Increase in population is a matter of great concern, because it needs more food grains. Increased demand for food results in intense agriculture which in turn need heavy use of either inorganic fertilisers or natural fertilisers-biofertilisers. The energy crisis has hindered fertiliser production and attention is being paid to alternate ways of increasing crop yields. The rapid increase in the rate of demand for food (70%) merely represents the more rapid increase in population which is far higher in the developing countries than in the rest of the world, and has in fact become faster in the last ten years or so.

It is estimated that the total food demand in developing countries will increase by approximately 150% by 2025. The continuing population increase will result in a decline of available cultivable land per capita world-wide from 0.3 ha in 1988 to 0.17 ha in 2025, with only 0.11 ha per capita in developing countries. Mahatma Gandhi mentioned as far back as 1930 that Indian agriculture will continue to stagnate unless brain and brawn are married in the countryside. He made his remarks because of the very low social prestige attached to rural professions with the consequent migration of the educated persons to town and to city.\footnote{1}

Recent predictions of the growing imbalance between our ability to produce food from diminishing land and water resources, and expanding
biotic and abiotic stresses point towards the incidence of serious famines if efforts in the area of population stabilisation do not bear fruit.

The poorer sections of the agricultural population must be drawn into the productive process which provides food, income and employment, if the overall target of development and betterment is to be achieved. This does not mean turning ones' back on modern inputs, despite the present scarcity of fertilisers and their high cost. It would be futile, however to allow a situation to continue to deteriorate whereby developing countries are unable to procure even the minor part of their fertiliser needs. Efforts are needed to bring about a better balance in the demand and supply of fertiliser backed by mechanisms which would ensure that the essential demands in the developing countries can be met with some degree of certainty. The long term solution lies at least to some extent, in the increase in fertiliser production in the developing countries themselves.

Production levels have to be further raised with the aim of attaining the production of 240 million tonnes by the turn of century. There is a good potential to achieve this target but it certainly is a big task before us. Fertilisers play a significant role in increasing the production of food and fibre. Therefore, their proper use is directly related to our efforts for ensuring food security. The interest of farmers and the fertiliser industry are interlinked in the sense that the growth of the industry will enable the farmers for adequate use of fertilisers for increased production. Apart from the chemical fertilisers it is very necessary to explore the possibilities of alternate sources of nutrients for use by farmers.

The well known areas in this regard are the mobilisation of the city and rural wastes, popularising green manure in appropriate area, production and popularisation of biofertilisers. Biofertilisers are cost-effective supplements to chemical fertilisers and can help us economise on the high cost of developing our fertiliser industry. It is therefore appropriate to understand the critical importance of bio-technology of which bio-fertiliser is
only one part. Biotechnology is the technology which will prepare us for the 21st century. It was coal and steam power which fuelled the first industrial revolution. The second revolution was sparkled by chemical and electrical industries. The third industrial revolution is bred by computers and biotechnology which are the frontier areas of development in India. The progress made so far in bio-fertilisers is at best an initial advance, and we are expecting much more to follow for stepping up productivity of our agriculture. It is perfectly possible to increase yields through higher fertiliser use provided it is done scientifically on the basis of soil tests. The nutrients can be supplied not only by chemical fertilisers but also by bio-fertilisers and manure. To meet the estimated requirements of food grains by 2000 AD at 225 million tonnes we may need fertiliser nutrients of the order of 200 to 240 lakh tonnes. We need to place very high emphasis on bio-fertilisers as a source of plant food by 2000 AD.3

Our policy is to help the farmers with plant food from cost-effective sources. The higher the yield, the greater the removal of nutrients from the soil and the greater need to replace such nutrients in the soil. We must encourage the farmers to provide balanced nutrient replacement through sources, namely, organic wastes from the farm, bio-fertilisers and chemical fertilisers.

One of the paradoxes of the industry is the abundance of nitrogen in the atmosphere and yet its non-availability to plants and animals. This nitrogen has to be provided if possible through organic wastes. But the concentration of nitrogen in such wastes is hardly 2-3%; and one needs enormous quantities to meet the nitrogen requirement of the soil. Three cart loads or 3 tonnes of manure is required for one hectare paddy or wheat. Bio-fertilisers are an inexpensive method of providing nitrogen to the soil. Cultivation of leguminous crops like pulses, groundnut or soybean can fix nitrogen of the order of 60 kg. of the value of about Rs. 300, with the assistance of 500 g rhizobium culture of the value of Rs. 20. Use of rhizobium
culture has now become very common in the US, Australia and New Zealand. Rhizobium in powder form is used to coat pulses seeds and legume oilseed in order to help the supply of nitrogen by biological fixation. In the case of wet land rice, specific strains of blue green algae have been identified which add about 25 kg of nitrogen per hectare of the value of about Rs. 125. It costs only about Rs. 15 per hectare to apply the algae.

The government of India has sanctioned a national project on the development of 'Biofertilisers'. We already have one national, six regional centres as well as 40 subcentres for the production of the blue green algae. The project also provides for the transfer of technology to the farmers and for quality control. The technology of bio-fertilisers is quite inexpensive and could be intensively adopted by small and marginal farmers. There is however, no time to relax or to rejoice, as population is growing tremendously and land availability for agriculture is getting reduced. Also the nature of the food security challenge is undergoing change. If during the first three decades of our Independence, physical access to food was the most important food security challenge, economic access to food has now become the most important cause of under nutrition and mal-nutrition. In the 21st century, ecological access to food might become the most important challenge owing to the damage now being done to land, water, flora, fauna and the atmosphere. Sustainable expansion will clearly involve considerable expenditure over a fairly long period of time. And there will in any case be a higher import bill for the raw-materials, including the oil which is an essential ingredient.

The explosive growth of the world's population has been supported by increased input of nitrogen into the world's agricultural soils, through chemical fertilisers, the overuse of which may result in serious consequences such as eutrophication, effects of atmospheric nitrogen oxides etc. The production of industrial 'N' fertiliser by Haber Bosch process requires high energy costs and is beyond the reach of Indian farmers. In this context,
nitrogen fixation has become a fascinating subject of immense practical importance.

Nitrogen is the nutrient most limiting plant growth, especially in agricultural systems. Plants normally acquire nitrogen from the soil in inorganic form (nitrite or nitrate or ammonium), made available by biological nitrogen cycle.

\[
\begin{align*}
\text{reduction} & \quad \xrightarrow{\text{N}_2 \text{ fixation}} \\
\text{dinitrogen} & \quad \xrightarrow{\text{nitrification}} \text{NO}_3^- \quad \xrightarrow{\text{denitrification}} \text{NH}_3 \quad \longrightarrow \quad \text{Plant and microbial protein} \quad \longrightarrow \quad \text{Animal protein}
\end{align*}
\]

The biological nitrogen cycle is the reduction of atmospheric dinitrogen (N\(_2\)) to ammonia with the subsequent reoxidation of ammonia to dinitrogen. Reoxidation of NH\(_3\) to dinitrogen leads to the depletion of the "fixed", biologically usable nitrogen pool. Besides the relatively small contribution from commercial ammoniacal fertiliser production, replenishing of the nitrogen pool falls mainly to a limited number of physiologically diverse microbes that contain the nitrogenase system.

In recent years, the use of \(^{15}\text{N}\) tracer and C\(_2\)H\(_2\) reduction method have enriched our knowledge regarding the biochemical pathway between nitrogen and ammonia but the exact nature of intermediate products is not known. The overall reaction in the enzymic reduction of atmospheric nitrogen to NH\(_3\) could be summarised as follows:

\[
\begin{align*}
\text{N}_2 + 2\text{H}^+ + 2\text{e}^- & \xrightarrow{2\text{H}^+ + 2\text{e}^-} \text{HN = NH} + \text{H}_2\text{N} - \text{NH}_2 \xrightarrow{2\text{H}^+ + 2\text{e}^-} 2\text{NH}_3 \\
\text{Dinitrogen} & \quad \text{D\(_3\)ide} \quad \text{Hydrazine} \quad \text{Ammonia}
\end{align*}
\]

Nitrogenase in addition to reducing atmospheric nitrogen to ammonia, can also reduce certain other compounds, as follows:
This property of nitrogenase provides a non-nitrogen based approach for the measurement of nitrogen fixation. Specifically, the reduction of \( \text{C}_2\text{H}_2 \) to \( \text{C}_2\text{H}_4 \) and its determination by gas chromatography was proposed as a sensitive method of assay.\(^5\)

Essential elements of nitrogenase reaction: \( \text{NH}_3 \) synthesis requires eight electrons. Six for the reduction of dinitrogen and two for the coupled obligatory synthesis of \( \text{H}_2 \). These reactions are catalysed by the terminal component of the complex, the Mo-Fe protein, so designated because it contains Mo and Fe as moieties. Electrons are transferred to the Mo-Fe protein from the Fe protein in a process coupled to the hydrolysis of two ATP per electron. Because a minimum of 16 ATP are hydrolysed for the reduction of one molecule of dinitrogen, the organisms carrying out nitrogen fixation, have a vigorous energy metabolism.

The nitrogenase Fe protein and Mo-Fe protein have been sequenced and/or characterised from a variety of nitrogen fixing organisms. Generally speaking, the structural and functional properties of these proteins are highly conserved among different organisms.

The protein is a \( \sim 60 \) KDa dimer with identical subunits bridged by a single \( 4\text{Fe}:4\text{S} \) cluster. It has the property of binding to the nucleotides, Mg ATP and Mg ADP. It is at the \( 4\text{Fe}:4\text{S} \) cluster that undergoes a one electron redox cycle. Mo-Fe protein is an \( \alpha_2\beta_2 \) tetramer with a total molecular weight of \( \sim 240 \) KDa.\(^5\) The active site of the enzyme for substrate reduction is believed to be composed of an Mo-Fe dinuclear site, bridged by sulphur having the proper size and electron characteristics to provide Mo-Fe distance of about 3.8 Å. This distance is specific so as to accommodate the various nitrogenase substrates and to exclude others.\(^5\)
The first reaction in nitrogen reduction is formation of a linear complex of nitrogen with Fe of nitrogenase. This is followed by transfer of electrons from Mo which is the end point of the electrons activating system, resulting in the formation of diimide which is stabilised by H-bonding from the protein as well as the metal N-bonds. Successive addition of electrons produce hydrazine followed by cleavage of N-N bond to yield two moles of ammonia.\(^5\)

This process of splitting the triple bond of nitrogen requires an enormous amount of energy and it is the most energetic reaction in the biological world. The nitrogen molecule is also split in the Haber Bosch process, used for ammonia manufacture, at a temperature of 500°C and a pressure of 250 atmospheres. Micro-organisms accomplish the same process at one atmosphere and at 0-40°C.\(^6\)

The vast reserves of atmospheric dinitrogen has been continuously trapped by microorganisms through biological nitrogen fixing process asymbiotically as well as symbiotically. The discovery of consistent nitrogen fixation in cell free extracts of *Clostridium pasteurianum* by Carnahan and others at the Dupont Laboratory in USA in 1960 was a landmark in the field of biological nitrogen fixation. Applying the knowledge obtained with asymbiotic nitrogen fixers, Bergerson in Australia elaborated the biochemistry of nitrogen fixation in legume root nodules in the sixties. During the same period, Fogg and Steward in U. K. intensified the work on nitrogen fixing blue green algae.

Ammonia is assimilated in two ways by glutamate dehydrogenase (GDH) and glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway. Glutamate dehydrogenase catalyses the formation of glutamate by the reaction.

\[
\alpha\text{-oxoglutarate} + \text{NH}_3 + \text{NAD(P)H} + \text{H}^+ \rightleftharpoons \text{Glutamate} + \text{NAD(P)} + \text{H}_2\text{O}
\]
Most bacterial glutamate dehydrogenases have a low affinity for ammonia which result in an unfavourable equilibrium for glutamate formation when intracellular $\text{NH}_4^+$ levels are low. In addition, in $\text{NH}_4^+$ limited chemostat cultures of *Klebsiella aerogenes* this enzyme is repressed. When such cultures are pulsed with $\text{NH}_4^+$ an initial rise in the concentration of glutamine is observed, followed by a rise in glutamate concentration. These observations were rationalised by the demonstration of glutamine synthetase (GS) and glutamate synthase (GOGAT) in extracts of these organisms. These two enzymes result in the formation of glutamate by the coupling of two reactions.

\[
\text{Glutamate} + \text{NH}_3 + \text{ATP} \rightarrow \text{glutamine} + \text{ADP} + \text{Pi}
\]
catalysed by GS with

\[
\text{Glutamine} + \alpha\text{-oxoglutarate} + \text{NAD(P)}H + \text{H}^+ \rightarrow 2 \text{glutamate} + \text{NADP}
\]
catalysed by GOGAT.

Glutamate dehydrogenase appears to be involved in $\text{NH}_4^+$ assimilation, but under N-limited conditions, where the GS/GOGAT pathway operates.

Ammonia is assimilated in many bacteria by glutamate dehydrogenase (GDH) when the extracellular concentration of ammonia is high and by glutamine synthetase and glutamate synthase (GOGAT) at low ammonia concentrations. GDH, GS and GOGAT activities have been reported in *Azotobacter chroococcum*. The level of GDH or GOGAT activity does not vary with the nitrogen source.

Bacteria containing fertilisers such as azotobacterin and phosphobacterin were extensively used in Russia to improve soil fertility. The extensive use of the assay procedure utilising the nitrogenase catalysed reduction of acetylene to ethylene coupled with sensitive gas chromatographic analyses may be regarded as a turning point in the field of biological nitrogen fixation.
The term biofertiliser has been coined to embody all such micro-organisms which add, conserve and mobilise the plant nutrients in the soil. Such micro-organisms have somehow come to be called as "bio-fertilisers", a term which is a misnomer, compared to commercial fertilisers manufactured on a large scale in factories. In other words, biofertilisers are based on renewable energy sources and eco-friendly compared to commercial fertilisers. Biofertilisers play a very significant role in improving soil fertility by fixing atmospheric nitrogen, both in association with plant roots and without it, solubilise insoluble soil phosphates and produce plant growth substances in the soil.

Depending upon the nutrient provided Verma and Battacharya have broadly classified biofertilisers as follows:

**Biofertilisers (BF)**

<table>
<thead>
<tr>
<th>Nitrogen Fixing Biofertiliser (NBF)</th>
<th>Phosphate Mobilising Biofertiliser (PMBF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBF for legumes</td>
<td>Phosphate absorber</td>
</tr>
<tr>
<td><em>Rhizobium</em></td>
<td>VA-Mycorrhiza (VAM) like <em>glomus</em></td>
</tr>
<tr>
<td><em>Azotobacter</em></td>
<td></td>
</tr>
<tr>
<td><em>Azolla</em></td>
<td></td>
</tr>
<tr>
<td>BGA</td>
<td></td>
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<tr>
<td>NBF for cereals</td>
<td>Phosphate solubiliser</td>
</tr>
<tr>
<td><em>Azospirillum</em></td>
<td><em>Bacillus</em></td>
</tr>
<tr>
<td><em>Aspergillus</em></td>
<td><em>Pseudomonas</em></td>
</tr>
</tbody>
</table>

According to Dr. M. S. Swaminathan, many of the ecological problems associated with the green revolution of the seventies and eighties stem from a greed revolution, which leads to the excessive use of mineral fertilisers and chemical pesticides. Thus various environmental factors adversely affect the growth and nitrogen fixation of biofertilisers to a large extent. In general, N-cycling process and especially nitrogen fixation, have been shown to be sensitive to pollutants of which majority are heavy metals with which we are mainly concerned.¹
Heavy metals are one of the major pollutants in various habitats. Environmental contaminants are defined as anthropogenic chemicals that have the potential to damage or injure organisms. A number of heavy metals are required as cofactors or prosthetic group of enzymes in a wide variety of metabolic pathways. But high concentrations of these metals are toxic to the living organisms. Nitrogen fixation by free living heterotrophic bacteria was considerably reduced in soils contaminated with heavy metals. The inhibition of cyanobacterial growth and nitrogen fixation in the soils severely contaminated by heavy metals was confirmed.

Central role in nitrogen fixation is played by the enzyme nitrogenase. Metals are toxic to nitrogenase in many different ways.

(a) There can be a direct action on enzyme complex

(b) There may be an effect on the supply of ATP or reductant pool which are the requisites for enzyme activity.

Heavy metals have been reported to inhibit nitrogenase activity. The toxic effect may be directly due to the inhibition of enzyme activity and biochemical process involved in nitrogen fixation.

Various reports are available on the heavy metal pollution in the southwest coast of India.

Ernakulam district in Kerala accounts for more than 70% of the chemical industries situated on the banks of the river Periyar and Chitrapuzha. The effluents from these industries reach the estuary by tides and fresh water discharges. Surveys of the Cochin estuary, confirmed the presence of high concentrations of heavy metals in the sediments, viz., 45-80 ppm Ni, 44-298 ppm Cu and 58-180 ppm Cd in particulate matter.

Estuaries and coastal waters all over the world are increasingly exposed to heavy metal contamination in recent times. The sediments in the
Princes Royal Harbour, Albany, W. Australia contained 12-180 ppm of Pb, 6-122 ppm of Zn and 6-122 ppm of Cu whereas heavy metal contamination in Cochin estuary is 30-165 ppm Pb, 60-380 ppm Zn and 40-160 ppm Cu. The input of Hg and other metals to the study area may be from the chemical industries situated near the banks of the river Periyar and Chitrapuzha.

Another instance is Kayamkulam estuary, one of the major backwater systems in the South west coast of India. Except for coconut husk retting and agricultural activities, the estuarine system is devoid of other major sources of pollution. However, effluents from a gas based thermal power plant which is commissioned can alter the pace of sedimentological and geochemical processes of this aquatic regime in several ways. Heavy metal concentrations in Kayamkulam estuary is reported to be very high, viz., 38-160 ppm Cu, 0.5-4.5 ppm Cd and 0.15-1.10 ppm Hg.

The Kuttanad wetland system in Kerala has suffered heavy metal pollution by various anthropogenic activities. George et al. has reported on the heavy metal concentrations in Kuttanad, the rice bowl of Kerala, viz., 0.006-1.44 mg of Ni, 0.002-0.14 mg Cu and 0.002-0.11 mg Cd. The most important contributors of heavy metals in Kuttanad are fertilisers, sewage disposal and industrial discharges.

Heavy metals such as copper and zinc are required by biological systems as structural and catalytic components of proteins and enzymes and as cofactors essential to normal growth and development. In excess, heavy metals such as Cd, Hg, Ni and Cu become extremely toxic to cells. While plant growth may be severely restricted by heavy metals, some plant species posses a unique ability to adapt rapidly and evolve tolerance to toxic or lethal levels of heavy metals.

The uptake and concentration of environmental contaminants are important in understanding the toxicity of various compounds and their
distribution into different biotic and abiotic compartments. The phenomenon of uptake and concentration of a chemical by organisms in commonly termed bioaccumulation or bioconcentration. Bioaccumulation of heavy metals by algae may be an important factor in the physical transport of toxic materials from one place to another and the biomagnification through the food chain to consumer in the upper trophic levels.\textsuperscript{15}

The sorption of metal ions into organisms is in the following forms:

1. Low molecular weight proteins called metallothioneins.
2. Low molecular weight iron binding proteins called siderophores.
3. Metal sequestering agents, e.g., organic acids and amino acids; and
4. High molecular weight polymers.

One mechanism used by organisms to alleviate stresses imposed by exposure to heavy metal is synthesis of heavy metal binding peptides, whose function is to sequester and detoxify excess metal ions. These heavy metal binding peptides are known as phytochelatins. These have been observed from the orchids, the most advanced group of higher plants to the red, green and brown algae.\textsuperscript{14}

Generally, metals exert their toxicological activity through the inappropriate non-specific binding of the metal to physiologically important target molecules such as small polypeptides, glutathione (GSH), metal containing enzymes such as Zn \textsuperscript{-}-containing enzymes-alkaline phosphatase, malate dehydrogenase, carboxypeptidase and RNA polymerase.\textsuperscript{16}

Ochiai\textsuperscript{16} proposed that toxicity was due to one of the following

(1) blocking the essential biological functional groups of biomolecules;
(2) displacing essential metal ions in biomolecules;
(3) modifying the active conformation of biomolecules.
Based on the functional definition of detoxification, a simple model was described by Manson et al.\textsuperscript{13}.

Non-essential metals cannot form MeE type complexes and therefore cannot be beneficial.

Heavy metals have been reported to inhibit nodulation and nitrogenase in some legumes. But lower concentrations of heavy metals may be tolerated by resistant species. The metal tolerance may be due to induction of metal-binding peptides which may sequester metal ions.\textsuperscript{14}

Keeping all the above pressing problems in mind, we have studied whether the nitrogen fixing organisms of our interest are metal tolerant and whether they produce thiols in response to various concentrations of heavy metals so as to suggest good biofertilisers even if the habitats are polluted with heavy metals so that they primarily act as biofertilisers and protect crops from heavy metal toxicity. These aspects are discussed in the ensuing chapters.