frankia* genome have been sequenced (e.g.

Frankia EAN1pec NC 009921; *Frankia* Eul1c NC 014666, *Frankia* NC 015657; *Frankia* ACN14a NC 008278; *Frankia* CcI3, NC 007777; and *Frankia* EUN1f, NZ ADGX00000000) (http:// www.ncbi.nlm.nih.gov/genome)

The criteria used in classifying *Frankia* include the morphology, chemistry, plant infectivity, serology and DNA homology (Lechevalier, 1984). Although in order to differentiate strains a few physiological tests have been developed (Lechevalier *et al.*, 1983; Horriere, 1984; Weber *et al.*, 1988). But limited work had done in this field of microbiology. The molecular biological work is also very limited in this field. Overall very limited work has been done in Indian *Frankia* and particularly in *Frankia* associated with *C. equisetifolia*. A few work has been done in the *Alnus* associated *Frankia* (Sen, 1996; Bajwa, 2004). So, there are mammoth work is left in the field of *Frankia* research in India. In short the difficulties are summarized under the following heads:

- After almost thirty years of its isolation in pure culture, the isolation of *Frankia* in pure culture still remains a very difficult job to do. Development of a suitable easy technique for isolation of *Frankia* in pure culture is one of the prime necessities of *Frankia* research.
- Physiological and morphological characterization of *Frankia* strains have not been done widely and in India this kind of characterization work is almost absent. Thus physiological and morphological characterization of *Frankia* is another important field of *Frankia* research which need immediate attention.
- The genetic diversity of *Frankia* is that kind of field where a considerable work has been done, but satisfactory result needs more rigorous work in this field with new primers and statistical methods.

Thus The Objective of The Present Study

- Survey and collection of germplasms from various parts of Darjeeling hills for *Alnus* based *Frankia* and different regions of coastal Bengal for *Casuarina* based *Frankia*.
- Isolation of *Frankia* in pure culture.
- Morphological characterization of pure culture of *Frankia* with field emission scanning electron microscopy.
- Biochemical characterization of

isolated *Frankia* found in hilly and coastal Bengal.

- Preparation of heavy metal stress resistance profile of *Frankia* found in hilly and coastal Bengal.
- Study of GC composition, codon usage profile, codon preferences and codon adaptation index of heavy metal resistance genes of *Frankia* of both *Alnus* and *Casuarina* based *Frankia* strains found in public domain along with *Elaeagnus* strain.
- Isolation of genomic DNA directly from nodules and strains.
- PCR amplification of parts of 16s rRNA gene of *Frankia* with newly designed primer and restriction digestion of the amplicon.
- Determination of diversity among cultured and uncultured *Frankia* strains from hilly and coastal Bengal from the data generated.