Chapter 6.

Summary
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The anthropogenic and geologic inputs of different heavy metals/metalloids have increased rapidly since the industrial revolution leading to environmental pollution. Bioremediation of heavy metals has emerged to be the major effective alternative approach for effective cleaning up of heavy metals from the contaminated sites. Looking into the broad prospect of bioremediation of heavy metal research for better tomorrow, isolation of potent microorganisms as a bioremediating agent for effective bioremediation purpose is being targeted. The impact of environmental pollution through raw waste disposal from various industrial effluents on living system is globally a major concern. Heavy metal pollution in water and soil directly hampers plant growth and reduce productivity. The other major concern is the entry of heavy metals into the food chain through plants and thereby exposing human to a range of heavy metal toxicity causing serious health hazards. Some heavy metals play important role acting as trace element in the life process but this when present at high concentrations, toxic to all branches of life. Upon entry into the cell these metal ions form complexes with the essential enzymes or molecules thereby preventing their normal function. Because 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. Therefore, the urgency lies in studying metal resistance mechanisms in microorganisms, which might shed light towards the development of suitable bioremediating candidate.

According to US EPA Nickel is one of the 17 toxic metals/metalloids posing hazard in the environment. Among the different heavy metal pollutions in soil and water Ni is one of the the priority pollutant as documented by US EPA and Ni pollution needs special attention because Ni has been identified as a significant pollutant due to its high toxicity and solubility in water. Contamination of soil and ground water due to the use of Ni in various anthropogenic activities has become a serious global threat. Nickel is released in to the environment by several routes, mainly through smelting of ores, battery and jewelry making industries etc. Although Ni in environmental pollution has emerged as significant global problem, it is most serious in the developing countries of
Asia including India. In India, battery industries play a prominent role. The Gangetic basin which includes states like Uttar Pradesh and West Bengal, the Godabari basin in Andhra Pradesh, the Kaveri Basin in Tamilnadu and other states like Maharashtra and Gujarat contribute major proportion of the total nickel contamination of soil in India from various industries operating at these locations. Among the different heavy metal pollutions in soil Ni ranked fifth.

This transition metal is the 24th most abundant metal found in the earth’s crust. The solubility and adsorption by soil and sediments depend on the form of nickel species and physico-chemical features of the contaminated site. 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 the 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.

The vision of this work was to isolate a Ni resistant and Niremoving bacterial strain which could be exploited as effective bioremediator and plant growth promoting agent in the rhizosphere of economically important plants viz. mustard and pumpkin, grown in waste-fed soil. The study was also aimed to evaluate the efficacy of the strain to reduce Ni accumulation in those plants.

Bacteria isolated from industrial waste fed soil which could survive at 12 mM of Niconcentration in the nutrient agar plates, designated as KUNi1, was used in this study. Biochemical tests identified the isolate to be a strain of \textit{Bacillus thuringiensis}. PCR amplification of the 16S rDNA sequence with bacterial specific primer set f27 and r1479 produced an amplicon of 1479-bp and subsequent analysis of the amplicon sequence using BLAST function at NCBI database showed 99% sequence homology to \textit{Bacillus thuringiensis}. The phylogenetic trees were constructed with KUNi1 and other closely related Bacilli having 99% sequence homology. Repeated subculturing of KUNi1 in Ni free medium and subsequently on Ni amended medium revealed that the resistance mechanism in this strain is a stable phenomenon and might be
chromosomally regulated. The strain was observed to be highly resistant to Ni and it could overcome Ni toxicity by intracellular accumulation. This property has been key feature for many microorganisms which is to be tested for bioremediation purpose. The strain was found to remarkably resistant to other heavy metals as well. The order of toxicity of the metals to the bacterium on both solidified and liquid medium was found to be Ni (10 mM) > Zn = Cu = Co (1 mM) > Cd (0.5 mM). Minimum inhibitory concentrations (MIC) of the test metals for the bacterium are less in minimal broth. The risk of spreading of antibiotic resistance through KUNi1 after implementation for bioremediation purpose was found to be less since KUNi1 exhibited sensitivity against many of the antibiotics tested except chloramphenicol, rifampicin, ampicilin and kanamycin and low resistance to colistin. The strain showed optimum growth at pH 7 and 37 ºC was found to be the optimum temperature for maximum growth. The growth of the strain in presence of different concentration of NaCl supplementation showed differential responses. When the Ni-fed medium was supplemented with 100 mM of NaCl salt, it showed shortening of the lag phase as compared to the growth of the strain in Ni-fed medium alone. However, with still higher doses of NaCl supplementation, the lag phase was observed to be lengthened in comparison with the set where the strain growing solely under Ni stress. The bacterial strain was found to accumulate Ni intracellularly and the amount of Ni accumulation was found to vary with the pH changes and incubation temperature. It showed maximum Ni removal in neutral pH and at 37 ºC of incubation temperature. Total thiol content in this strain when grown under Ni stress, did not show any marked difference between the control and Ni treated set. Therefore, it was concluded that thiol did not take part in Ni sequestration by KUNi1. Subcellular fractionation of KUNi1 cells coupled to quantitative estimation of Ni in each fraction showed that a major fraction (~78%) of total accumulated Ni retained in the cytoplasm portion followed by membrane component (~21.5%). To localize the intracellular deposition of accumulated Ni, the Ni treated and untreated cells of KUNi1 were observed under transmission electron microscope. The transmission electron micrographs showed distinct electron dense area within the cytoplasm which was thought to be the sites for Ni deposition. Very few of such electron dense bodies were seen in untreated control cells. The electron dense bodies seen under TEM have been
further analyzed taking advantage of EDXS equipped with SEM. The analysis of the electron dense area showed distinct peaks for Ni in the treated set and no such peaks were observed in the control set. The powdered X-ray diffraction data of the Ni loaded cells revealed the chemical nature of the accumulated cadmium within the cell. The broadened nature of the peaks in the diffractrogram indicates the dominant existence of amorphous phase(s) in the sample. The D value obtained from the diffractogram, of both the treated and untreated sample strongly suggested presence of nickel phosphides in the cytoplasm of the treated cells. Since, the strain KUNi1 accumulated Ni within the cells by converting it to phosphides, therefore, it was obvious that KUNi1 should be tested for its efficacy to remove Ni from growth condition and finally from contaminated soil. To an extent it fulfilled the objective and was found to be handful for implementation of soil reclamation. Under experimental condition, it removed more than 84% of 2.5 mM soluble nickel from growth medium after 48 hrs.

The other most interesting feature of this strain was observed to be multimetal bioaccumulation capacity of KUNi1 as it might be exploited even in the agricultural field contaminated with various toxic metals. This possibility is encouraging to start further investigation. Another important finding of this study is the elucidation of the mechanistic influence of magnesium in nickel influx and energy dependent nature of nickel uptake in this strain, which opens up another aspect of research field of mechanism of nickel bioremediation in this strain and other organisms as well. The strain was also found to antagonize the growth of many phytopathogens. But the exact mechanism of its antifungal activity is yet to be concluded.

The strain survived in the sterilized soil and rhizosphere of chili and pumpkin plants. The population size remained acceptably steady in the sterilized soil around 10^5-10^7 CFU/gm fresh soil under experimental condition and increased considerably in case of rhizospheric environment. The strain exhibited plant growth promoting potentiality both in chili and pumpkin plants tested through successful colonization in the rhizosphere. It increased shoot length, root length, plant biomass and chlorophyll content. Seed inoculation by KUNi1 enhanced all the growth parameters tested in the experiment except chlorophyll content over the control plants measuring Iron content in
the leaves of the test plants revealed the positive influence of KUNi1 in iron mobilization from soil and consequently improved iron status in the leaves. KUNi1 colonization in the rhizospheric environment of both the plants reduced Ni load significantly on the different plant parts.

It might be concluded that the strain might be of potential candidate for bioremediation in nickel contaminated soil provided with little growth supportive condition. This study demonstrated that the bacterial strain when applied to the rhizosphere soil of plants, it served two major purposes. Firstly, bioinoculation improved the growth parameters of plants under Ni stress condition at least in pot experiment and secondly, the more important, it reduced the Ni uptake in plants. Therefore, the strain could possibly be applied in the Ni contaminated agricultural land as a good bioremediation agent, although the extent of stimulation of plant growth and reduction in Ni uptake under actual metal-polluted field conditions warrants further study. The exhibition of in vitro antibiosis of this strain against phytopathogenic fungi has opened other possible avenue to be used as potential biocontrol agent as crop protectant besides its plant growth promoting features.