Chapter 1.

Introduction
1. INTRODUCTION

1.1. Heavy metal pollution - a global concern

Heavy metals are metals with a density above 5 gm/cm$^3$. Because they can form complex compounds, some heavy metal ions are essential trace elements, but, essential or not, most heavy metals are toxic at higher concentrations. Some heavy-metal cations, e.g. Hg$^{2+}$, Cd$^{2+}$ and Ag$^+$, form strong toxic complexes, which makes them too dangerous for any physiological function. Even highly reputable trace elements like Zn$^{2+}$ or Ni$^{2+}$ and especially Cu$^{2+}$ are toxic at higher concentrations. Industrial activities and anthropogenic influences have been a serious concern for concentrating these toxic heavy metals in the environment to a hazardous extent for decades. The relatively reckless handling of heavy metals and their compounds can partly be explained by the fact that their effects were unknown till the mid of 20th century. However, with the advent of current analytical detection procedures, now it is possible to measure even at ppb level of heavy metals for certain environmental samples obtained from water, soil and air. This in turn helped toxicologists to pursue biological experiments to follow up the effects of individual substances down to the smallest concentrations. Their warnings, particularly with regard to the effects on health in case of chronic consumption and the accumulations to which this leads, have made the common man concerned. All this has taken place against the background of a steady increase in the processing of all types of heavy metals in industry and the household. Therefore, proper disposal, recycling and the regulation of the application of sewage to agricultural land, have assumed great importance.

1.2. Sources of heavy metals in environment

Heavy metal contamination in environment is being resulted mainly from natural weathering processes and anthropogenic activities. Ross (1994) classified the anthropogenic sources of metal contamination into five main groups, viz. (i) metalliferous mining and smelting (As, Cd, Pb and Hg), (ii) industry (As, Co, Cd, Cr, Cu, Zn, Ni and Hg), (iii) atmospheric deposition (Cr, Cd, As, Pb Cu, , Hg and U), (iv) agriculture (As, Pb, Cd, Cu, Se, U and Zn) and (v) waste disposal (As, Cr, Cd, Cu, Zn, Pb and Hg).
Major sources of heavy metal pollution are entry of toxic heavy metals into food chain from industrial and mining processes, metallotherapy (e.g. gold from gold drugs used in arthritis, platinum from anti-cancer Pt-complexes, iron from Fe-containing pills taken by anaemic patients, lithium from Li$_2$CO$_3$ used in treatment of mental diseases etc), drink (e.g. arsenic from As-contaminated ground water, Se-induced toxicity in some areas where Se-content is very high in soil) etc.

1.3. General mechanisms of heavy metal toxicity on biota

The growth and development of life process involves metals. The essential metals include Ca, Co, Mo, Cu, Fe, K, Mg, Mn, Na, Ni and Zn which serve as micronutrients and are used for redox-processes, to stabilize molecules through electrostatic interactions, as components of various enzymes and for regulation of osmotic pressure (Bruins et al., 2000). Among the 19 metals or metalloids As, Cd, Hg, Pb and Cr have been known to be extremely toxic at low concentration (Waalkes et al., 1992; Bruins et al., 2000), although they have no significant biological function so far reported. Toxicity of nonessential metals occurs through the displacement of essential metals from their native binding sites or through ligand interactions (Nies, 1999; Bruins et al., 2000). For example, Hg$^{2+}$, Cd$^{2+}$ and Ag$^{2+}$ tend to bind to SH groups, and thus inhibit the activity of some enzymes (Nies, 1999). In addition, at high levels, both essential and nonessential metals can damage cell membranes, alter enzyme specificity, disrupt cellular functions and damage the structure of nucleic acid (Bruins et al., 2000). Cadmium is extremely toxic even in low concentration and induces DNA breakage (Mitra and Bernstein, 1978). It is a divalent cation and possibly exerts toxicity by binding to the essential respiratory proteins (Vallee and Ulmer, 1972) along with oxidative damage (Stohs and Bagchi, 1995). Cadmium ion also causes cessation of respiration by binding to sulphhydryl groups in proteins (Foster, 1983). These potent toxicity causes reduced growth rate, long lag phase, lowered cell density and even may cause cell death of microbes at levels below 1 ppm of Cd (Mitra and Bernstein, 1978; Aiking et al., 1982; Rachlin et al., 1982; Les and Walker, 1984; Shapiro and Keasling, 1996).
1.4. Nickel pollution

Nickel (Ni) is the 24th most abundant element in the Earth’s crust, comprising about 3% of the composition of the earth. Nickel is the 28th element of the Periodic table. It is a silver-white metal found in several oxidation states, ranging from -1 to +4. However, the +2 oxidation state of Ni is the most common form of nickel in biosystems. Chemical and physical forces (e.g. erosion, leaching, and precipitation) constantly redistribute nickel between land, water, and air. Elementary nickel is water insoluble at 20 °C and pressure of 1 bar. However, nickel compounds may be water soluble. Nickel chloride is most water soluble; 553 gm/l at 20 °C, to 880 gm/l at 99.9 °C. Nickel carbonate has a water solubility of 90 mg/l, whereas other nickel compounds, such as nickel oxide, nickel sulphide and nickel tetra carbonyls are water insoluble. The main sources of nickel contaminations are anthropogenic. Nickel and nickel compounds have many industrial and commercial uses. Nickel is mostly used for the production of stainless steel and other nickel alloys with high corrosion and temperature resistance. Nickel metal and its alloys are used widely in the metallurgical, chemical and food processing industries, especially as catalysts and pigments. The nickel salts of greatest commercial importance are nickel chloride, sulphate, nitrate, carbonate, hydroxide, acetate and oxide. Nickel is one of many trace metals widely distributed in the environment, being released from both natural sources and anthropogenic activities, with input from both stationary and mobile sources. It is present in the air, water, soil and biological materials. Natural sources of atmospheric nickel levels include wind-blown dust, derived from the weathering of rocks and soils, volcanic emissions, forest fires and vegetation. Nickel finds its way into the ambient air as a result of the combustion of coal, diesel oil and fuel oil, the incineration of waste and sewage, and miscellaneous sources (Grandjean, 1984; Cempel, 2006). Environmental sources of lower levels of nickel include tobacco, dental or orthopaedic implants, stainless steel kitchen utensils and in expensive jewellery (Cempel, 2006). Tobacco smoking is another, not negligible, source of non-occupational exposures to nickel. It has been estimated that each cigarette contains nickel in a quantity of 1.1 to 3.1 μg and that is about 10-20% of the nickel inhaled is present in the gaseous phase. According to some authors, nickel in tobacco smoke may be present in the form of nickel carbonyl, a form which is extremely
hazardous to human health. Pipe tobacco, cigarettes and other types of tobacco products do not greatly differ one from another in the content of nickel (Bencko, 1983).

1.4.1. Ni pollution in soil. Most nickel on Earth is inaccessible because it is locked away in the planet's iron-nickel molten core, which is 10% nickel. Nickel is generally distributed uniformly throughout the soil profile but typically accumulates at the surface from deposition by industrial and agricultural activities. Organic matter has a strong ability to absorb the metal which is why coal and oil contain considerable amounts of nickel. The nickel content in soil can be as low as 0.2 ppm or as high as 450 ppm in some clay and loamy soils. The average is around 20 ppm (Bencko, 1983). Nickel may present a major problem in land near towns, in industrial areas, or even in agricultural land receiving wastes such as sewage sludge. Nickel can exist in soils in several forms: inorganic crystalline minerals or precipitates, complexed or adsorbed on organic cationic surfaces or on inorganic cation exchange surfaces, water soluble, free-ion or chelated metal complexes in soil solution. Nickel occurs combined with sulfur in millerite, with arsenic in the mineral niccolite, and with arsenic and sulphur in nickel glance. Most ores from which nickel is extracted are iron-nickel sulphides, such as pentlandite. The metal is mined in Russia, Australia, New Caledonia, Cuba, Canada and South Africa, and the annual production exceeds 500,000 tons.

1.4.2. Ni pollution in water. The total amount of nickel dissolved in the sea has been calculated to be around 8 billion tons. Seawater contains approximately 0.5-2 ppb of nickel, and rivers contain approximately 0.3 ppb. Phytoplankton contains 1-10 ppm nickel (dry mass), resulting in a $10^3$-$10^4$ bioconcentration factor compared to seawater. Benthic algae can be found both in freshwater and salt water, and may contain between 0.2 and 84 ppm nickel. Lobsters contain 0.14-60 ppm nickel, molluscs 0.1-850 ppm, and fishes between 0.1 and 11 ppm (all values based on a dry mass). Nickel occurs in water as $\text{Ni}^{2+} (\text{aq})$ and sometimes as $\text{NiCO}_3$. It may be either dissolved, or complexed with inorganic ligands. Nickel may also be bound to particles. Nickel may be found in slate, sandstone, clay minerals and basalt. The main nickel source is pentlandite. The element accumulates in sediments and is a part of various biological cycles. Nickel may end up in water from both point and non-point sources. Nickel is directly emitted from various industries through discharge on surface waters.
1.4.3. Ni pollution in air. Nickel concentrations in ambient air vary considerably and the highest values have been reported from highly industrialized areas. Typical average levels of airborne nickel are: $10^{-5}$–$3 \times 10^{-2} \mu g/m^3$ in remote areas; 0.003-0.03 μg/m$^3$ in urban areas having no metallurgical industry; 0.07-0.77 μg/m$^3$ in nickel processing areas (Bencko, 1983).

1.5. Utility of nickel in biological system

Nickel is an essential trace element for at least four biological processes: (i) oxidation and evolution of molecular hydrogen, (ii) hydrolysis of urea, (iii) carbon monoxide dehydrogenase-mediated acetate metabolism in methanogens and in homoacetogenic bacteria, and (iv) reduction of methyl coenzyme-M to methane in methanogenic archaea (Mergeay et al., 1985). In some microorganisms nickel is essential for nickel dependent processes. Nickel is required for transporting divalent cations like Fe$^{2+}$ into the cell. For example, nine Ni-dependent enzymes are now recognized, with seven being structurally characterized by crystallographic or nuclear magnetic resonance (NMR) approaches. In several cases, activation of these enzymes has been shown to require complex cellular processes utilizing a host of accessory proteins to specifically insert Ni (and other components) into the apoproteins (Hausinger, 1997; Watt et al., 1999; Mulrooney et al., 2003). Nickel-specific sensing and transport systems also have been characterized in numerous microorganisms (Eitinger and Mandrand-Berthelot, 2000).

1.6. Nickel toxicity and its molecular mechanism

Although Ni is vital for the function of many organisms, concentrations in some areas from both anthropogenic release and naturally varying levels may be toxic to living organisms. Inhalation exposure in occupational settings is a primary route for nickel-induced toxicity, and may cause toxic effects in the respiratory tract and immune system. High levels of Ni exert toxic effects both in bacteria (Babich et al., 1983; Nies, 1999) and higher organisms including human (Malkoc et al., 2005; Aksakal et al., 2008). According to WHO there are five forms of Ni which are toxic to human, of which NiCl$_2$, NiSO$_4$ and NiCO$_3$ are class I carcinogens when inhaled. It has been reported that
Ni is mutagenic and it causes genetic abnormalities due to DNA strand break, nucleotide excision, DNA-protein cross link etc. (Coogan et al., 1989; Costa, 1991). Ni also reported to have toxic effect on the development of foetus (Chashchin et al., 1994). Therefore, huge release of Ni salts into the environment from different industries and consequent accumulation in food chains is causing a global concern.

Depending on the soil type and pH, mobility of nickel varies in soil. At pH > 6.7, most nickel exists as insoluble hydroxides, whereas at pH < 6.5, the compounds are relatively soluble. The range of nickel concentrations in plants averages 0.05-5 mg/kg dry weight with concentrations above 50 mg/kg dry weight being toxic for most plants. Based on laboratory studies, nickel probably does not accumulate in fish and there is little evidence for the biomagnification of nickel in the food chain (Valko et al., 2005). Occupational exposure occurs via mining, alloy production, electroplating, refining and welding. Epidemiological studies revealed an increased risk of respiratory tract and nasal cancers in miners and workers in nickel refineries (Valko et al., 2005).

Nickel can enter the body via inhalation, ingestion and dermal absorption. The amount of nickel absorbed by the gastrointestinal tract depends on the type of nickel species in the food, the content and the absorptive capacity. Normally, only 1-2% of ingested nickel is absorbed. The daily intake of nickel has been estimated to be in the range of 35 – 300 μg per day (Valko et al., 2005). The chemical form of nickel determines the route by which nickel enters the cells. Soluble nickel, for example, nickel carbonyl, is fat soluble and can freely cross cell membranes, most probably by diffusion or through calcium channels (Valko et al., 2005). Based on the data obtained, two mechanisms for nickel-induced oxidative DNA damage have been proposed: i) all the nickel compounds used induced indirect damage through inflammation, and ii) Ni$_3$S$_2$ also showed direct oxidative DNA damage through H$_2$O$_2$ formation.

This double action may explain the relatively high carcinogenic risk of Ni$_3$S$_2$. This implies that a high content of nickel and its clearance from tissue is directly proportional to nickel carcinogenic activity. Calcium is maintained at a very high gradient (~104 μM) between the extracellular and intracellular compartments of cells and is recognized as an important intracellular second messenger. Several studies related toxic and carcinogenic effect of nickel with changes in calcium metabolism. Soluble nickel
compounds are known to enter the cell through the calcium ionophore channel ionomycin, which increases the uptake of nickel by a factor of 4-5 (Valko et al., 2005). This decrease in influx of Ca\(^{2+}\) to intracellular space is compensated by an increase of free Ca\(^{2+}\) from intracellular stores which in turn have been shown to alter gene expression pattern associated with cell growth, differentiation and apoptosis (Valko et al., 2005).

1.7. Effects of nickel on plant

Although Ni is an essential metal and plays important roles in plant metabolism (Eskew et al., 1983), under Ni stress conditions, many common Ni-detoxification responses appear in plants. These responses include the formation of Ni\(^{2+}\)-organic acid and Ni\(^{2+}\)-NA complexes, the overproduction of NA and its synthase (Burd et al., 2000), and high levels of free histidine. Other responses include the induction of MTs and thiol glutathione, and high concentrations of glutathione, Cys and O-acetyl-L-serine (OAS) (Eskew et al., 1984). In addition, some enzyme activities may be enhanced, such as serine acetyltransferase (SAT) and glutathione Reductase (Ali et al., 2008). However, under excess Ni conditions, toxicity symptoms in plants will develop, such as metabolic changes, reduces growth of plant roots and shoots, inhibits root branching and germination. Also it disturbs plant mineral nutrition, water regime, photosynthesis as well as morphogenesis.

1.8. Metal-microorganism interactions – a possible prospect of bioremediation

Microorganisms cannot destroy metals but they can alter their chemical nature via a surprising array of mechanisms. Most microorganisms have a negative charge owing to the presence of negatively charged groups of atoms on the cell membrane and cell wall. The charged groups or ligands are responsible for the adsorption of positively charged metal ions in solution. The adsorption is typically rapid, reversible and independent of temperature and metabolic energy. The deposition of insoluble metals has been observed at the surface of some microorganisms (Brierley, 1982). Heavy metals are increasingly found in microbial habitats due to natural and industrial processes, microbes have evolved several mechanisms to tolerate the presence of heavy metals or to use them as terminal electron acceptors in anaerobic respiration in addition, owing to
large SA/V and high metabolic activities microbes are important vector of biochemical cycling of those metal ions, because the influx and subsequent efflux of heavy metal ions by microbes usually includes redox reaction involving the metal. This is important implication of microbial heavy metal tolerance because the oxidation state of a heavy metal relates to the solubility and toxicity of the metal itself. Since the oxidation state of a metal ion may determine its solubility. Many scientists have been trying to use microbes that are able to oxidized or reduce heavy metals in order to remediate metal contaminated sites. Simultaneously, microbes that immobilize heavy metals by binding to their membrane passively or accumulate heavy metals within the cell by complexation with metal binding protein are of immense importance in the perspective of bioremediation research. The microbiological processes or mechanisms for the removal of metals from solution have been observed in the laboratory and in natural environments where conditions are suitable for specific types of biological activity. The mechanism can be divided into three categories: the adsorption of metal ions onto the surface of microorganism; the intracellular uptake of metals and the chemical transformation of metals by biological agents (Brierley, 1982). Therefore, the urgency lies in studying metal resistance mechanisms in microorganism, which might shed light towards the development of effective bioremediation candidate.

1.9. Measures

Realizing the potential hazards of Ni contamination in soil and consequently in food and its well-known phytotoxicity, it was necessitated to invent some strategy for remediation of Ni from environment. The need of ecofriendly fertilizers, which will not build further Ni load on soil, was also felt simultaneously. Alleviation of metal contamination in soil can be done by any of the physical, chemical and biological means. Again these can be grouped into two major heads (Baker and Walker, 1990).

1.9.1. Ex situ method. In this process contaminated soil is removed for treatment on or off site, and returning the treated soil to the resorted site. The conventional ex-situ methods applied for alleviating the polluted soils relies on excavation, detoxification and/or destruction of contaminants physically or chemically; as a result the contaminants undergo stabilization, solidification, immobilization, incineration or
destruction. The main setbacks of this process are substantial. Firstly, the process is expensive and the treatment costs around $10 to 1000 per cubic meter of soil. Secondly, which is even worse is the total destruction of all biological activities, including useful microbes such as nitrogen fixing bacteria, mycorrhiza, fungi, as well as fauna in the process of decontamination (Burns et al., 1996).

1.9.2. In situ method. This method includes exploitation of microbes and plants in the contaminated site for reclamation of heavy metal and organic pollutants. This utilization of living organism to combat environmental pollution is broadly termed as bioremediation. Again, bioremediation involving exploitation of plants has been commonly designated as phytoremediation. This refers to the use of green plants and their associated micro biota for the in situ treatment of contaminated soil and ground water (Sadowsky, 1999). The idea of using metal accumulating plants to remove heavy metals and other compounds was first introduced in 1983, but the concept has actually been implemented for the past 300 years (Henry, 2000). This technology can be applied to both organic and inorganic pollutants present in soil (solid substrate), water (liquid substrate) or the air (Raskin et al., 1994; Salt et al., 1998). The other method includes bioremediation by microorganism such as algae, fungi and bacteria. Microbes remove heavy metals from the soil or water by mainly two methods. One method relies on the physical adherence of the metals to the microbial cell mass, a phenomenon commonly referred to as biosorption (Gadd, 1988; McHale and McHale, 1994) and thus providing a cost-effective solution for industrial wastewater management (Volesky and Holan, 1995). The other method is the bioaccumulation of heavy metals inside the cells, which is an active process, called bioaccumulation. The basic difference between biosorption and bioaccumulation is that only living organism could accumulate metals whereas both living and non-living cells could exhibit biosorption. The microbes that are used in the bioremediation purpose may be of indigenous origin (Burd et al., 1998) or may be bioengineered bacteria expressing proteins with metal binding motifs (Pazirandeh et al., 1998; Ueki et al., 2003). Exploitation of indigenous flora is often safe because it probably does not hamper the normal microflora of the environment and henceforth does not violate the law of release of genetically modified organism in the environment. Heavy metal resistant microbes prevent biomagnification of several toxic metals in
plants by mainly two ways. The first group of microorganism accumulates or physically adsorbs heavy metal in their body, thereby minimizing the bioavailability of heavy metals and subsequently restricts its accumulation in the plant parts (Tripathi et al., 2005). The other group of microbes alleviates heavy metal pollution indirectly through phytoremediation. Principally, the latter group of microbes increases the solubility of the heavy metals in the soil and hence, facilitating its uptake by plants (Sheng and Xia, 2006). Therefore, in the present context of increasing heavy metal pollution globally, microorganism might be the magical solution to this unavoidable problem. Many laboratories and companies all over the world are now engaged in finding suitable microorganism or microbial biomass, which could be effective in decontaminating soil and water.

1.10. Microbes as potential biofertilisers

Microbes also opened another avenue towards the development of cost effective and ecofriendly biofertilizers. Many microbes promote plant growth and productivity by various ways. These microbes exhibit their plant growth promoting property either being associated with the rhizosphere of plants or as free-living microbes. These beneficial microbes of the former type have been collectively termed as plant growth promoting rhizobacteria or simply PGPR. Again these microorganisms not only differ in their classes, they belong but also the way they execute. There are some microorganisms, which could produce plant growth promoting substances such as phytohormones like auxin (Mordukhova et al., 1991, Lambrecht et al., 2000), gibberelaline (Joo et al., 2005), Cytokonin (de Salamone et al., 2001) and ethylene (Arshad and Frankenberger, 1991). Microbes often found to convert insoluble mineral salts into soluble forms and therefore, its easy absorption by plants. For example, phosphate solubilising bacteria promote plant growth by making phosphate available to the plants (Pandey et al., 2006) and hence could act as an alternative to phosphate fertilizers. Siderophores are small iron chelating compounds produced by microorganisms are of immense importance. Siderophores chelate iron and prevents the growth of many other pathogens and hence behave as antagonist to many phytopathogenic fungi (Seuk et al., 1988; Yang et al., 1994; Xiao and Kisaalita, 1998).
Siderophores play active role in plant growth promotion (Kloepper et al., 1980) since bacterial iron-sideophore complex from soil is being used up by certain plants to survive in iron scarce condition (Reid et al., 1986; Crowley et al., 1988; Bar-Ness et al., 1991; Wang et al., 1993). Exploitation of siderophore overproducing mutants has been a major strategy to overcome deleterious effects of heavy metal pollution in soil (Burd et al., 2000). In the early 1970s several researchers identified microbial populations in the rhizosphere as constituting the first barrier to pathogen infection. Nowadays, it is well known that some soils are naturally suppressive to some soil-borne plant pathogens including *Fusarium*, *Gaumannomyces*, *Rhizoctonia*, *Pythium*, and *Phytophthora*. Although this suppression relates to both physicochemical and microbiological features of the soil, in most systems the biological elements are the primary factors in disease suppression and the topic of ‘biological control of plant pathogens’ gained feasibility in the context of sustainable development. Inhibition of the growth of the plant pathogens by bacteria mainly result from the production of several secondary metabolites such as antibiotics, siderophores, HCN, several enzymes such as chitinase, cellulase etc. (Whipps, 2001). Rhizobacteria from the genus *Pseudomonas* provide an excellent example of a combination of multiple mechanisms for effective biocontrol including direct antagonism and induction of plant’s disease resistance. Since, *Pseudomonas* spp. produces several metabolites with antimicrobial activity towards other bacteria and fungi (Haas and Keel, 2003), have rendered the strain a very important biocontrol agent.

### 1.11. Aim and objectives

In the present perspective of heavy metal pollution round the world including India, there is a growing need for a suitable microorganism, which could act as an ecofriendly bioremediating agent. Scientists are now engaged in characterizing microbes for the development of successful bioremediating candidate along with potent biofertilizer traits. Therefore, opportunity and temptation were taken to explore the microbial diversity in Ni-contaminated site for identifying a potential bacterial strain, which could be applied in the environment in the hope of a healthy future.
In spite of various limitations in and out of our laboratory, this is a valiant attempt towards answering to the global problem of Ni contamination in the environment. The objectives of the work were as follows:

i) Isolation, identification and characterization of a bacterial strain having high Ni tolerance property.

ii) Characterization of the mechanism of Ni tolerance of the isolated bacterial strain.

iii) Evaluation and optimization of its Ni accumulation under culture condition.

iv) Novel possibility for use this strain as a candidate of Ni bioremediation.

v) Analyze the plant growth promoting potentiality of this strain.