2. REVIEW OF LITERATURE

2.1. TECHNIQUES TO STUDY THE MICROBIAL DIVERSITY

2.1.1. Culture dependent techniques

Non-culturale microorganisms represent one of the most important problems currently associated with microbial ecology (Zengler et al., 2002; Leadbetter, 2003). Most of the microorganisms occurring in a nature, in principle, should be culturable, as between 50% - 90% of the bacterial cells in natural samples appear to be metabolically active (Fry, 1990; Bartscht et al., 1999). The intensive application of molecular techniques to describe microbial diversity in natural environments is yielding a large amount of data as indicated by the large number of sequences available in the public databases (Baker et al., 2003a; Baker and Cowan, 2004; Ludwig et al., 2004). However, comparisons between classical culture dependent and molecular methods have revealed that only a small fraction, about 1% of the prokaryotic diversity, appears to be amenable to culture (Rappe and Giovannoni, 2003) and less than 0.1% of archaenal species have been cultivated from soil (Bintrim et al., 1997).

Cultivation and subsequent isolation of microorganisms in pure culture is required to gain a comprehensive understanding of microbial physiology, their interaction with one another in their environment and to provide access to genes encoding metabolic pathways which may be dispersed throughout the genome (Schleifer, 2004; Green and Keller, 2006). Traditional culturing approaches are based on complex nutrient rich media that supply excessive amounts of nutrients to the system as the specific
requirements of many uncultured microorganisms are unknown. However, these approaches allow the enrichment of faster growing microorganisms that are capable of colony or biofilm formation (Leadbetter, 2003; Ferrari et al., 2004). These organisms are not necessarily the most abundant species in the environmental samples, but their fast growth allows them to compete other microorganisms present in the culture (Saul et al., 1999).

Many reasons such as lack of necessary symbionts or nutrients, excess of inhibitory compounds, incorrect combinations of temperature, pressure or atmospheric gas composition, accumulation of toxic waste products from their own metabolism for cultivating the microorganisms leading to slow growth rate or rapid dispersion from colonies (Caruge et al., 2004). In addition, the microbial compositions of the enrichment cultures are also influenced by the concentration of inoculum as the cultures resulting from inoculation at low concentration seem to be dominated by species that have superior growth capabilities in that medium, while high concentrations of inoculum result in cultures of those species whose growth was inhibited when using a lower concentration of inoculum (Jackson et al., 1998). No single method or medium is suitable for the cultivation of the majority of microorganisms from environmental samples (Green and Keller, 2006).

Laboratory techniques for successful cultivation and isolation of environmental microorganisms are required to mimic and reproduce the specific nutritional and physical conditions of their natural habitat (Kaeberlein et al., 2002). Novel techniques and culture media have been developed to address these issues (Frohlich and Konig, 2000; Bruns et al., 2003). Cultivation and isolation of new microorganisms has been achieved
by the use of media with low concentrations of nutrients containing polymeric growth substrates and long incubation times (Stevenson et al., 2004; Davis et al., 2005). For example, diluted nutrient broth has been used successfully to isolate novel soil bacteria microorganisms within the divisions Actinobacteria, Acidobacteria, Proteobacteria and Verrucomicrobia (Janssen et al., 1997; Janssen et al., 2002).

Many environments are very complex with respect to their chemical composition and physical parameters. These environments are difficult to reproduce under laboratory conditions. However, novel techniques have been developed to mimic the natural habitat and supply the media with the essential trace elements, such as the use of sterilized sample material (Alef, 1995; Anitori and Bergquist, 2006) and in situ chambers and membrane systems which allow the direct uptake of nutrients from the environment and the exchange of metabolites (Kaeberlein et al., 2002; Moissl et al., 2003; Svenning et al., 2003; Ferrari et al., 2005). For example, ubiquitous microorganisms, such as the SAR11 clade found in nearly every pelagic marine bacteria plankton community studied by culture independent techniques, was not cultured successfully and isolated until low nutrient media based on sterilized natural sea water was used (Rappe et al., 2002). Despite these developments, the majority of microorganisms remain to be cultured and isolated.

2.1.2. Culture independent techniques

Soil is probably the most challenging of all natural environments for microbiologists, with respect to the microbial community size and the diversity of species present. One gram of forest soil contains an estimated 4
x $10^7$ prokaryotic cells (Richter and Markewitz, 1995), whereas one gram of cultivated soils and grasslands contains an estimated $2 \times 10^9$ prokaryotic cells (Paul and Clarke, 1989). Based on the reassociation kinetics of DNA isolated from various soil samples, the number of distinct prokaryotic genomes has been estimated to range from 2,000 to 18,000 genomes per gram of soil (Torsvik et al., 2002). The extreme spatial heterogeneity, multiphase nature (including gases, water and solid material) and the complex chemical and biological properties of soil environments are thought to contribute to the microbial diversity present in soil samples.

Biodiversity describes the number of prokaryotic species and their relative abundance in a community (Torsvik et al., 2002). For a long period of time, the identification of microbial species was limited to pure cultures or defined co-cultures (Amann et al., 1995; Pace, 1997). The development of molecular biological methods has revolutionized the field of environmental microbiology by allowing the analysis of microbial diversity without the need to isolate individual species (Olsen et al., 1986; Hugenholtz et al., 1998). Techniques such as the application of universal primers for direct PCR amplification of diverse 16S rRNA genes from total community DNA combined with cloning and sequencing technologies have generated a vast quantity of data that has redefined the microbial diversity (Pace, 1997; Gans et al., 2005). These molecular techniques mainly focus on the small-subunit (16S) ribosomal RNA (rRNA) (Woese, 1987). The 16S rRNA gene has several characteristics that explain why it is so widely used to study bacterial diversity: ubiquitous distribution among prokaryotes, relatively slow evolution rate, and the coexistence of highly variable and conserved regions. The variable regions enable a comparison between very
divergent bacteria, while the highly conserved domains serve as templates for designing specific PCR amplification primers or specific nucleotide probes. Thereby, the diversity of a bacterial community in a natural environment can be investigated without any culture, solely based on molecular phylogeny (Giovannoni et al., 1990; Santos and Ochman, 2004).

Potentially, most of the sequences present in the environment can be detected by PCR. Consequently, there is a tremendous difference in the estimation of bacteria diversity based on culture-independent and culture-dependent approaches since cultivation has inherent selection towards certain bacteria. Environmental sequencing projects targeting 16S rDNA have revealed a large number of new phylotypes previously undetectable by culture dependent techniques (DeLong and Pace, 2001; Tringe et al., 2005). Culture independent techniques normally are based on PCR amplification of DNA extracted from environmental samples. These techniques include the ribosomal intergenic spacer analysis (RISA), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single strand conformation polymorphism (SSCP), random amplified polymorphic DNA (RAPD) and amplified ribosomal DNA restriction analysis (ARDRA) (Kirk et al., 2004; Zhong and Cai, 2004; Fierer and Jackson, 2006). Although, these techniques have allowed the study of microbial diversity from many habitats, these procedures have several limitations, becoming inefficient for the detection of certain populations of microorganisms.

The extraction of community genomic DNA from environmental samples represents the initial step for most culture-independent techniques.
The extraction efficiency can be influenced by various factors and strongly depends on the characteristic of the samples (Krsek and Wellington, 1999). For example, the DNA extraction from acidic sulphur rich environments such as White Island has many difficulties as the high content of heavy metals and minerals that strongly bind to the nucleic acids and the acidity of the sample not only increases the binding of the DNA to the clay minerals but also decreases the amount of extracted DNA, as nucleic acids are easily degraded by depurination under acidic conditions (Roh et al., 2006; Henneberger, 2008).

Sequence based analyses of microbial communities also can be influenced by several factors such as contaminants and artefacts which may occur during PCR amplification having significant impact on the resulting data and leading to misinterpretation of the results obtained (Rossello Mora and Amann, 2001; Acinas et al., 2005; Osborne et al., 2005). Different approaches have been developed to improve detection and resolution of amplified DNA fragments. These include length heterogeneity PCR (LH-PCR) (Ritchie et al., 2000) and terminal restriction fragment length polymorphism (T-RFLP) (Marsh, 1999). However, variables that affect the efficiency of culture independent techniques for the study of microbial diversity of unknown habitats, such as genome size or the number of the rRNA operons present in the different microorganisms, cannot be estimated and the accuracy of the resulting data cannot be confirmed (Farrelly et al., 1995). Fluorescent in situ hybridisation (FISH), as a culture independent technique, allows simultaneous visualization, identification, enumeration and localization of individual microbial cells in their natural habitat. FISH also provides insights into community structure and diversity, spatial
distribution and abundance of specific types of microorganisms (Amann et al., 1990; Amann et al., 1995). FISH has been used to visualize uncultured microorganisms from a wide range of environments (Eilers et al., 2000; Gonzalez Toril et al., 2003) as well as to study microbial communities and biofilms (Daims et al., 2001; Ferrari et al., 2006). For example, a microbial community comprising novel archaeal and bacterial species living in the cold sulphurous marsh water of Sippemauer Moor (Germany) were discovered to form strings of pearls-like morphologies using FISH (Rudolph et al., 2001).

Initially, Woese (1987) primarily classified the domain Bacteria into 12 related phyla, which were tripled in the following decade up to 36 divisions (Hugenholtz et al., 1998; Santos and Ochman, 2004), based on 8,000 bacterial 16S rRNA gene sequences at that time available as obtained by cloning environmental DNA directly, or after amplification by PCR. A high proportion of the bacterial divisions was predominantly represented by uncultured organisms. Even 13 divisions were entirely characterized by environmental sequences and hence named as ‘candidate divisions’. In the following years, the number of bacterial divisions increased to 52 (Rappe and Giovannoni, 2003; Handelsman, 2004), from which 25 divisions were still represented by only uncultured organisms. At present, almost 80 phyla including 54 ‘candidate-divisions’ are recognized (Kamagata and Tamaki, 2005). Due to the progress in the molecular techniques, the number of phyla doubled in the last few years (from 36 to 80), whereas the number of phyla with cultivated representatives failed to increase significantly (from 23 to 26).
2.1.2.1. Metagenomics and its applications

It is widely accepted that upto 99% of the microbes in the environment can’t be readily cultivated (Rappe and Giovannoni, 2003; Kamagata and Tamaki, 2005; Sekiguchi, 2006). Studies have revealed that only 0.001-0.1% of the total microbes in seawater, 0.25% in freshwater, 0.25% in sediments and only 0.3% of soil microorganisms could be cultivable in vitro (Amann et al., 1995; Singh et al., 2008). To overcome the difficulties and limitations associated with cultivation techniques, different DNA based molecular methods have been developed for characterizing microbial species and assemblages, and these have significantly influenced our understanding of microbial diversity and ecology. A recently developed metagenomic approach clones the total microbial genome (the metagenome), which is directly isolated from natural environments, in culturable bacteria such as Escherichia coli (Handelsman et al., 1998; Rondon et al., 1999; Beja et al., 2000; Rondon et al., 2000) to discover novel microbial resources (Handelsman, 2004).

The metagenomic approach originated from the molecular analysis of microbial communities, which revealed that the majority of microorganisms in nature were not cultivable by standard culturing techniques (Boreman et al., 1996; Pace, 1997; Bintrim et al., 1997; Hugenholtz et al., 1998). The discipline of metagenomics, defined as the culture independent genomic analysis of all the microorganisms in a particular environmental niche (Handelsman et al., 1998), evolved as an effort to discover more about the microbial diversity of natural environments such as soil, marine water and the gastrointestinal tracts of vertebrates and invertebrates (Lopez Garcia and
Moreira, 2008). Metagenomics is a new and increasingly sophisticated field which in its simplest terms is concerned with the direct isolation of DNA from a defined habitat, followed by cloning (in a surrogate host such as *Escherichia coli*) of the complete genomes of the entire microbial population in that habitat (Langer *et al.*, 2006). The resulting DNA library is then analyzed for functions and sequences of interest.

Metagenomics can be divided into sequence based and function driven analysis of uncultured microorganisms (Gabor *et al.*, 2007). Functional metagenomics involves screening metagenomic libraries for a particular phenotype, e.g. salt tolerance, antibiotic production or enzyme activity, and then identifying the phylogenetic origin of the cloned DNA (Dinsdale *et al.*, 2008). Sequence based approaches, on the other hand, involve screening clones for the highly conserved 16S rRNA genes for identification purposes and then sequencing the entire clone to identify other genes of interest or large scale sequencing of the complete metagenome to search for phylogenetic anchors in the reconstructed genomes (Riesenfeld *et al.*, 2004; Hoff *et al.*, 2008).

Tyson *et al.* (2004) chosen a much simpler community in acid mine drainage (AMD) in the Richmond mine at Iron Mountain, California, one of the most extreme environments on earth. In this environment, the microbiota exists as a pink biofilm that forms on the surface of the mine water. The biofilm has a pH of 0.83, a temperature of 43°C and contains high concentrations of iron, zinc and copper. An extended random shot-gun sequencing approach revealed 384 16S rRNA genes and the 5’ and 3’ ends of these genes were sequenced (Baker *et al.*, 2003b). The AMD microbiota
was found to contain three bacterial and three archaeal species (Schoss and Handelsman, 2005). The three dominant bacterial genera included *Leptospirillum, Sulfobacillus, Acidomicrobium* and the dominant archaeal species was *Ferrophilum acidomicrobium* (Handelsman, 2004). The simplicity of the community structure allowed Tyson et al. (2004) to sequence almost the entire microflora with a high degree of coverage. It was noted that the G + C content of the genomes of the dominant taxonomic groups differed to a large extent and thus provided a means to source each of the clones (Bond et al., 2000). Metagenomic analysis of the AMD community resulted in the reconstruction of nearly complete genomes of *Leptospirillum* group II and *Ferroplasma* type II. Perhaps not surprisingly, all of the genomes in the AMD were found to be plentiful in genes that function in the removal of elements that would otherwise be toxic to the cell (Handelsman, 2004).

The marine environment has recently been pursued as a target of metagenomic studies (Li and Qin, 2005; Pedros Alio, 2006). It is the largest contiguous ecosystem on earth, occupying 71% of the earth’s surface with an average depth of 4 km (Karl, 2007). It is no surprise then that the earth’s oceans represent one of the most significant yet least understood microbial driven natural environments on the planet (Martin Cuadrado et al., 2007). Two large scale metagenomic analyses of deep sea communities have been carried out to date: the Pacific gyre water column at the A Long-Term Oligotrophic Habitat Assessment station (ALOHA; 22°45’N, 158°00’W – located 100 km north of Oahu, Hawaii), and more recently a single depth of 3000 m was sampled in the Ionian Sea located south east of Sicily in the
deep Mediterranean (Martin Cuadrado et al., 2008). A fosmid library was constructed from the 3000 m deep Mediterranean plankton and analyzed by phylogenetic analysis of 16S rRNA genes and fosmid end sequencing. Sequence analysis revealed a high similarity with genomes from Rhizobiales within the Alphaproteobacteria, Cenarchaeum symbiosum, Planctomycetes, Acidobacteria, Chloroflexi and Gammaproteobacteria (Martin Cuadrado et al., 2007).

2.2. MICROBIAL DIVERSITY IN COAL MINE ENVIRONMENT

The microbial world is immense and ubiquitous in both natural and many artificial environments (DeLong, 2002). There are more microorganisms per ton of soil ($10^{16}$) than stars in our galaxy ($10^{11}$) (Curtis and Sloan, 2005). Microorganisms are responsible for maintenance of the biosphere by playing crucial roles in many geochemical processes (Madsen, 2005). Microbial communities consisting of bacteria, actinomycetes, fungi, yeast and algae occupy important niches in mining ecosystems. These organisms play key roles in the earth’s biogeochemical cycle (Dave and Natarajan, 1987; Dave et al., 2002). The knowledge of structure and functions of this community is very limited.

The most well documented type of water pollution associated with mining is that which results from the accelerated oxidative dissolution of exposed minerals, principally sulfides, giving rise to acidic, metal enriched waters generally referred to as “acid mine drainage” (AMD). Due to its acidic nature, as well as its elevated concentrations of dissolved metals, AMD is often considered to be devoid of life. While this is generally true for higher life forms, it has been known for nearly half of a century that
microorganisms inhabit AMD (Colmer and Hinkle, 1947). The first microbe isolated by Colmer et al. (1950) from AMD was an iron-oxidizing microbe called *Thiobacillus ferrooxidans* (now known as *Acidithiobacillus ferrooxidans*). It is now recognized that a considerable diversity of microbial life may be found in acidic, metal rich effluents such as mine spoils, tailings and AMD. Many of these are obligate acidophilic microorganisms and grow poorly. Microbial community structure and interactions in these environments are as complex and diverse as those found in more ‘normal’ environments (Johnson, 1998). The last two decades have been marked by growing interest in deep terrestrial biosphere studies. During this period, the description of microbial communities originating from varied deep sub-terrestrial settings showed that the subsurface microbial communities could represent the greatest reservoir of living organisms on our planet (Whitman et al., 1998) and could possibly be analogous to extraterrestrial forms of life (Fredrickson and Balkwill, 2006). Original and varied microbial populations were characterized from different types of geological contexts, with most of the studies using culture independent methods based on 16S rRNA gene analysis. On the other hand, culture techniques for the enumeration, isolation and characterization of cultivable bacteria were rarely used for the characterization of deep terrestrial biodiversity. Various acidophilic autotrophs and heterotrophs, prokaryotic and eukaryotic microorganisms are documented from mine ecosystems (Gross and Robbins, 2000; Bhattacharya et al., 2006). In the Lusatia region of eastern Germany, there are an estimated 200 acidic (pH < 3) lakes of a surface area >1 ha, which have arisen as a result of the infill of voids following open cast mining of open lignite coal. A large amount of research has been carried out on the
geochemistry, microbiology and remediation strategies of these water bodies (Geller et al., 1998) Photosynthetic (e.g., Ochromonas, Chlamydomonas) and lithotrophic (e.g., Acidithiobacillus spp.) as well as heterotrophic (e.g., Acidiphilium and Ferrimicrobium spp.) microorganisms have been identified in these acid mining lakes.

Coal spoil heaps are susceptible to ignite spontaneously and may smolder for many years. One of the first thermophilic acidophiles to be discovered (Thermoplasma acidophilum) and isolated from such a site (Darland et al., 1970). One isolate (Sulfolobus BC) was isolated from a self heating coal spoil in central England (Norris and Owen, 1992). In other work, Kusel et al. (2001) carried out a series mesocosm experiment using sediment samples taken from a coal mining impacted acid lake in Lusatia. Bacteria that were able to couple the oxidation of elemental sulfur to the reduction of ferric iron were estimated to be 1% of the total microbial population. In addition, a sulphate reducing bacteria (SRB) was isolated from a sample with an in situ pH of 5.2. Phylogenetic analysis revealed that this was again most closely related to the spore former Desulfosporosinus orientis and that it had a optimum pH of 5.5, but could not grow at pH < 4.9. A fungus, Hypocrea lixii was isolated from coal mine soil at the Fushunxi colliery, Liaoning Province, North of China (Xiu Xiang Tao et al., 2010).

Mitesh et al. (2009) isolated the yeast Candida digboiensis from an extreme acid mine drainage of the lignite mine. Acidithiobacillus ferrooxidans and Acidithiobacillus thiooxidans were identified by 16S rRNA sequencing from lignite mine (Siebert et al., 2009). Soomro et al. (2010) isolated spores of two fungal species Dicellaesporinies bolharensis sp. nov.
and *Lacrimasporonites sondensis* sp. nov. from brown coals. Species of *Agrobacterium, Xanthomonas, Corynebacterium* were isolated from coal mine area by enrichment culture (Magda Constanti et al., 1996). *Thiobacillus ferrooxidans* was isolated from *in situ* oil shale coal mine samples by Vrvc et al. (1987). *Thiobacillus ferrooxidans* was isolated from coal samples by Acharya et al. (2001). The fungal species *Penicillium chrysogenum* was isolated and identified on the basis of fungal ITS sequences from a core sample of coal (Rizwan Haider et al., 2012).

Duongruitai Nicomrat et al. (2008) reported *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans*, species of *Bordetella* and *Alcaligenes* from a constructed wetland that receives acid drainage from underground coal mine. A Gram positive bacterium related to *Desulfosporosinus orientis* was isolated from acidic coal mine lake (Kusel et al., 2001). In another study, Richard Pokorny et al. (2005) isolated microorganisms from freshly excavated lignite in the Zahorie coal mine (south western Slovakia). The isolates represented both Prokaryotes and Eukaryotes (fungi). Bacteria belonged to the genera *Bacillus, Staphylococcus* and *Rhodococcus* according to both morphological criteria and ITS sequences. Fungal isolates were typified by using their ITS sequences. They belonged to the genera *Trichoderma, Penicillium, Epicoccum, Metarhizium* and *Cladosporium*. Temple and Colmer (1951b) confirmed the presence of sulphur oxidizing bacterium in acid drainage waters from a West Virginia coal mine and its ability to oxidize ferrous iron. Gram-negative bacterium *Flavobacterium acidurans* was isolated from acid water of a closed deep coal mine (Miller, 1973). Heavy metal resistant bacteria isolated from coal mining environment by Marcus Adonai et al. (2003).
2.3. HEAVY METAL CONTAMINATION OF SOIL BY DIFFERENT INDUSTRIAL EFFLUENTS

Soil is a complex mixture of minerals, nutrients, organic matter and living organisms upon which all other terrestrial trophic systems are dependent (Perezde Mora et al., 2005). It is a vital resource for sustaining basic human needs such as food, fibre and shelter (Branzini et al., 2009). Significant increases in soil metal content are found in areas of high industrial activity where, accumulation may be several times higher than the average content in non contaminated areas. Additionally, areas distant from industrial centers also show increased metal concentrations due to long range atmospheric transport. This fact has been observed by numerous authors (Jonathan et al., 2004; Omar and Al Khashman, 2004). In low and medium contaminated soils, concentration of metals in crops is mostly not high enough to cause acute toxicity but in the long run, it may cause chronic damage to human and animal health (Puschenreiter et al., 2005). Long term application of waste water to agricultural lands often causes the build-up of the toxic metals in soils, the extent of which depends on the period of application (Nagajyoti et al., 2008).

Various industries have been continuously adding lot of waste water containing nutrients, heavy metals and hazardous substances to the cultivable land (Srivastava et al., 2000). Due to scarcity of good water for irrigation, the farmers have started to irrigate their agricultural crops by using the industrial waste water (Rathore et al., 2000). Irrigation with wastewater is said to have both beneficial and harmful effects as it contains substantial amounts of beneficial nutrients and toxic heavy metals (Singh et al.,
2004a; Chen et al., 2005). Using waste water possess several threats both to environment and to human as well as livestock health. The contaminants present in the industrial waste water are sequestered in the soils and thereby possess environmental problems. In developing countries including India, farmers are irrigating their crop plants with industrial effluents having high level of several toxic metals (Cu, Cd, Cr, Zn, Fe, Ni, Mn, Mg and Pb) due to the non availability of alternative sources of irrigation water (Chandra et al., 2009). The presence of heavy metals in waste water irrigated areas has been a problem in several countries like Germany, France and India (Ingwersen and Streit, 2006; Dere et al., 2006; Singh and Kumar, 2006). In many developing countries, waste water used for irrigation, is often inadequately treated. For example, WHO/UNICEF (2000) estimated the median percentage of waste water treated by effective treatment plants to be 35% in Asia, 14% in Latin America and the Caribbean, 90% in North America and 66% in Europe. Globally, around 20 million ha land is reported to be irrigated with waste water and at least 10% of the world’s population is thought to consume foods produced by irrigation with wastewater (WHO, 2006a, b; Hamilton et al., 2007).

Sharma et al. (2004) reported that the soil samples collected from the agricultural land irrigated with effluents had high level of several toxic metals as Cu, Cd, Cr, Zn, Ni, Fe, Mn and Pb. Similarly, soil analysis showed higher values of Zn, Fe, Mn, Cd, Ni and Pb and plant samples had greater concentration of many heavy metals than recommended values in areas where industrial effluent was used as irrigation for vegetable cultivation (Saleem Saif et al., 2005). The excessive accumulation of heavy metals in agricultural soils through waste water irrigation, may not only result in soil
contamination but also lead to elevated heavy metal uptake by crops and thus affect food quality and safety (Muchuweti et al., 2006).

Butt et al. (2005) found high concentrations of the heavy metals Fe, Cu, Zn, Mn, Ni, Pb and pathogens in the soil and plants intensively irrigated with waste water at Faisalabad, Pakistan. In another study, Gupta et al. (2008) reported that municipal waste water irrigation was the cause of heavy metals (Pb, Zn, Cd, Cr, Cu and Ni) accumulation in soils and vegetables. They concluded that vegetables grown with waste water possess concentrations of heavy metal (Pb, Zn, Cd, Cr and Ni) in all the sampled vegetables beyond safe limits, and there is great health risk in consuming such vegetables. Similarly, Murtaza et al. (2008) observed accumulation of Co, Cd and Mn in vegetables grown with sewage water irrigation. In all plant samples, Cd and Mn were above critical limits whereas, Co was within permissible limit. Root crops such as carrot, radishes and onion grown using waste water irrigation were unsuitable for marketing because of development of several short roots instead of a single straight root. Similarly, metals addition from waste water also resulted in severe burning of sorghum and wheat crops leading to expensive re-planting. Moreover, long term application of waste water resulted in build-up of soil salinity and damage to soil structure.

Investigations regarding the heavy metal contents in soil and vegetables irrigated with Hudiara drain water at Lahore, Pakistan revealed that this water was unfit for irrigation with regard to heavy metals contents (Kashif et al., 2009). Soils irrigated by this water were alkaline and showed very high concentration of trace metals. The vegetables grown using this
water also had metal contents far higher than the Indian standards for safe consumption of vegetables. In India, Rattan et al. (2005) reported that sewage effluents contained substantial amounts of nutrients such as S, P, K, Zn, Fe, Cu, Mn and Ni along with organic C contents and heavy metal concentrations in all the plants tissue grown using these effluents were below the critical level of phytotoxicity, probably due to the addition of organic C through waste water in the soil that resulted in less metal accumulation in plant tissues. They further suggested that humans can consume these vegetables safely; however, the accumulation of heavy metals particularly the Cu, Ni and Zn in soils should be periodically monitored considering their chances of accumulation in plants.

Many researchers have observed higher concentrations of toxic metals in crops irrigated with waste water sufficient to cause disorders in plants and clinical problems in both humans and animals consuming these plants (Madhava and Sresty, 2000; Ensink et al., 2004; Hussain et al., 2006; Nagajyoti et al., 2008). The heavy metal polluted water when used for irrigation resulted in increased contents of Cu and Cd after the harvest of rice crop (Allah Nawaz et al., 2006).

2.4. HEAVY METAL CONTAMINATION OF SOIL BY COAL MINING AND ITS EFFLUENT

Mining and combustion of coal is often associated with environmental contamination and pollution (Liores et al., 2001; Finkelman et al., 2002; Finkelman, 2004) and different types of coals can cause different trace element pollution (David, 2002; Fang et al., 2003). There is concern that coal mine operations can mobilize trace elements into aquatic
ecosystems in greater quantities than would normally occur in a natural setting (Johnson, 2003).

AMD is defined as drainage flowing from or obtained from surface mining, deep mining or coal refuse piles. The drainage is usually highly acidic (pH 2.3 – 6.5) and contains elevated levels of dissolved metals, including iron, manganese and aluminium (Johnson, 1995). The most severe and common problem caused by coal mining is water pollution, caused by heavy metal contaminated water flowing from coal mines. The most important trace elements that occur in mine water and that have a high probability of being regulated are arsenic, barium, cadmium, copper, manganese, molybdenum, nickel, lead, selenium and zinc (Sullivan and Yelton, 1988). Mine water includes water draining through dewatering adits, mine dewatering pumps, open pit mines, or raw material handling facilities on the site of a mining operation (ERMITE Consortium, 2004). The rain water mixed with newly mined soil, grease and oil used in machines also discharged along with mine waste water. The water used in the cooling towers of power plants is released into rivers, causing deterioration in water quality (Koumantakis, 1999; ELIMEIA, 1999; Dimitrakopoulos et al., 2000; Grigorakou et al., 2002; EC-GPPC, 2005). Elevated concentrations of Fe and Mn are commonly associated with coal drainage (Rose and Cravotta, 1998a). Heavy metal analysis of lignite mine water, fly ash pond and the natural reservoirs which receiving effluent from lignite mines reveals that Co, Cr and Hg are above the recommended irrigation water quality standards in 17%, 75% and 100% of the samples, respectively (Khan et al., 2005).
Coal operated thermal power plant can be a source of pollution, because ash derived from burning of coal containing heavy metals such as arsenic (As), cadmium (Cd), lead (Pb), mercury (Hg) and zinc (Zn), can contaminate water in the drainage system, presenting a potential hazard to the environment (Kanungo and Mohapatra, 2000). Lignite combustion generates emissions of potentially toxic trace elements (Rubin, 1999; Reddy et al., 2005a; Petaloti et al., 2006; Nelson, 2007; Guo Xin et al., 2007). Studying the distribution of heavy metals in soils near coal mines and power plants consuming coal is very important to determine coal related contamination (Popovic et al., 2001; Tsikritzis et al., 2002; Snezana et al., 2013). Investigations have been made of the extent of heavy metal pollution of surface water, groundwater, soils, air and vegetation by mining and associated industrial activities, particularly thermal power plants and open cast mines (Sahu, 1998; Liorens et al., 2001; Mohanty et al., 2001; Coulthard and Macklin, 2003; Fang et al., 2003).

The research community has a diverse view regarding whether coal mining and combustion increase trace element concentrations in nearby soils. Chadwick et al. (1987) believed that this kind of contamination from power plants consuming coal with average trace element concentrations is too small and not detectable while others believe that the deposition of fly ash from power plants can lead to higher trace element concentrations in nearby soils. Many researchers reported that pollution of soil by coal mining activities (Klein et al., 1975; Patel and Pandey, 1986; Jyoti Singh et al 1995; Stalikas et al., 1997; Orem and Finkelman, 2005; Snezana et al., 2013).
2.5. EFFECT OF DIFFERENT CONCENTRATIONS OF VARIOUS HEAVY METALS ON PLANTS

Contamination of agricultural soil by heavy metals has become a critical environmental concern due to their potential adverse ecological effects. Such toxic elements are considered as soil pollutants due to their widespread occurrence and their acute and chronic toxic effect on plants grown of such soils. The regulatory limit of cadmium (Cd) in agricultural soil is 100 mg/kg soil (Salt et al., 1995). But, this threshold is continuously exceeding because of several human activities. Cadmium is non-essential but poisonous for plants, animals and humans (Gupta and Gupta, 1998). In plants, Cd inhibits root and shoot growth, affects nutrient uptake and homeostasis, and frequently is accumulated by agriculturally important crops. Cd is consumed by animals and humans in their diet and can cause diseases (Belimov et al., 2005). Plants exposed to high levels of Cd causes reduction in photosynthesis, water uptake and nutrient uptake. Plants grown in soil containing high levels of Cd show visible symptoms of injury reflected in terms of chlorosis, growth inhibition, browning of root tips, and finally death (Wojcik and Tukiendorf, 2004; Mohanpuria et al., 2007). Soil is also contaminated with zinc (Zn) in addition to Cd by the sewage sludge or urban composts, fertilizers, emissions from municipal waste incinerators, residues from metalliferous mining, the metal smelting industry and other human activities.

Zn is an essential nutrient for higher plants. But, the high level of Zn found in contaminated soils frequently exceed to those required as nutrients and may cause phytotoxicity. In polluted soil, the higher concentration of Zn
(150 to 300 mg/ kg) have been measured (De Vries et al., 2002; Warne et al., 2008). High levels of Zn in soil inhibit many plant metabolic functions; result in retarded growth and cause senescence. Zinc toxicity in plants limited the growth of both root and shoot (Choi et al., 1996; Ebbs and Kochian, 1997; Fontes and Cox, 1998). Zinc toxicity also causes chlorosis in the younger leaves, which can extend to older leaves after prolonged exposure to high soil Zn levels (Ebbs and Kochian, 1997). Another typical effect of Zn toxicity is the appearance of a purplish red color in leaves, which is ascribed to phosphorus (P) deficiency (Lee et al., 1996). Excess Zn can also give rise to deficiencies of Mn and Cu in plant shoots. Such deficiencies have been ascribed to a hindered transfer of these micronutrients from root to shoot. This hindrance is based on the fact that the Fe and Mn concentrations in plants grown in Zn rich media are greater in the root than the shoot (Ebbs and Kochian, 1997).

Copper (Cu) is considered as a micronutrient for plants (Thomas et al., 1998). It plays important role in CO₂ assimilation and ATP synthesis and acts as an essential component of various proteins like plastocyanin of photosynthetic system and cytochrome oxidase of respiratory electron transport chain (Demirevska Kepova et al., 2004). Higher level of Cu is added to soils from different human activities including mining and smelting of Cu containing ores. Excess of Cu in soil leads to plant growth retardation and leaf chlorosis (Lewis et al., 2001), oxidative stress (Stadtman and Oliver, 1991). Oxidative stress causes disturbance of metabolic pathways and damage to macromolecules (Hegedus et al., 2001).
The large input of mercury (Hg) into the arable lands has resulted in the widespread occurrence of mercury contamination in the entire food chain. Hg released to the soil mainly remains in solid phase through adsorption onto sulfides, clay particles and organic matters. Increasing evidence has shown that Hg can readily accumulate in higher and aquatic plants (Kamal et al., 2004; Wang and Greger, 2004; Israr et al., 2006). High level of Hg is strongly phytotoxic to plant cells and induced visible injuries and physiological disorders in plants (Zhou et al., 2007). For example, Hg can bind to water channel proteins, thus inducing leaf stomata to close and physical obstruction of water flow in plants (Zhang and Tyerman, 1999). High level of Hg interfere the mitochondrial activity. This leads to the disruption of biomembrane lipids and cellular metabolism in plants (Israr and Sahi, 2006; Cargnelutti et al., 2006).

Chromium (Cr) is a heavy metal that causes serious environmental contamination in soil, sediments and groundwater (Shanker et al., 2005). The tanning industry is one of the major consumers of water and most of it is discharged as waste water which contains high amount of Cr (1.07–7.80 mg/L). Worldwide anthropogenic discharge of Cr in fresh water bodies has been estimated to be 3550 MT (Nriagu, 1990). Toxicity of Cr has been studied in many plants. Excess of Cr causes inhibition of plant growth, chlorosis in young leaves, nutrient imbalance, wilting of tops and root injury (Chatterjee and Chatterjee, 2000; Dixit et al., 2002; Sharma et al., 2003; Scoccianti et al., 2006). Inhibition of chlorophyll biosynthesis has also been reported in terrestrial plants (Vajpayee et al., 2000). For example, barley seedlings grown in 100 μM Cr showed 40 % inhibition of growth (Skeffington et al., 1976). Toxic effects of Cr on plant growth and
development include alterations in the germination process as well as in the growth of roots, stems and leaves. Cr also causes deleterious effects on plant physiological processes such as photosynthesis, water relations and mineral nutrition. Metabolic alterations by Cr exposure have also been described in plants either by a direct effect on enzymes and metabolites (Shanker et al., 2005). Hence, exposure to high level of Cr affected total dry matter production and yield of plants (Shanker et al., 2005). The Cr is not only toxic to plants. It is a very toxic, powerful epithelial irritant and a proven human carcinogen established by International Agency for Research on Cancer (IARC), the Environmental Protection Agency (EPA) and the World Health Organization (WHO).

Lead (Pb) is one of the ubiquitously distributed most abundant toxic elements in the soil. The toxic level of Pb in soil results from disposal of municipal sewage sludge, mining and smelting activities, Pb containing paints, paper and pulp, gasoline and explosives. It exerts adverse effect on morphology, growth and photosynthetic processes of plants. High level of Pb also causes inhibition of enzyme activities, water imbalance, alterations in membrane permeability and disturbs mineral nutrition (Sharma and Dubey, 2005). High level of Pb also induces oxidative stress by increasing the production of reactive oxygen species (ROS) in plants (Reddy et al., 2005b).

Arsenate (As) is an analog of phosphate (P) and competes for the same uptake carriers in the root plasmalemma of plants (Meharg and Macnair, 1992). The As tolerance has been identified in a number of plant species (Meharg, 1994; Sharples et al., 2000). The As tolerance in grasses results from suppression of a high affinity P/ As uptake system (Meharg and
Macnair, 1992). This suppression reduces As influx to a level at which plant can easily detoxify it, presumably by constitutive mechanisms (Meharg, 1994). The As also undergoes transformation within plant cells to other less phytotoxic As species (Meharg, 1994). In phytoplankton and macroalgae, As is converted to arsenite, dimethylarsinic acid (DMA) and monomethylarsonic acid (MMA). Such methylated forms of As are then metabolized to organophospholipids and arenosugars (Phillips, 1990). Previously, terrestrial plants have been documented only for the presence of arsenate and arsenite (Meharg, 1994; Van den Broeck et al., 1998). However, a later study on a range of terrestrial plants has also reported low concentrations of methylated As species such as MMA and DMA (Koch et al., 2000).

Copper is an important micronutrient for plants and its concentration in soils is increased by deposition from the burning of fossil fuels, wearing of Co containing alloys and spreading of sewage sludge and manure (Barceloux, 1999). However, environmental risks of Co are managed through the establishment of environmental quality criteria and standards. Plants can accumulate small amount of Co from the soil. The uptake and distribution of Co in plants is species dependent and controlled by different mechanisms (Kukier et al., 2004; Bakkaus et al., 2005). Very little information is available regarding the phytotoxic effect of excess Co. Phytotoxicity study of Co in barley (Hordeum vulgare L.), oilseed rape (Brassica napus L.) and tomato (Lycopersicon esculentum L.) has recently shown the adverse effect on shoot growth and biomass (Li et al., 2009). In addition to biomass, excess of Co restricted the concentration of Fe, chlorophyll, protein and catalase activity in leaves of cauliflower. Further, high level of Co also affected the translocation of P, S, Mn, Zn and Cu from
roots to tops in cauliflower. In contrast to excess Cu or Cr, Co significantly decreased water potential and transpiration rate. While, diffusive resistance and relative water content increased in leaves of cauliflower upon exposure to excess Co (Chatterjee and Chatterjee, 2000).

Nickel (Ni) is a transition metal and found in natural soils at trace concentrations except in ultramafic or serpentinic soils. However, Ni concentration is increasing in certain areas by human activities such as mining works, emission of smelters, burning of coal and oil, sewage, phosphate fertilizers and pesticides (Gimeno Garcia et al., 1996). Ni concentration in polluted soil may reach 20 to 30 fold (200–26,000 mg/kg) higher than the overall range (10–1000 mg/kg) found in natural soil (Izosimova, 2005). The higher concentration of Ni in soil causes chlorosis and necrosis in different plant species (Zornoza et al., 1999; Pandey and Sharma, 2002; Rahman et al., 2005), including rice (Samantaray et al., 1997). The impairment of nutrient balance and disorder of cell membrane functions was observed in plant grown in soil containing higher concentration of nickel. Thus, Ni affected activity of the plasma membrane as reported in Oryza sativa shoots (Ros et al., 1992). Exposure of wheat to high level of Ni enhanced Malondialdehyde (MDA) concentration (Pandolfini et al., 1992). Moreover, Gonnelli et al. (2001) reported an increase in MDA concentration of Ni sensitive plants compared to a Ni tolerant Silene. Such changes might disturb membrane functionality and ion balance in the cytoplasm, particularly of K⁺, the most mobile ion across plant cell membrane. A decline in water content of dicot and monocot plant species was induced by high uptake of nickel. The decrease in water uptake
is used as an indicator of the progression of Ni toxicity in plants (Pandey and Sharma, 2002; Gajewska et al., 2006).

The symptoms of reduced root growth, reduced seed sprouting and seedling stunting, necrosis and chlorosis appear in susceptible plants grown in soils contaminated with heavy metals (Gemmell, 1977; Foy et al., 1978; Wong and Bradshaw, 1982) and using with industrial effluents (Sharma et al., 2002; Dixit, 2003; Kaushik et al., 2005; Soundarajan and Pitchai, 2007; Yadav and Meenakshi, 2007; Muthalagi and Mala, 2007; Kalaiselvi et al., 2009). Some crop plants growing in highly contaminated soil contain heavy metals at concentration levels hazardous to human health (Kim et al., 1998, 2002). Cultivation of crops for human or livestock consumption on contaminated soil can potentially lead to the uptake and accumulation of trace metals in the edible plant parts with a resulting risk to human and animal health (Gupta and Gupta, 1998; Monika and Katarzyna, 2004; McBride, 2007).

Potential health risks to humans and animals from consumption of crops can be due to heavy metal uptake from contaminated soils via plant roots as well as direct deposition of contaminants from the atmosphere onto plant surfaces (McBride, 2003). Accumulation of heavy metals in crops grown in metal-polluted soil may easily cause damage effect on human health through food chain. Fu et al. (2008) contacted an investigation on heavy metal contents in rice sampled from Taizhou city in Zhejiang province, China and found that the geometric mean of Pb in polished rice reached 0.69 mg kg$^{-1}$, which was 3.5 folds higher than the maximum allowable concentration (MAC) (0.20 mg/kg) of the safety criteria for milled
rice. Cd contents in 31% of the rice samples exceeded the national MAC (0.20 mg/kg) and the arithmetic mean also slightly exceeded national MAC. The carryover of toxic trace and heavy metals in grain depends on several factors such as pH, Organic carbon, microorganisms, concentration of metals and their form of occurrence, and the mobility of such elements to the root, their transport from root surface to root interior and their translocation from root to shoot (Chaney and Giardino, 1977) plays a significant role.

2.6. BIOREMEDIATION OF HEAVY METAL CONTAMINATED ENVIRONMENT

Soil health is defined as the continued capacity of soil to sustain its biological productivity, maintain the quality of the surrounding air and water environments and promote plant, animal and human health (Doran et al., 1996). Soil health is threatened by various materials derived from human activities, which include industrial pollutants, pesticides, livestock waste water, mine drainage and petroleum contamination (Thornton, 1983; Alloway, 1990; Yeo and Kim, 1997; Kim et al., 2002). Among these, contamination of soil, especially by heavy metals from mines, is one of the most important causes of soil health decrease, as excess amounts of heavy metals are detrimental to both human health and plants (Jarup, 2003; Li and Yang, 2008).

Agricultural soils in many parts of the world are slightly to moderately contaminated by heavy metal toxicity such as Cd, Cu, Zn, Ni, Co, Cr, Pb and As. This could be due to long term use of phosphatic fertilizers, sewage sludge application, dust from smelters, industrial waste and bad watering practices in agricultural lands (Bell et al., 2001; Schwartz et al., 2001;
Passariello et al., 2002). In the past decade, many countries have spent billions of dollars trying to clean up contaminated ground water and soil. Heavy metal soil contamination is a significant environmental problem due to the increased release of metals to the environment. Methods for the removal of heavy metals from the environment can be divided into two groups: 1) biotic methods, which are based on the accumulation of heavy metals by plants or microorganisms and 2) abiotic methods, which are based on the removal of heavy metals using physiochemical processes such as precipitation, coprecipitation, ion exchange and adsorption of heavy metals by suitable adsorbent (Celis et al., 2000; Vijayaraghavan and Yun, 2008). Conventional physico-chemical methods such as electro chemical treatment, ion exchange, precipitation, reverse osmosis, evaporation and sorption (Kadirvelu et al., 2001; Kadirvelu et al., 2002) for heavy metal removal are being economically expensive and have disadvantages like incomplete metal removal, higher reagent, energy requirements and generation of toxic sludge, thereby resulting in merely the transfer of the metal from one form into less mobile and available form, but not providing a definitive solution (Kratochvil and Volesky, 1998). Biological processes have long been considered cost effective environmentally friendly methods for the remediation of heavy metal contaminated soils (Congeevaram et al., 2007).

Microorganisms are nature’s original recyclers, converting toxic organic compounds to harmless products, often carbon dioxide and water. Bioremediation is defined as the process of using microorganisms to degrade or to remove hazardous components of the wastes from the environments (Hazen, 1997). Evidence of kitchen middens (ancient household garbage dumps) and compost piles dates back to 6000 B.C. (NABIR, 2003),
demonstrating that some form of bioremediation was practiced by humans since the beginning of recorded history. Bioremediation was used over 100 years ago with the opening of the first biological sewage treatment plant in Sussex, UK, in 1891 (NABIR, 2003). However, the word “bioremediation” is fairly new; first appearing in a peer-reviewed scientific literature in 1987 (NABIR, 2003). Bioremediation technologies can be broadly divided into two categories: *ex situ* and *in situ* bioremediation (Boopathy, 2000). *In situ* techniques are defined as the treatment of a pollutant without the removal of the contaminated site. In contrast, *ex situ* technologies are treatments that involve the physical removal of the contaminated material for treatment process.

Microbial populations in metal polluted environments adapt to toxic concentrations of heavy metals and become metal resistant (Viti and Giovannetti, 2001; Prasenjit and Sumathi, 2005). Microorganisms able to survive well in high concentrations of heavy metals are of great interest as bioremediation agents because they can achieve different transformation and immobilization processes. Specifically, they conduct bioaccumulation based on the incorporation of metals inside the living biomass or biosorption, in which metal ions are adsorbed at the cellular surface by different mechanisms (Vijayaraghavan and Yun, 2008). Biological approach has the great potential that contributes for the achievement of this goal and is economical.

**2.7. REMOVAL OF HEAVY METALS BY UTILIZING BACTERIA**

Chemical approaches like precipitation (Xia and Liyuan, 2002), ion-exchange method (Addour *et al.*, 1999), electrochemical cells (Cossich *et
and reverse osmosis (Xia and Liyuan, 2002) are available for remediation, but are often expensive to apply and lack the specificity required to treat the effluents and soil. In addition, such approaches are not applicable to a cost-effective remediation of large-scale subsurface contamination in situ. As an alternative to these methods, recently, the method of removal of contaminants by means of microorganism has been focused on.

Heavy metal bioremediation involves removal of heavy metal from waste water and soil through metabolically mediated or physico-chemical pathways. This natural and environmental friendly technology is cost effective, aesthetically pleasant, soil organism-friendly, diversity enhancer, energy derivation from sunlight (Susarla et al., 2002; Huang et al., 2004; Chaney et al., 2005) and more importantly, it is able to retain the fertility status of the soil even after the removal of heavy metals (Kirkham, 2006). Bioremediation of toxic industrial effluents and soil by using bacteria serves as an effective method to substitute the conventional recovery and removal process.

Hanife Buyukgungor, (2000) studied the bioaccumulation of lead from aqueous solutions by immobilized cells of *Citrobacter freundii*. The bacterial Extracellular polymeric substances (EPS) isolated from a species of *Marinobacteria* selectively bound more amount of copper per mg of EPS than lead. Both copper and lead were sorbed more at near neutral pH than acidic pH (Bhaskar and Narayan Bhosle, 2003).

Hussein et al. (2004) studied on the biosorption for Cr, Cu, Cd and Ni using non-living biomass of different *Pseudomonas* sp. isolated from rice field. The maximum adsorption capacity was found to be the highest for Ni
followed by Cd, Cu and Cr. Maximum Cr removal reached around 38% and its removal increased with the increase of Cr influent. Cu removal was at its maximum value in presence of Cr as a binary metal, which reached 93% of its influent concentration. Concerning to Cd and Ni similar removal ratios were obtained, it was ranged between 35 to 88% and their maximum removal were obtained in the case of individual Cd and Ni. The ability of the co-immobilized culture of microalgae *C. vulgaris* and *Azospirillum* to clean wastewater revealed that almost all of the ammonium ions were removed within 48 hours, although the removal of phosphorus was low and resulted in significant increased growth of the microalga (Bashan et al., 2004).

Semra Ihan et al. (2004) studied on the selective biosorption of chromium, lead and copper ions by microorganisms from industrial waste waters. Liang Ming Whang et al. (2004) investigated the potential application of two biosurfactants, surfactin produced by *Bacillus* sp. and rhamolipid produced by *Pseudomonas aeruginosa*, for enhanced biodegradation of contaminated water and soil and reported that application of surfactin and rhamnolipid in stimulating indigenous microorganisms for enhanced bioremediation confirmed their enhancing capability on both efficiency and rate of diesel biodegradation in soil systems.

Belimov et al. (2005) isolated eleven cadmium tolerant bacterial strains of *Bacillus* sp., *Azotobacter* sp., *Flavobacterium* sp. and *Rhodococcus* sp. which offer promise as inoculants to improve growth of the metal accumulating plant *B. juncea* in the presence of toxic Cd and for the development of plant inoculant systems useful for phytoremediation of polluted soils.
Van Nostrand et al. (2006) isolated four aerobic Ni tolerant, Gram positive heterotrophic bacteria from sediments contaminated with high levels of Ni and U. These isolates were identified as Arthrobacter oxydans NR-1, Streptomyces galbus NR-2, Streptomyces aureofaciens NR-3, and Kitasatospora cystarginea NR-4 based on partial 16S rDNA sequences. Further characterization of S. aureofaciens NR-3 and K. cystarginea NR-4 demonstrated that both isolates expressed Ni tolerance constitutively. In addition, both were able to grow in higher concentrations of Ni at pH 6 as compared with pH 7 (42.6 and 8.5 mM Ni at pH 6 and 7, respectively.

Debabrata Bera et al. (2006) investigated the feasibility of Bacillus cereus immobilized in different carriers as a biosorbent for chromium removal from aqueous solutions in batch mode; optimum conditions were determined. Experimental results showed the bacterial strain immobilized in calcium alginate gel matrix was most effective in removing Cr ion from solution. The uptake of metal was very fast initially, and equilibrium was attained within 80 minutes. The highest value of Cr uptake by Bacillus cereus M1 16 (6.0g/L, dry basis) immobilized in 3% calcium alginate was 92.5% at 25°C, when initial chromium concentration was 50 mg/L. The bacteria Alcaligenes, Bacillus and Corynebacterium isolated from sago industry effluent and effluent contaminated soil were found efficient in starch degradation and recorded 63% of degradation of starch in sago industry effluent. The effluent treated by aerobic microorganisms had no negative impact on the seed germination and shoot length, root length, fresh weight, dry weight and chlorophyll content showed an increase. Hence, the bioremediated effluent can be effectively used for irrigation (Ayyasamy et al., 2008). Diana et al. (2008)
tested on adsorption properties of bacterial biomass for Cd removal from liquid effluents. Comte et al. (2008) assessed the influence of pH on the metal biosorption of extracellular polymeric substances (EPS) extracted from two different activated sludges.

Rehman et al. (2008) isolated two mercury resistant bacterial strains, *Pseudomonas aeruginosa* and *Pseudomonas* sp. from industrial wastewater. Both *Pseudomonas aeruginosa* and *Pseudomonas* sp. could reduce 90% of mercury from the medium after 40 hours of incubation at 37°C. Both bacterial strains have shown remarkable ability to uptake metal ions from the culture medium. *Pseudomonas aeruginosa* was observed to uptake 75% and *Pseudomonas* sp. 65% of Hg$^{2+}$ from the medium after 24 hours of incubation at 37°C.

Abou Shanab et al. (2008) examined four bacterial isolates for their ability to increase the availability of water soluble Cu, Cr, Pb and Zn in soils and for their effect on metals uptake. Random Amplified Polymorphic DNA analysis was used to show that the bacterial cultures were genetically diverse. Bacterial isolates S3, S28, S22 and S29 had 16S rRNA gene sequences that were most similar to *Bacillus subtilis*, *Bacillus pumilus*, *Pseudomonas pseuodocaligenes* and *Brevibacterium halotolerans* based on 100% similarity in their 16S rDNA gene sequence, respectively. Filtrate liquid media that had supported *Bacillus pumilus* and *Bacillus subtilis* growth significantly increased Cr and Cu extraction from soil polluted with tannery effluent and from Cu-rich soil, respectively. The highest concentrations of Pb, Zn and Cu were accumulated in shoots of *Zea mays* grown on Cu-rich soil inoculated with *Brevibacterium halotolerans*. The
highest concentration of Cr was accumulated in *Sorghum bicolor* roots grown in polluted soil by tannery effluent inoculated with a mixed inoculum of bacterial strains.

Eight isolates, which can grow on LB agar containing 500 mg/L of Cr, were isolated from soil samples of iron mineral area. The bacterial isolates were identified as *Bacillus* sp. by the 16S rRNA gene sequences. Phylogenetic tree analysis indicates the isolates can be divided into two groups. The bacterial isolates can be resistant to other heavy metals and reduce Cr at different levels. One bacterial isolate (MDS05), which can tolerate 2500 mg/L Cr and was able to reduce almost 100% of Cr at the concentration of 10 mg/L in 24 hours (Guojun Cheng and Li, 2009).

Lina Velasquez and Jenny Dussan (2009) tested Colombian *Bacillus sphaericus* native strains for the tolerance to As, Hg, Co, Fe and Cr, as well as the biosorption and bioaccumulation in living biomass and reported that the live and dead cells of *Bacillus sphaericus* OT4b31 and *Bacillus sphaericus* IV(4)10 showed biosorption of Cr and had the capacity to accumulate between 6 and 47% of Co, Hg, Fe and As.

Efficiency of utilizing petroleum refinery effluents under aerobic condition was demonstrated by *Pseudomonas* sp. and *Acinetobacter* sp. However, the greatest efficiency was by the consortium of both microorganisms (Atuanya *et al.*, 2009). Deepika Lakshmipath *et al.* (2010) stated that the *Bacillus* sp. isolate with its biosurfactant production and heavy metal resistant activity could be used as potential strain and could be used as bioremediating agent. *Pseudomonas* sp. is better microbial tool for bioremediation of heavy metal and can be explored to remove heavy metal
load, present even in low concentration, in waste water of pulp and paper mill effluent by using indigenous microorganisms (Arti and Smita, 2010).

Selvarathi et al. (2010) proposed that the inoculation of Serratia sp. significantly increased the root biomass of Zea mays under Cd or Cu contaminated conditions. Similarly, the culture of Bacillus sp. was able to increase the level of degradation rate and degrade the dye from textile effluent (Murugalatha et al., 2010).

Immobilized biosurfactant producing bacteria (Bacillus subtilis and Pseudomonas fluorescens) were assessed for survival in heavy metal contaminated soil and for their ability to remove cadmium and zinc from contaminated soil. The strain P. fluorescens G7 was considered to be a good candidate for bioremediation of heavy metals. The results of soil remediation showed that approximately 19% of Zn and 16.7% of Cd could be removed by this immobilized biosurfactant producing bacteria after incubation for 2 weeks (Charoon Sarin and Siripun Sarin, 2010).

Many researchers reported that the living cells of bacterial species had the capacity to remove heavy metals from soil (Voss and Thomas, 1998; Burd et al., 2000; Lebeau et al., 2002; Jeyasingh and Philip, 2005) and to remove different pollutants from soil (Singh et al., 2004b; Karpouzas and Singh, 2006; Hong et al., 2007; Lakshmi et al., 2008; Liang et al., 2011; Mariusz Cycon et al., 2013).

Bacterial species resistant to heavy metal Cu, Cd and Pb were isolated from the effluent samples of an electroplating industry. The biosorption capacity of immobilized bacterial isolates like Bacillus sp.,
*Pseudomonas* sp. and *Micrococcus* sp. were 69.34, 90.41 and 84.27% of Cu, Cd and Pb respectively. But, the dried biomass of the same species adsorbed only 44.73, 86.66 and 79.22% of Cu, Cd and Pb respectively (Johncy Rani *et al.*, 2010a).

The *P. aeruginosa* and *Thiobacillus ferrooxidans* showed high resistant to all metals and *T. ferrooxidans* reduced/ absorbed some heavy metals from mines (Cd, Ca, Zn, Cr, Mn and Pb) and *P. aeruginosa* was effectively absorbing the most of the metals than *T. ferrooxidans* (Narayanan Mathiyazhagan and Devarajan Natarajan, 2011).

Sahar Alzubaidy (2012) evaluated the resistance of *Serratia marcescens* obtained from soil and water to metals chlorides (Zn$^{2+}$, Hg$^{2+}$, Fe$^{2+}$, Al$^{3+}$ and Pb$^{2+}$). Two isolates, identified as *Serratia marcescens* and *Serratia marcescens* (S4) were selected for this study according to their resistance to five heavy metals. The ability of *Serratia marcescens* (S4) to grow in different concentrations of metals chloride (200-1200 μg/mL) was tested; the highest concentration that *Serratia marcescens* (S4) tolerate was 1000 μg/mL for Zn$^{2+}$, Hg$^{2+}$, Fe$^{2+}$, Al$^{3+}$ and Pb$^{2+}$ and 300 μg/mL for Hg$^{2+}$ through 24 hours incubation at 37°C. The isolates showed the ability to grow in different pH values (4, 7 and 9) in presence of four metals in all pH values (1000 μg/mL) and inability to grow with 300 μg/mL Hg$^{2+}$. The highest Zn$^{2+}$ removal ratio was 75% then Pb$^{2+}$ 55% while Fe$^{2+}$ has the lowest removal ratio (48%).

Munees Ahemad and Malik, (2011) isolated the most promising zinc resistant bacteria from heavy metal contaminated soils and further, to assess their metal accumulating ability. A total of 34 bacterial isolates from
agricultural soils irrigated with metal polluted wastewater were characterized and identified as *Pseudomonas*, *Bacillus* and *Staphylococcus*. The zinc resistant bacteria (*Pseudomonas* isolate SN7, *Pseudomonas* isolate SN28 and *Pseudomonas* isolate SN30) were selected because of exhibiting co-resistance against Cu$^{2+}$, Hg$^{2+}$, Cd$^{2+}$, Ni$^{2+}$, Pb$^{2+}$, Cr$^{3+}$ and Cr$^{6+}$ in addition to Zn$^{2+}$ and displaying high values of Minimum Inhibitory Concentrations (MIC) for each heavy metal. Further, the three isolates were assessed for their ability to remove zinc and copper from medium amended with these metals. The zinc resistant bacterial isolates SN7, SN28 and SN30 accumulated zinc maximum 29, 25 and 26 mg g$^{-1}$ dry weight of cells, respectively at the zinc concentration of 1.6 mM. Similarly, bacterial isolates SN7, SN28 and SN30 accumulated copper maximum 20, 25 and 22 mg g$^{-1}$ dry weight of cells, respectively at 2.92 mM of copper.

### 2.8. UTILIZATION OF BIOREMEDIATED EFFLUENT AND SOIL FOR AGRICULTURE

In developing countries including India, farmers are irrigating their crop plants with industrial effluents having high level of several toxic metals (Cu, Cd, Cr, Zn, Fe, Ni, Mn, Mg and Pb) due to the non-availability of alternative sources of irrigation water. Untreated effluent used for irrigation is highly toxic to the plant, fish and other aquatic life. The need for economical, effective and safe methods for disposal of pollutant in effluent has resulted in the search for unconventional materials that may be helpful in reducing the pollutant in the effluent (Bako *et al.*, 2008). Reusage of treated effluent that is normally discharged to the environment is receiving an increasing attention as a reliable water resource (Akram Tamini, 2008).
Hulugalle et al. (2004) reported that the treated sewage effluent using *Aspergillus niger* contained considerable amounts of nitrogen, phosphorus and high concentrations of sodium salts. Effluent water was moderately saline, and compared with river water, had higher concentrations of Na, nitrate-N and K, and lower concentrations of Ca and Mg. Irrigation with treated sewage effluent caused large increase in nitrate-N, small increase in exchangeable Mg, Na and K, and small decrease in heavy metals.

Experimental effects of untreated (Raw) distillery effluent, discharged from a distillery unit (based on fermentation of alcohol from sugarcane molasses) and the post-treatment effluent from the outlet of conventional anaerobic treatment plant (Treated effluent) of the distillery unit were studied in mung bean (*Vigna radiata*, L.). The germination percentage, growth characters and seedling enzyme activity of Mung bean was high even in 20% concentration of treated effluent compared with raw effluent (Prasad, 2008).

Seed germination, shoot length, root length, fresh weight, dry weight and chlorophyll content of maize and green gram showed an increase when treated effluent by aerobic bacterial consortium composed of *Alcaligenes*, *Bacillus* and *Corynebacterium* was used, whereas, a decrease of growth was noticed in untreated effluent tested seedlings (Ayyasamy et al., 2008). Similarly, germination of kidney bean (*Phaseolus aureus*) and Bhendi (*Abelmoschus esculentus*) seeds were affected adversely when raw textile effluent was used, whereas the bioremediated effluent increased the seed germination, total sugars, starch, reducing sugars and chlorophyll content of the plant than control (Priya Kaushik et al., 2004). The dry weight of root
and shoot of *Phaseolus radiatus* exhibited progressive increase from the control upto 9.25% concentration when irrigated with *Aspergillus terreus* treated dairy effluent (Ramana *et al.*, 2002).

Ragen *et al.* (2001) experimented on the sugar factory effluent using a pilot upflow anaerobic sludge blanket reactor. After bioremediation of sugar mill effluent using *Trichoderma* sp., the physico-chemical parameters of the effluent was found to be within the permissible limit and the seed germination of green gram and maize was effective even at 100% bioremediated effluent whereas in untreated effluent the seed germination stopped at 50% (Ajmal and Khan, 2008). Similarly the amount of amino acid, protein and chlorophyll content gradually increased with increasing concentration of the bioremediated effluent in tomato (*Lasa et al.*, 2000) and in barely, the plumule length and number of lateral roots significantly increased from 50% (Chandra *et al.*, 2004).

Biodegradation of sugar mill effluent using white rot fungus (*Phanerochaete chrysosporium*) showed successful efficiency in reduction of BOD, COD, TDS and lignin by over 50- 90% there by satisfying CPCB standard for effluent discharge (Prabakar *et al.*, 2005). Marine cyanobacteria such as *Oscillatoria boryna* degraded melanoidin in the distillery effluent and increased the yield of Sorgum and millet crops (Patel *et al.*, 2001). Likewise, *Pseudomonas, Enterobacter, Stenotrophomonas* and *Aeromonas* reduced the level of COD in the effluent. The effluent induced the growth parameters like root length, shoot length, number of leaves and nodules in *Phaseolus mungo* (Ram Chandra *et al.*, 2008). Similarly, untreated paper mill effluent and sugar mill effluent application had a significant effect on
*Lycopersicum esculentum* when compared to bioremediated effluents (Paul Sebastian *et al.*, 2009).

Nath and Sharma (2002) found that the lower concentration of sugar factory effluent increased the seedling growth, chlorophyll and amylase contents in green gram seedlings, while the higher concentration of the treated effluent using fungi (*Aspergillus niger*) had no effect on seed germination of *Triticum aestivum*. In another study, Kamlesh Nath *et al.* (2007) studied on *Phosphobacterium* treated distillery and sugar factory mixed effluent to investigate its effect on seed germination and seedling growth in wheat and black gram. Among the effluent analysed, the treated sugar mill effluent exhibited better performance in growth rate of wheat and black gram.

The lower concentration of sugar mill effluent showed promoting effect on seed germination, seedling growth, dry matter production and biochemical parameters, whereas germination percentage and seedling growth was inhibited at 100% concentration (Nagda *et al.*, 2006). The sugar factory effluent drastically reduced the growth and germination of wheat, Jowar, Raddish and bhendi (Yadav and Minakshi, 2007). Albino Wins and Murugan (2010) has stated that the sugar mill effluent can be safely used for irrigation purposes with proper treatment and dilution at 25% for growing blackgram *Vigna mungo*.

Vijayaragavan *et al.* (2011) studied the effect of sugar mill effluent on plant growth and biochemical constituents of *Raphanus sativus* in a pot culture experiment. The radish plants were grown upto 60 days, in the soil irrigated with different concentrations of sugar mill effluent (0%, 20%, 40%,
60%, 80% & 100%v/v). All pots were irrigated (500 mL) with respective concentration of test solutions daily. Plants were thinned to a maximum of three per pots, after a week of germination. The higher sugar mill effluent concentrations were found to affect plant growth and decreased total chlorophyll, caroteinoids, total sugar, amino acids and protein contents, but diluted effluent favoured the plant growth and biochemical contents.

Siva Santhi and Suja Pandian (2012) assessed the waste water quality parameters of treated sugar effluent and their effect at various concentrations like 0%, 25%, 50%, 75% and 100% on germination, speed of germination, peak value and germination value of two selected seeds i.e. peanut (Arachis hypogaea) and green gram (Vigna radiata). Germination percentage decreases with increasing concentration of effluent in all the tested seeds, whereas the germination speed, peak value and germination value increases from control to 25% and 50% concentration and decreases from 50% to 75% and 100% effluent. The germination percentages and germination values decrease with increasing concentration of effluent in all the seeds tested.

Tiwari et al. (2006) conducted an experiment. In which, they used coal mine effluent at different concentration for rice crop (Oryza sativa L.) cultivation and the results showed that the raw effluent affect all the growth characters including plant height, leaf area, root weight, dry biomass and seed weight, while the reduced concentration of effluent increase the above parameters.

Many researchers reported that the biologically treated soil enhance the growth and yield and of plants. Laine and Jurgensen (2007) found that mixing of Bacillus megaterium, along with nutrient addition degraded the
heavy metals in the sugar mill contaminated soil and increased the germination percentage of Onion (Allium cepa). Similarly, distillery contaminated soils were degraded using Termiomyces clypeatus and used in cultivation of olives, the length and number of new shoots, diameter of the main trunk, leaf area and dry weight of the plants was enhanced under the influence of treated soil (Absi, 2008). Baskaran et al. (2009a) reported that treating the soil using Rhizobium showed a decreased level in pollutants but showed a increased level in shoot length, leaf area and number pods in black gram.

Lakshmi and Sundaramoorthy (2010) has explained that the microbes (PGPR) mixed polluted soil showed good results in morphological, biochemical and yield parameters of blackgram than the untreated soil. Bacteria viz., B. cereus and B. thuringensis played an important role in degrading Cd, Ni and Zn in soil and increasing soil fertility by removal of heavy metals (Cd, Cu, Ni, Pb and Zn) for Millet and Green gram plants (Ajaz Haja Mohideen et al., 2010). Reclamation of sugar mill effluent contaminated soil using Penicillium sp. showed good percentage of pollutant reduction and best germination of green gram (Baskaran et al., 2009b).

Ajithkumar et al. (1998) demonstrated that chlorobenzoates adversely affect the seed germination and seedling vigor of tomato. However, the bioremediation of the soil with P. aeruginosa strain 3mT protected the tomato seeds, resulting in normal germination and seedling vigor. Ogboghodo et al. (2004) studied the effects of crude oil polluted soil on maize growth. They showed that percent survival rate and plant high decreased with increase in crude oil contamination; however when soils
were amended with poultry manure, plant highs increased significantly. In another study, Baskaran *et al*. (2009c) experimented on the reclamation of sugar mill effluent contaminated soil using earthworm. The physico-chemical analysis of sugar mill effluent and the polluted soils revealed that they are toxic in nature because they contain higher amount of micronutrients and macronutrients, organic and inorganic chemicals and heavy metals. The presence of pollutants in the soil mainly affected the plant metabolism which leads to growth and yield reduction. In order to enrich the soil quality, earthworm was cultured upto 60 days in polluted soil. After that, the various soil properties were analysed and good percentage of pollutant reduction was observed. Germination studies were conducted in bioreclamated soil and best germination was noticed under bioreclamated soil when compare with polluted soil.

Indira *et al*. (2010) determined Mycorrhizal fungi intimately associate with plant root forming a symbiotic relationship. The mycorrhizal symbiosis in effluent polluted soils was documented and the effect of dual inoculation with AM fungus on the host plant green gram (*Vigna radiata* L.) in pot culture experiments were investigated at six concentrations of tannery effluent *viz.*, control (AM only), 50% effluent, 50%+AM, 100% effluent, 100%+AM inoculated plants significantly increased in all morphological parameters. When compared with uninoculated plants the morphological parameters such as root length, shoot length, fresh weight and dry weight, root nodules and phosphorous content of green gram were increased in AM fungi inoculation soil. This study provides evidence for benefits of AM fungi protection of host plants and symbiosis could be a new approach increase in the effluent tolerance to legumes plants under effluent stress. In
soil bioremediated with *Streptomyces* sp. M7, normal germination (100%) and an increased in the seedling vigor were observed, compared to the control maize seeds (Benimeli *et al.*, 2008). In a similar study by Krueger *et al.* (1987), soybean and pea seedlings susceptible to the herbicide dicamba (3- dichloro-2-methoxy benzoic acid) were protected from its deleterious effect by inoculating soils with dicamba degrading microorganisms.