PHYLOGENETIC ANALYSIS OF BACTERIAL DIVERSITY OF HEAVY METAL AFFECTED SOIL OF BATHINDA REGION (SOUTH WEST PUNJAB)

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In

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BY

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CERTIFICATE

I declare that the dissertation entitled "PHYLOGENETIC ANALYSIS OF BACTERIAL DIVERSITY OF HEAVY METAL AFFECTED SOIL OF BATHINDA REGION (SOUTH WEST PUNJAB)" has been prepared by me under the guidance of Dr. Sanjeev K. Thakur, Assistant Professor, Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

Phylogenetic Analysis of Bacterial Diversity of Heavy Metal Affected Soil of Bathinda Region (South West Punjab)

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The soil of Bathinda region is affected by heavy metals due to anthropogenic activity, industrial effluent and fly ash. Three soil samples in triplicate were collected from three different places of Bathinda region, abandoned soil, agricultural soil, thermal power plant fly ash soil. Based on 16S rRNA gene cloning and sequence analysis, phylogentic analysis of bacterial diversity was performed. 366 clones were picked and through colony PCR 128 positive clones were chosen. Out of 128 clones, 126 clones were sequenced in three fragments and all three fragments aligned using SeqMan Lasergene ver.10.0 (DNA STAR) software. BLAST analysis of sequenced and aligned samples was carried out at NCBI to find out the homology with different groups of bacteria. All 126 clones aligned for phylogenetic analysis by MEGA 5 (Neighbor Joining method).

32 genus belong to 8 phyla were observed in all three samples. Most dominant phylum is Proteobacteria followed by Bacteriodates. Pseudomonas spp. is dominant in agricultural and thermal power plant soil. Abandoned soil showed maximum diversity followed by thermal power plant soil. In agricultural soil minimum diversity was observed. So with this investigation it can be concluded that lands without anthropogenic activities are rich in bacterial diversity as compared to more anthropogenic active areas.

(Rajiv Kumar) (Dr. Sanjeev K. Thakur)

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LIST OF ABBREVIATIONS

S. No.	Abbreviation	Full Form
1.	US	Unused/Abandoned Soil
2.	AS	Agricultural Soil
3.	TS	Thermal Power Plant Soil
4.	rRNA	Ribosomal Ribonucleic acid
5.	S	Sedimentation Coefficient
6.	bp	Base pairs
7.	G+C	Guanine + Cytosine
8.	HCl	Hydrochloric acid
9.	HNO_3	Nitric acid
10.	ICAP-AES	Inductively Coupled Argon Plasma- Atomic Emission Spectrophotometry
11.	DNA	Deoxyribonucleic acid
12.	CTAB	Cetyltrimethyl ammonium bromide
13.	M	Molar
14.	mg/Kg	Milligram/kilogram
15.	^0C	Degree Celsius
16.	ml	Milliliter
17.	ng	Nanogram
18.	µl	Micro liter
19.	F	Forward
20.	R	Reverse
21.	PCR	Polymerase Chain Reaction
22.	U	Unit
23.	BLAST	Basic Local Alignment Sequence Tool
24.	pH	Hydrogen ion concentration
25.	S	Siemens
26.	EC	Electrical Conductivity
27.	OC	Organic Carbon
28.	MilliQ H_2O	Distilled water
29.	dNTP	Deoxynucleoside triphosphates
30.	~	Approximately
31.	NCBI	National Centre for Biotechnology Information

Chapter I
Introduction

Microorganisms are the unseen majority on earth and their diversity is enormous. They are the most important component of biodiversity, and their activities are essential for survival of other forms of life. Soil microbes play pivotal role of biogeochemical cycles on earth, and are responsible for cycling of organic matter. These soil microbes influence soil fertility, soil structure and of above ground ecosystem by contributing plants nutrition. Microbes also help in removing toxins, soil formation, to suppress soil borne diseases of plants, in promoting growth factors and changes in vegetation of particular site (Garbeva et al., 2004; Kirk et al., 2004). They also help in biotransformation, metals and minerals transformation, decomposition, bioweathering and soil formation in biosphere. Both prokaryotes and eukaryotes and their association involve in biogeochemical cycling (Gadd, 2010). Environmental factors like pH, oxygen concentration, particle size of soil, organic carbon content, nutrition availability and water content influence the microbial diversity. Microbial diversity and community vary by physiological and metabolic function of microbes along with the soil profile and location (Hansel et al., 2008).

Some of the heavy metals are essential and required by the organisms as micronutrients (trace elements) (Co, Cr, Ni, Fe, Mn and Zn). They are involved in redox processes and also act as cofactor in enzymatic reactions and regulating osmotic balance. Some other heavy metals (Pb, As, Cd, Hg etc.) do not play role in biological system so they are detrimental for organisms even at very low concentration. At high level both essential and nonessential heavy metals become toxic to all organisms (Gadd, 2010; Rathnayake et al., 2009). These metals (Cu, Zn, Pb, Co, Ni, Cd, Cr etc.) have large effects on processes of microbial diversity, soil fertility and its structure. Soil processes like mineralization of organic matter, nitrogen fixation and other geochemical cycles are inhibited by contamination of heavy metals even in minor level increment. Heavy metal contamination reduces microbial biomass and decreases number of specific population of microbes. Some bacteria have capability that can grow on heavy metal contaminated area by adapting according to their environment.

Human activities have extensively altered the global environment changing global biogeochemical cycle, transforming land and enhancing the mobility of biota (Chapin III et al., 2000). Excessive use of pesticides, herbicides and inorganic fertilizers in agricultural land and industrial effluent in rivers and industrial waste dispose in open area without any treatment causes water, soil and air pollution. These are main contributory sources for heavy metal accumulation in soil. There is need to remove the heavy metal contamination from environment and two methods are mainly employed to remove heavy metal pollution i.e. abiotic and biotic. Abiotic method is by chemical treatments and other filtration methods which are very costly. Whereas biotic method includes bioremediation (removal of pollutant by microbes) and phytoremediation (removal of pollutant by plants), which is cheap and helpful method to maintain ecosystem processes (Fierer & Jackson, 2006; Kirk et al., 2004; Velusamy & El Azeem, 2011). So there is strong need to explore the bacterial diversity which has capability to reduce the heavy metals contamination and maintain the fertility as well as structural integrity of soil. Healthy soil maintains the diverse communities of microbes that help to control plant disease, recycling of plant nutrients, improve soil structure and form symbiotic relationship between plant roots (Arias et al., 2005).

One gram of soil sample may have 10 billion microorganism and thousands of different species, but less than 1% microbes can only be observed under microscope (Kirk et al., 2004; Torsvik & Ovreas, 2002). Most of the bacteria are generally unculturable, only 0.1-1% bacteria are culturable in vitro (Torsvik et al., 1998). Most of the bacteria remain in community and die if they grow separately (Garbeva et al., 2004). Direct DNA based methods offer the possibility to assess the present microbial diversity; these methods bypass the limitations of culture-based studies. Recent years have seen the rapid development of such culture-independent methods for analyzing the microbial communities in soil. It has been shown that molecular techniques are fast and effective technology to study microbial diversity in different environment. A number of molecular techniques have been developed to study the microbial diversity like 16S rRNA sequencing, DGGE (denaturant gradient gel electrophoresis), TGGE (temperature gradient gel

electrophoresis), ARDRA (amplified ribosomal DNA restriction analysis), T-RFLP (terminal- restriction fragment length polymorphism) and RISA (ribosomal intergenic spacer analysis) (Garbeva et al., 2004). In present study we have employed 16S rRNA gene sequencing for phylogenetic analysis of Bathinda region soil. 16S rRNA represent ecological marker of prokaryotes. It is highly conserved gene of ~1500 bp length.

South west Punjab particularly Bathinda region is suspected of heavy metals affected due to effluent coming from increasing industries, thermal power plant, oil refinery and most importantly agricultural practices often used by farmers to increase production of cash crops like cotton. This environmental pollution of heavy metals is affecting human health, agriculture crop yield and soil ecosystem. The expected development would definitely increase the level of these industrial pollutant as well as agricultural pollutants which would affect the human health and soil ecosystem more adversely. Soil health and human health is interrelated, therefore better strategy is required for rehabilitation of soil ecosystem.

So there is need to understand the bacterial diversity to find some novel species which have potential of bioremediation of heavy metals.

OBJECTIVES

With special reference to the Bathinda (South West Punjab) soil which is suspected of heavy metals contamination and their direct effects on plants and human health, the present study is planned with following objectives:

To study bacterial diversity by 16S rRNA gene sequencing technique from different soil samples of unknown metals and metalloids:-

i. Industrial/thermal power plant land affected by heavy metal contamination by effluent
ii. Agriculture land affected by heavy metal contamination by excessive use of fertilizers
iii. Non agriculture land/ barren land without anthropogenic activity.

Chapter II
Review of Literature

2.1 Biodiversity and Phylogenetics

Biodiversity is the whole population of all living organisms present in the biosphere. It includes all different types of plants, animals and microbes. Biodiversity explains the variety of life in all its forms, levels and combinations. Biodiversity has been defined as “the range of significantly different types of organisms and their relative abundance in an assemblage or community” (Torsvik et al., 1998).

Phylogenetics is the study of evolutionary relatedness between groups of organisms which is discovered through molecular sequencing data and morphological data matrices. Microbial diversity is the study of all microbes like bacteria, fungi and archea in particular environment.

2.2 Soil and Microbial Diversity

Microorganisms in soil ecosystem are important to maintain their function, structure, fertility, removal of toxic material, mineralization of organic material, reduction of heavy metals and geochemical cycling of C, N, S, P. Microbe also play pivotal role to provide healthy environment to plants by providing minerals, suppressing soil borne diseases, in promoting plant growth and in changes in vegetations. Both plant and soil can direct the structure of microbial community because diversity of microbes depends upon the soil type (Garbeva et al., 2004). The gradual accumulation of heavy metals may inhibit the degradation of organic pollutants or humic substances in the environment. The maintenance of soil quality is main factor by removing pollutant from it for improving the crop yield and productivity of soil. In India most of the industries shed their effluent without proper treatment and this causes pollution in environment which directly affects the human health (Mohideena et al., 2010).

Soil microbes closely associated with soil particles. They are present in single cell or as micro colonies in soil. Their metabolism and interactions with other organisms and with soil particles is dependent on the conditions at the microhabitat level, which often differ between microhabitats even over very small distances. The genetic complexity of microbial soil communities has been

estimated by re-association of community DNA (Daniel, 2004; Ghazanfar et al., 2010). Approximately 10^7cells were counted in 1 g of soil, but only 0.1% of the cells were culturable. Thus, 99.9% of the genetic diversity present in this population was lost owing to difficulties in enriching and isolating microorganisms (Daniel, 2004).

Soil is fundamental and irreplaceable; it governs plant productivity of terrestrial ecosystems and it maintains biogeochemical cycles. The living population inhabiting soil includes macrofauna, mesofauna, microfauna and microflora. 80–90% of the processes in soil are reactions mediated by microbes (Nannipieri et al., 2003).

Heavy metals and metalloids concentration in soil show influence on microbial diversity, composition and activity of soil microbes that maintain the soil ecosystem. These metals are required in very minute concentration for their metabolism and provide mineral nutrition to plants. If the concentration increases it becomes toxic and reduces microbial diversity and altered biochemical processes that underlie ecosystem function. Microbial population can adapt to metal pollution, but reduces the microbial diversity and changes the biochemical function and reduces soil resilience (Crowley, 2008). Microorganisms have ability to reduce heavy metals and metalloids to their lower redox state. They use these metals as terminal electron acceptor. When microbes alter the redox state of contaminants, they bind with heavy metals and make them more soluble. This can aid in leaching of this contaminants from soil (Lovley & Coates, 1997).

2.3 Bacteria and fungi help in Bioremediation

Microorganisms and plants are usually used for the removal of heavy metals. Mechanisms by which microorganisms act on heavy metals include biosorption (metal sorption to cell surface by physiochemical mechanisms), bioleaching (heavy metal mobilization through the excretion of organic acids or methylation reactions), biomineralization (heavy metal immobilization through the formation of

insoluble sulfides or polymeric complexes) intracellular accumulation, and enzyme-catalyzed transformation (redox reactions)(Vargas-Garcaa et al.).

Facultative anaerobic Gram-negative rods (Enterobacteriaceae), Gram-negative aerobic rods (Pseudomonadaceae and Moraxellaceae), and a Gram-positive rod (Corynebacteriaceae) are arsenic resistant bacteria. The dominant As-resistant members included Acinetobacter, Edwardsiella, Leclercia, Pseudomonas, Salmonella and Serratia species. The A. radioresistens, A. baumannii, P. chlororaphis and P. syringae strains are resistant to high concentrations of both As(V) and As(III) (Turpeinen et al., 2004).

Microbial U^{6+} reduction can effectively remove from contaminated site. Citrobacter sp. has ability to precipitate uranium. Geobacter metaflireducens and Shewanella putrefaciens are capable of reducing highly soluble Tc^{7+} (Technetium) to less-soluble, reduced forms of technetium. Thauera selenatis removed over 98% of the selenium in irrigation drainage water while simultaneously removing nitrate via denitrification. Modified bacterial mercury reductase gene inserted gene in Arabidopsis thaliana plant, this plant can grow in mercury contaminated site. Geospirillum anenophihs (formerly strain MIT-13), Geospirillum barnseii (formerly strain SES-3) and Chysiogenes arsenatis bactrerial strains reduces Arsenic (Lovley & Coates, 1997).

Enzymic reduction of plutonium Pu(IV) to the more soluble Pu(III) under anaerobic conditions was demonstrated for Geobacter metallireducens GS-15 and Shewanella oneidensis MR-1. Methylation of Hg, Sn and Pb, and the metalloids As, Se and Te can be mediated by a range of microbes, including clostridia, methanogens and sulfate-reducing bacteria under anaerobic conditions, and principally fungi under aerobic conditions, such as Penicillium and Alternaria spp., as well as a variety of bacteria, including Pseudomonads. There is also evidence for methylation of Sb by diatoms. Methyl groups are enzymatically transferred to the metal, and a given species may transform a number of different metal(loid)s. Methylated metal compounds formed by these processes differ in their solubility, volatility and toxicity. Volatile methylated species are often lost from the soil.

Sulfur- and sulfate-reducing bacteria are particularly important in reductive precipitation of U (VI), Cr (VI), Tc (VII) and Pd (II). Some sulfate-reducing bacteria such as Desulfotomaculum reducens share physiological properties of both sulfate- and metal-reducing groups of bacteria, and can use Cr (VI), Mn (IV), Fe (III) and U (IV) as sole electron acceptors. Some bacteria like Acidithiobacillus ferrooxidans, Leptospirillum ferrooxidans, Sulfolobus spp., Acidianus brierleyi and Sulfobacillus thermosulfidooxidans can oxidize ferrous iron enzymatically with the generation of energy. Ectomycorrhizal fungi (Suillus granulatus and Paxillus involutus) can release elements from apatite and wood ash (K, Ca, Ti, Mn, Pb) and accumulate them in the mycelia. Ericoid mycorrhizal and ectomycorrhizal fungi can dissolve a variety of cadmium, copper, zinc and lead-bearing minerals, including metal phosphates. Acinetobacter johnsonii was effective in removing lanthanum from solution. For ericaceous mycorrhizas, clear host protection has been observed, e.g in. Calluna, Erica and Vaccinium spp. growing on Cu- and Zn-polluted or naturally metalliferous soils, the fungus preventing metal translocation to plant shoots (Gadd, 2010).

Leptospirillum ferrooxidans acidophile resistant to copper and can grow at concentration 5mM Cu (II). Acidiphilium symbioticum KM2 is Zn (II) and Cd (II) resistant. Sulfolobus acidocaldarius and S. solfataricus are cadmium resistant archeal species that can grow at 10mM concentration. Acidophiles resistant to Ni (II) include Acidocella multivorum, A. aminolytica and Acidocella strain GS19. Sulfobacillus acidophilus and Acidimicrobium ferrooxidans are tolerant to Fe (III). Acidophiles must be resistant to heavy metals due to the selective pressures that metal-rich acidic environments pose (Dopson et al., 2003).

Bacterial mercury resistance has been described for many phyla, such as Firmicutes, Actinobacteria, and Proteobacteria. A number of these organisms can reduce Hg(II) to Hg(0) and/or degrade MeHg, but Hg methylation is thus far restricted to sulphate reducing bacteria (SRB) and iron reducing bacteria (IRB) of the Deltaproteobacteria (Vishnivetskaya et al., 2011).

Heavy metal contaminated soil was analyzed and found it has three sub division of Proteobacteria: the Cytophaga-Flavobacterium division, the gram-

positive high G+C division, and the gram-positive low G+C division. Genus Sphingomonas increase in community if site is contaminated by pollutant pentachlorophenol. Burkholderia- like organisms increases in chlorinated benzoate infected area. Phenyl urea herbicides decrease the number of culturable heterotrophic bacteria and decline the number of unculturable Acidobacterium (Kent & Triplett, 2002).

Table 2.1:- Heavy metal and metalloid reducing and tolerant bacterial species

Metals	Metal Reducer and Tolerant Microbes	References
Arsenic (As)	Acinetobacter, Edwardsiella, Leclercia, Pseudomonas, Salmonella, Serratia, Acinetobacter radioresistens, A. baumannii, Pseudomonas chlororaphis and P. syringae	(Turpeinen et al., 2004)
	Chysiogenes arsenates	(Lovley & Coates, 1997) (Gadd, 2010; Macy et al., 1996)
	Geospirillum anenophihs, Geospirillum barnseii	(Gadd, 2010)
	Pseudomas stutzeri	(Lalucat et al., 2006)
Uranium (U)	Citrobacter,	(Lovley & Coates, 1997)
	Desulfotomaculum reducens	(Gadd, 2010)
	Pseudomas stutzeri	(Lalucat et al., 2006) (Geibler et al., 2004)
	Thiobacillus denitrificans	(Beller et al., 2006)
	Geobacter metallireducens	(Beller et al., 2006; Lovley et al., 1993; Ortiz-Bernad et al., 2004)
	Pseudoma aeruginosa	(Strandberg et al., 1981)
Copper (Cu)	Pseudomas stutzeri	(Lalucat et al., 2006)
	Thiobacillus denitrificans	(Beller, 2005)
	Pseudomonas syringae, Escherichia coli	(Kamal et al., 2010)
	Pseudoma aeruginosa	(Mullen et al., 1989)
	Leptospirillum ferrooxidans	(Dopson et al., 2003)
Chromium (Cr)	Pseudomas stutzeri	(Lalucat et al., 2006)
	Thiobacillus denitrificans	(Beller, 2005)
	Acinetobacter haemolyticus	(Pei et al., 2009)
	Desulfotomaculum reducens	(Gadd, 2010)
Manganese (Mn)	Desulfotomaculum reducens	(Gadd, 2010)
	Pseudomas stutzeri	(Lalucat et al., 2006)
	Geobacter metallireducens	(Lovley et al., 1993; Ortiz-Bernad et al., 2004) (Beller et al., 2006)
Mercury (Hg)	Pseudomonas spp.	(Gadd, 2010)
	Phylum Firmicutes, Actinobacteria, Proteobacteria	(Vishnivetskaya et al., 2011)
	Thiobacillus denitrificans	(Beller, 2005)
Nickel (Ni)	Pseudomas stutzeri	(Lalucat et al., 2006)
	Acidophile multivorum, Acidocella aminolytica, Acidocella	(Dopson et al., 2003)

	strain GS19,	
	Alcaligenes xylosoxidans	(Kamal et al., 2010)
	Thiobacillus denitrificans	(Beller, 2005)
Iron (Fe)	Thiobacillus denitrificans	(Beller, 2005)
	Desulfotomaculum reducens, Acidithiobacillus ferrooxidans, Leptospirillum ferrooxidans, Sulfolobus spp., Acidianus brierleyi, Sulfobacillus thermosulfidooxidans	(Gadd, 2010)
	Sulfobacillus acidophilus, Acidimicrobium ferrooxidans	(Dopson et al., 2003)
Cadmium (Cd)	Ralstonia eutropha, Alcaligenes xylosoxidans, Staphylococcus, Bacillus	(Kamal et al., 2010)
	Acidiphilium symbioticum, Sulfolobus acidocaldarius, S. solfataricus	(Dopson et al., 2003)
Cobalt (Co)	Ralstonia eutropha, Alcaligenes xylosoxidans,	(Kamal et al., 2010)
	Pseudomas stutzeri	(Lalucat et al., 2006)
Zinv (Zn)	Pseudomas stutzeri	(Lalucat et al., 2006)
	Acidiphilium symbioticum	(Dopso et al., 2003)
Lead (Pb)	Thiobacillus denitrificans	(Beller, 2005)
	Pseudomas stutzeri	(Lalucat et al., 2006)
Selenium (Se)	Thauera selenatis	(Lovley & Coates, 1997)
	Azospira oryzae	(Hunter, 2007)
	Pseudomas stutzeri	(Lalucat et al., 2006)
Aluminum (Al)	Pseudomas stutzeri	(Lalucat et al., 2006)
Silver (Ag)	Pseudomas stutzeri	(Lalucat et al., 2006)
	Thiobacillus denitrificans	(Beller, 2005)
Technetium (Tc)	Geobacter metaflireducens, Shewanella putrefaciens	(Lovley & Coates, 1997)
Plutonium (Pu)	Geobacter metaflireducens, Shewanella putrefaciens	(Gadd, 2010)
Vanadium (V)	Geobacter metallireducens	(Ortiz-Bernad et al., 2004)
Lanthanum (La)	Acinetobacter johnsonii	(Gadd, 2010)

- **Heavy metal resistance system in bacteria**

Bacteria have developed several efficient systems for detoxifying metals. These mechanisms can be grouped into five categories: **(A)** intracellular sequestration; **(B)** export; **(C)** reduced permeability; **(D)** extracellular sequestration, and; **(E)** extracellular detoxification. Almost all known bacterial resistance mechanisms are encoded on plasmids and transposons, and it is probably by gene transfer or spontaneous mutation that bacteria acquire their resistance to heavy metals (Dopson et al., 2003; Kamal et al., 2010).

In Gram-negative bacteria (e.g., Ralstonia eutropha), the czc system is responsible for resistance to Cd, Zn, and Co. The czc genes encode for a cation–proton antiporter (CzcABC) that exports Cd, Zn, and Co. A similar mechanism,

called the ncc system, has been found in Alcaligenes xylosoxidans, which is resistant to Ni, Cd, and Co. In contrast, the Cd resistance mechanism in Gram-positive bacteria (e.g., Staphylococcus, Bacillus or Listeria) is a Cd-efflux ATPase. The two most well-studied Cu resistance systems are cop from Pseudomonas syringae and pco from Escherichia coli. The cop genes encode for different Cu-binding proteins that allow the sequestration of Cu in the periplasm or in the outer membrane. In contrast, the pco system is expected to be an ion-dependent Cu antiporter (Kamal et al., 2010).

There are two basic strategies for a microbe to function in metal-contaminated environment. One, a system of transmembrane metal pumps has evolved in a number of bacteria, for example, system encoded by sil and mer operons, conferring resistance to silver and mercury, respectively. Those pumps scavenge metals on the inside of the cell membrane and remove them from the cell, thus protecting the internal cell structures from toxic metal effects. As denitrification related enzymes are generally located within the cell membrane or periplasmic space, expelling heavy metal ions out of the cell would place them in the immediate contact with denitrification related enzymes, thus limiting utility of such a resistance strategy. The second mechanism of microbial resistance to metals is evolution of enzyme forms resistant to metals. This resistance pathway is expected to be the predominant in the denitrifying bacteria. The metal resistant forms of enzymes present in metal-stressed denitrifying community are expected to be readily identifiable by their gene sequence and therefore their genetic signature. Disruption of denitrification by heavy metals could lead to a number of undesirable consequences, influencing the human health at both global and local levels (Sobolev & Begonia, 2008).

Bacterial resistance properties can be used for different purposes: in the case of mercury pollution, the insertion of the microbial mercury reductase into a transgenic plant improved significantly the phytoextraction process. Another example was the inoculation of heavy metal resistant bacteria into a contaminated

soil, which seemed to protect the indigenous, sensitive, ammonia-oxidizing bacteria from metal toxicity (Kamal et al., 2010).

2.4 Heavy metal toxicity to Humans

Since the industrial revolution, metals have increasingly been redistributed in the environment, with accumulation in terrestrial and aquatic habitats being associated with adverse effects on the biota and human health. Thirteen trace metals and metalloids (Ag, As, Be, Cd, Cr, Cu, Hg, Ni, Pb, Sb, Se, Tl, Zn) are considered priority pollutants (Gadd, 2010). Most of heavy metals cause toxicity to human health; these have ability to cause mutation and potential of carcinogenicity (Prozialeck et al., 2006). Heavy metals and mettaloids effect on human physiology shown in table 2.2. Toxicity of metallic ions could be the result of competition with or replacing a functional metal as well as causing conformational modification, denaturation and inactivation of enzymes and disruption of cellular and organelles integrity (Anyanwu et al., 2011). These heavy metals and metalloids damage the DNA and disrupt the synthesis of nucleic acid and proteins.

Table 2.2:- Heavy metals and metalloids with their effects on human physiology

Metal	Effect on human	References
Cadmium	Inhibitor of DNA mismatch repair system in animals	(Wang & Crowley, 2005)
	Nephrotoxicity	(Goyer, 1995; Sobolev & Begonia, 2008)
	Hypertension	(Duruibe et al., 2007; Prozialeck et al., 2006)
Lead	Effect on nervous, renal, endocrine, skeletal system, seizures, mental retardation and behavioral disorders	(Goyer, 1995; Sobolev & Begonia, 2008)
	Central nervous system, neurological function, Cardiovascular system, bone, nephrotoxicity, Carcinogenicity reproductive effect	(Goyer, 1993)
	Hypertension	(Duruibe et al., 2007; Prozialeck et al., 2006)
Zinc	Kidney and Liver failure	(Duruibe et al., 2007; Prozialeck et al., 2006)
	Sideroblastic anaemia, hypochromic microcytic, anaemia, leucopenia, lymphadenopathy, neutropenia, hypocupraemia, hypoferraemia	(Nriagu, 2010)
Arsenic	Causes acute and chronic dose dependent effects in organs	(Duruibe et al., 2007; Prozialeck et al., 2006)
	Cancer, gastrointestinal distress, Dermal effects in humans, altered pigmentation, hyperkeratosis and skin cancer, reduce angiogenesis	(Hughes et al., 2011)
Mercury	Central Nervous system	(Goyer, 1995; Sobolev & Begonia, 2008)

Uranium	Brain, Kidney, Bones of mature animals	(Briner, 2010)
	Renal toxicity, cardiovascular system, muscle, liver, nervous system, cancer	(Taylor & Taylor, 1997)
	Nephrotoxicity, genotoxicity, developmental defects	(Brugge & Buchner, 2011)
Iron	Atherosclerosis, Alzheimer's disease	(Brewer, 2007)
Copper	Atherosclerosis, Alzheimer's disease	(Brewer, 2007)
Cobalt	Goiter, reduced thyroid activity, heart failure, asthma, allergy	(Barceloux & Barceloux, 1999)
Chromium	Cancer in gastrointestinal and central nervous system	(Costa & Klein, 2006)
Manganese	Central nervous system, lung, cardiac, liver, reproductive, fetal toxicity	(Crossgrove & Zheng, 2004)
Selenium	Hyperchromic anaemia, leucopenia, Gastrointestinal disturbance, hair and nail change, Neurological manifestation	(Tinggi, 2003)
	Heart disease and cancer	(Longnecker et al., 1991)
Nickel	Haematotoxic, immunotoxic, neurotoxic, genotoxic, reproductive toxic, pulmonary toxic, nephrotoxic , hepatotoxic and carcinogenic	(Das et al., 2010)
Aluminium	Neurotoxic, neurodegenerative	(Goyer, 1993)
	Bone, kidney, hematopoietic tissue	(Jeffery et al., 1996)
Silver	Skin, gastrointestinal tract, Central nervous system, development, reproduction, respiration	(Faust, 1992)
Vanadium	Respiration, Central nervous system, kidney, digestive tract, skin	(Venkataraman & Sudha, 2005)

2.5 Approach to study bacterial diversity

The number of microbial species present on earth may be in millions. But only 1% of species are culturable under laboratory conditions (Kirk et al., 2004). Under laboratory condition most of bacteria are unable to grow because many unknown factors required for microbial growth and many bacteria can grow only with community. When they remain together they can grow but in isolated conditions, they die because they show symbiosis. 99% bacteria in an ecosystem are unculturable. So cultivation- independent methods are beneficial to study the microbial diversity.

Molecular studies of both cultured and uncultured microbial assemblages will improve our understanding of the role of microbes in geochemical cycles and of the mechanisms of adaptation of ecologically and medically important groups of bacteria (Mes, 2008).

Traditionally, methods to analyze soil microorganisms have been based on cultivation and isolation; a wide variety of culture media has therefore been designed to maximize the recovery of diverse microbial groups. Cultivation based methods are limited in that only a small fraction of the microbial cells in soil are accessible to study, although a recent study claimed that this percentage can be raised substantially by using special cultivation techniques (Garbeva et al., 2004).

2.5.1 PCR based approaches

PCR targeting the 16S rDNA has been used extensively to study prokaryote diversity and allows identification of prokaryotes as well as the prediction of phylogenetic relationships. 18S rDNA and internal transcribed spacer (ITS) regions are increasingly used to study fungal communities. A number of community "fingerprint" methods are commonly used to assess differences in community composition between samples or treatments or to assess changes in microbial communities over time. Such techniques as clone libraries, ribosomal intergenic spacer analysis (RISA), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single-strand-conformation polymorphism (SSCP), ITS-restriction fragment length polymorphism (ITS-RFLP), random amplified polymorphic DNA (RAPD), or amplified ribosomal DNA restriction analysis (ARDRA) yield complex community profiles that do not directly offer phylogenetic information but do allow analysis and comparisons of community composition (Arias et al., 2005; Garbeva et al., 2004; Hill et al., 2000; Kent & Triplett, 2002; Kirk et al., 2004). Different methods used to study microbial diversity from environmental samples showed in table 2.3.

(i) Cloning Libraries

Clone libraries are useful to identify and characterize the dominant bacterial or fungal types in soil and thereby provide a picture of diversity. A clone library generated by amplified 16S rRNA genes from soil DNA and inserted in suitable vector and cloned. Later this can be sequenced aligned by neighbor joining method for phylogenetic analysis by bootstrap analysis with 500 iterations to know

about the robustness of the phylogeny. The sequences analysis is done by different software (Clustal W, BioEdit package, MEGA) (Ellis et al., 2003; Garbeva et al., 2004; Giloteaux et al., 2011; Rastogi et al., 2010; Shivaji et al., 2004; Van Elsas & Boersma, 2011; Wang et al., 2011).

(ii) Denaturing gradient gel electrophoresis (DGGE)/ Temperature gradient gel electrophoresis (TGGE)

PCR-DGGE is probably the most widely used among the methods to study microbial communities in environmental samples. The DGGE profiles of microbial communities in soil and rhizosphere are often very complex when analyzed with universal (bacterial) primers, and as a result, less abundant organisms may escape detection (Garbeva et al., 2004).

DNA is extracted from soil samples and amplified using PCR with universal primers targeting part of the 16S or 18S rRNA sequences. The 5' -end of the forward primer contains a 35–40 base pair GC clamp to ensure that at least part of the DNA remains double stranded. Theoretically, DGGE can separate DNA with one base-pair difference. TGGE uses the same principle as DGGE except the gradient is temperature rather than chemical denaturants. The partial community level fingerprints derived from DGGE/TGGE banding patterns have been analyzed for diversity studies based on the number and intensity of the DNA bands as well as similarity between treatments. DGGE/TGGE has been used to assess the diversity of bacteria and fungi in the rhizosphere (Dong & Reddy, 2010; Holben et al., 2004; Kirk et al., 2004; Loisel et al., 2006; Nannipieri et al., 2003; Sobolev & Begonia, 2008; Van Elsas & Boersma, 2011).

(iii) Single strand conformation polymorphism (SSCP)

Another technique that relies on electrophoretic separation based on differences in DNA sequences is single strand conformation polymorphism (SSCP). Single-stranded DNA is separated on a polyacrylamide gel based on differences in mobility caused by their folded secondary structure. When DNA fragments are of equal size and no denaturant is present, folding and hence

mobility will be dependent on the DNA sequences. Also, some single-stranded DNA can form more than one stable conformation. Therefore, one sequence may be represented by more than one band on the gel. However, it does not require a GC clamp or the construction of gradient gels and has been used to study bacterial or fungal community diversity (Kirk et al., 2004; Loisel et al., 2006; Schwieger & Tebbe, 1998; Stach et al., 2001).

(iv) Ribosomal intergenic spacer analysis (RISA)/Automated ribosomal intergenic spacer analysis (ARISA)

In RISA and ARISA, the intergenic spacer (IGS) region between the 16S and 23S ribosomal subunits is amplified by PCR, denatured and separated on a polyacrlyamide gel under denaturing conditions. This region may encode tRNAs and is useful for differentiating between bacterial strains and closely related species because of heterogeneity of the IGS length and sequence. In RISA, the sequence polymorphisms are detected using silver stain while in ARISA the forward primer is fluorescently labeled and is automatically detected. ARISA increases the sensitivity and reduces time. RISA has been used to compare microbial diversity in soil, in the rhizosphere of plants and in contaminated soil (Garbeva et al., 2004; Kent & Triplett, 2002; Kirk et al., 2004; Martin-Laurent et al., 2001; Nannipieri et al., 2003; Van Elsas & Boersma, 2011).

(v) Restriction fragment length polymorphism (RFLP)/Amplified ribosomal DNA restriction analysis (ARDRA)

The method is based on DNA polymorphism and involves digestion of genomic DNA/ PCR amplified 16S rRNA gene sequences using restriction enzymes (usually a four-cutter). Fragments are then separated on agarose or polyacrylamide to generate a profile of microbial community. This method has been used most frequently to screen clones or to measure bacterial community structure. Although this method cannot be used for quantifying diversity or following specific ribotypes but it has found its application for detecting structural changes in the microbial community. A single species can give many restriction fragments thus sometimes complicating study in complex communities when

analyzed by RFLP (Garbeva et al., 2004; Kent & Triplett, 2002; Kirk et al., 2004; Martin-Laurent et al., 2001; Torsvik et al., 1998).

Table 2.3:- Different method used to study microbial diversity

Method	References
Cloning library	(Ellis et al., 2003; Garbeva et al., 2004; Giloteaux et al., 2011; Rastogi et al., 2010; Shivaji et al., 2004; Van Elsas & Boersma, 2011; Wang et al., 2011)
Denaturing gradient gel electrophoresis (DGGE)/ Temperature gradient gel electrophoresis (TGGE)	(Dong & Reddy, 2010; Garbeva et al., 2004; Holben et al., 2004; Kirk et al., 2004; Loisel et al., 2006; Nannipieri et al., 2003; Sobolev & Begonia, 2008; Van Elsas & Boersma, 2011)
Single strand conformation polymorphism (SSCP)	(Kirk et al., 2004; Loisel et al., 2006; Schwieger & Tebbe, 1998; Stach et al., 2001)
Ribosomal intergenic spacer analysis (RISA)/Automated ribosomal intergenic spacer analysis (ARISA)	(Garbeva et al., 2004; Kent & Triplett, 2002; Kirk et al., 2004; Martin-Laurent et al., 2001; Nannipieri et al., 2003; Van Elsas & Boersma, 2011)
Restriction fragment length polymorphism (RFLP)/Amplified ribosomal DNA restriction analysis (ARDRA)	(Garbeva et al., 2004; Kent & Triplett, 2002; Kirk et al., 2004; Martin-Laurent et al., 2001; Torsvik et al., 1998)

Chapter III
Materials and Methods

3.1 Soil Samples

To study microflora of this region, soil samples were collected in triplicate around Bathinda region, suspected to have heavy metal contamination. Sample one: Abandoned land (City campus of Central University of Punjab, Bathinda which was free from human activity from last 15 years), sample two: Agricultural land (agricultural fields outside the city near Goniana, Bathinda) and sample three: Thermal power plant's (TPP) ash soil (collected from very near to TPP backside and outside of its boundary). Soil (10 cm diameter and 20 cm depth) in August 2011 were collected from all three places shown in map. Distance between triplicate sample collections was about 100 m.

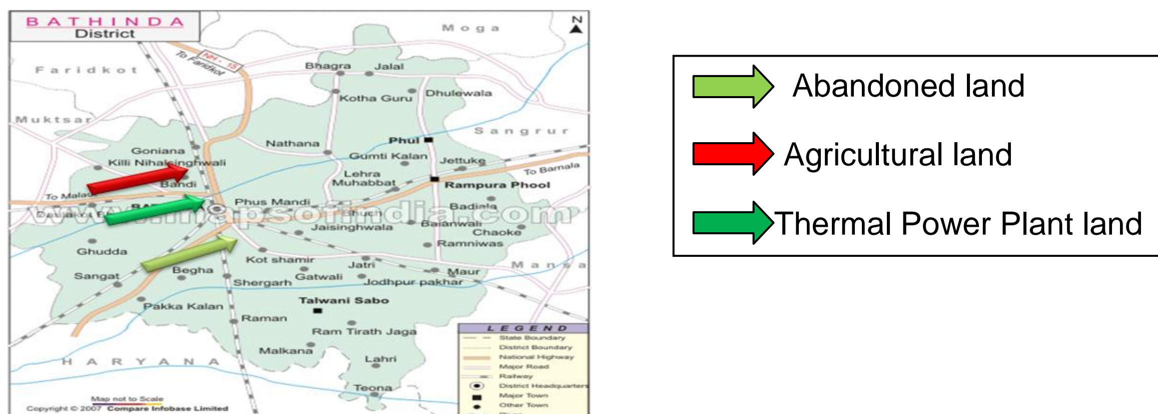

Fig. 3.1: Map of Bathinda showing sites of sample collection

3.2 Physiochemical analysis of soil

Physical and heavy metal analysis of soil samples were done at Department of Soil Sciences, Punjab Agricultural University, Ludhiana. For heavy metal analysis soil samples were digested with aqua-regia (Radojevic & Bashkin, 2006). The samples were air dried ground and sieved. Triplicate sample mixed in equal amounts for processing. Processed 1 gm sample placed inside kjeldahl flask. 15 ml aqua regia (3:1; conc. HCl: conc. HNO_3) was added, swirl to wet sample and allowed to stand overnight. The next day, flasks were placed in the heating block and heat at 50° C for 30 minutes and gradually raised the temperature to 120° C for continues heating for 2 hour. Samples were cooled at room temperature and

10 ml of 0.25 M HNO_3 was added. Digested sample were filtered through filter paper. Flasks were washed with small aliquots of 0.25 M HNO_3. The filtrates were transferred to a 50 ml volumetric flask and volume was made up with 0.25 M HNO_3. The extracted samples were analysis on ICAP-AES.

3.3 DNA extraction from soil samples

Soil microbial DNA was extracted from three different sieved mixed soils by CTAB method with minor modifications (Ellis et al., 2003). The quality of DNA was checked on 0.8% agarose gel and the quantity of DNA was checked with spectrophotometer. Isolated DNA stored at -20°C.

3.4 Amplification of 16S rRNA gene

16S rRNA gene of prokaryotes was amplified by universal primers 27F and 1492R (Table 3.1). The PCR mix consisted of deoxynucleoside triphosphates at 2.5 mM each 1 µl, primer 2 µl (10 ng/ µl) each (27F and 1492R), 1µl 10X PCR buffer including $MgCl_2$,, and 0.2 µl 5U/ µl of DNA Taq polymerase (Applied Biosystem, USA) and 1.8 µl MilliQ water in a total volume of 10 µl. Approximately 2 µl of target DNA (25 ng/ µl) was added to each reaction. The following PCR conditions were used for 16S rRNA genes: 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 48°C for 2 min. and 72°C for 1.30 min.; and a final extension at 72°C for 8 min.

Table 3.1:- Primers of 16S rRNA

Primer name	Sequence (5' ⟶ 3')	References
27 F	AGAGTTTGATCMTGGCTCAG	(Lane, 1991)
515 F	GTGCCAGCMGCCGCGGTAA	(Turner et al., 1999)
895 F	CRCCTGGGGAGTRCRG	(Hodkinson & Lutzoni, 2009)
519 R	GWATTACCGCGGCKGCTG	(Turner et al., 1999)
907 R	CCGTCAATTCMTTTRAGTTT	(Lane, 1991)
1492 R	GGTTACCTTGTTACGACTT	(Turner et al., 1999)

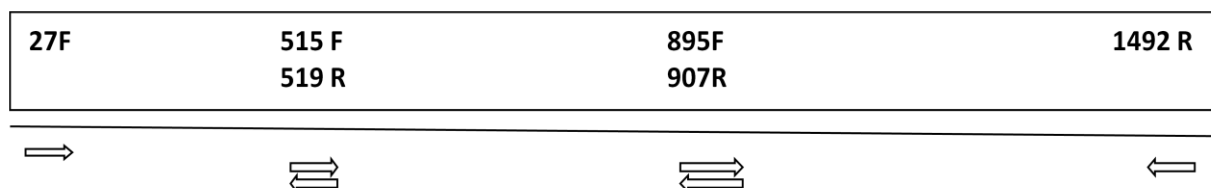

Fig 3.2 : Primers of 16S rRNA gene with its internal fragments

3.5 Ligation and Cloning

Amplified PCR product was used for ligation. PCR product ligated into TOPO TA 2.1 vector (Invitrogen, USA) and transformation in competent cells E. coli (strain TOP10, Invitrogen, USA). Cloning was performed as per manufacturer's instructions. Blue white screening used to isolate fragment inserted colonies.

3.6 Colony PCR

After blue white screening, white colonies were sub-cultured on Petri plates containing ampicillin (100mg/ml). Colony PCR performed to check inserted fragment in vector. The PCR mix consisted of deoxynucleoside triphosphates at 2.5 mM each 1 µl, 10 ng/ µl each primer 2 µl (M13F and M13R), 1µl 10X PCR buffer including $MgCl_2$,, and 0.2 µl 5U/ µl of DNA Taq polymerase (Applied Biosystem, USA) and 2.3 MilliQ water in a total volume of 10 µl. Approximately 1 µl of colony suspension (loopful colony dissolved in 10 µl MilliQ water) was added to each reaction. The following PCR conditions were used for 16S rRNA genes: 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec., 55°C for 45 sec. and 72°C for 1.30 min; and a final extension at 72°C for 8 min. Positive clones grew in LB broth 5ml overnight in shaker incubator at 37°C.

3.7 Plasmid Isolation

Positive culture grown at 37°C overnight and plasmid isolated using manual method (Sambrook & Russell, 2001). Isolated plasmid DNA stored at -20°C.

3.8 Sequencing and Sequence Analysis

Sequencing was performed in 10 µl of sequencing reaction consisted of Ready reaction mix 1 µl, 2 µl 5X buffer (Applied Biosystem, USA), 2 µl universal primer

M13F, 515F and 895F (10ng/ µl), Plasmid DNA 2 µl and 3 µl sterile water. The PCR conditions are 96°C for 1 min. followed by 35 cycles of 96°C for 1 min., 52°C (for M13F) 55°C (for 515F) 50°C (for 895F) for 40 sec. and 60°C for 4 min. For cleaning of sequencing reaction Bigdye X terminator kit (Applied Biosystem, USA) used according to manufacturer's manual. Sequencing was performed using ABI 3730 xl DNA Analyzer (Applied Biosystem, USA). Post processing of the sequence data was done using DNA STAR (Lasergene ver. 10 SeqMan) to compile three fragments of gene 16S rRNA. Genome walking performed to align all three fragments (M13F, 515F and 895F) of 16S rRNA gene shown in fig. 3.3. MEGA 5 software (Neighbor joining method) used to align sequences and to predict phylogenetic tree of bacteria of all soil samples. Sequences were analyzed on BLASTN [NCBI].

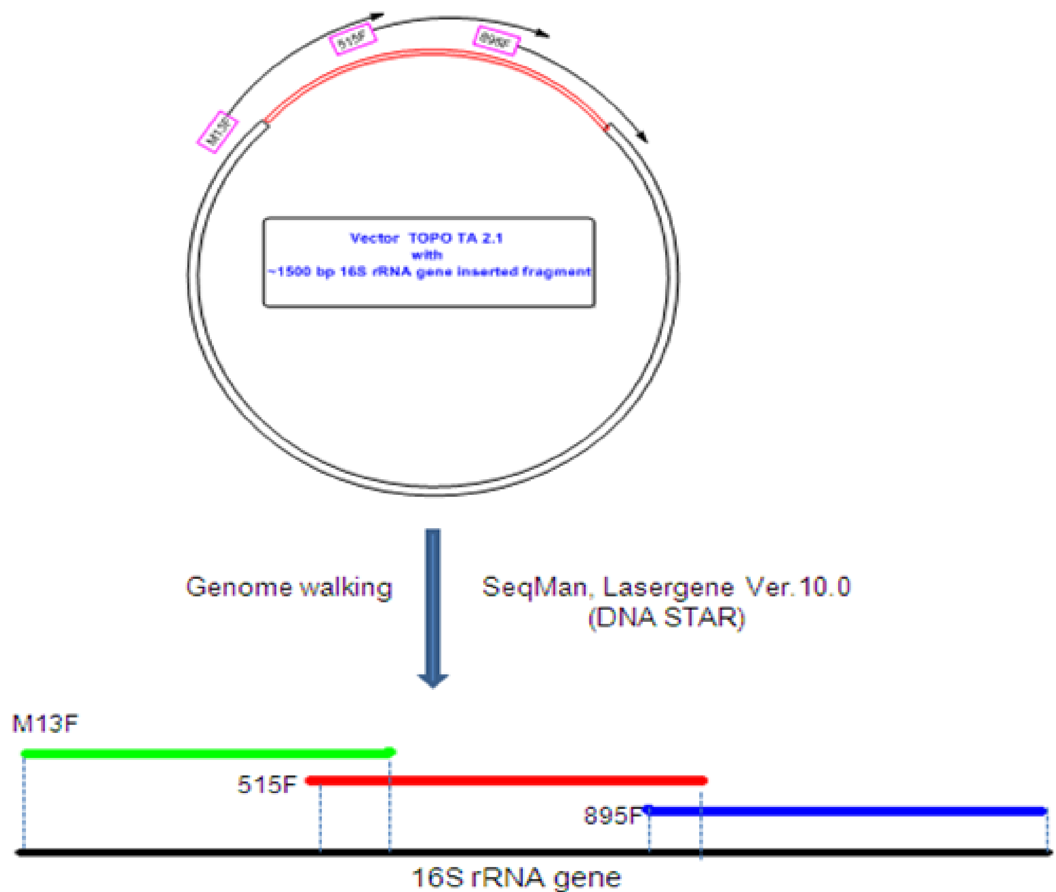

Fig 3.3: Genome walking to align sequenced three fragments of 16S rRNA gene

Chapter IV
Results

Malwa region is highly affected by heavy metal toxicity that causes mutation and potential carcinogenicity in animals and plants. Soil is getting polluted and its consequences are reflected as health hazards like cancer, kidney failure, birth abnormalities etc., especially in south west Punjab. This environmental pollution of heavy metals cause effects on human health, agriculture crop yield and soil ecosystem. Heavy metals have effects on soil fertility by affecting microbial community.

4.1 Physiochemical and heavy metal analysis of soil samples

Physiochemical analysis and heavy metals analysis report of soil samples showed in table 4.1 and 4.2. pH of agricultural soil (AS) was less as compared to abandoned soil (US) and thermal power plant soil (TS) but in range which is 6.5-8.7. Electronic conductivity (EC) of AS sample was higher as compared to US and TS samples and also out of range (<0.8Seimens). Organic Carbon (OC) and phosphorus of AS sample was also higher than US and TS sample and also out of range. Potassium level was much higher in all three soil samples. All three soil samples texture was sandy loam.

Heavy metal analysis of all three samples digested by Aqua regia tested on ICAP-AES (Thermo, USA). Heavy metals of agricultural soil (AS) and thermal power plant soil (TS) samples compared with abandoned soil (US) sample because it abandoned land from last 15 years and away from anthropogenic activities. In agricultural soil (AS) sample arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), cupper (Cu), iron (Fe), manganese (Mn), nickel (Ni) and lead (Pb) was higher than US sample. In TS sample cadmium (Cd), cobalt (Co), chromium (Cr), cupper (Cu), iron (Fe), nickel (Ni), lead (Pb) and zinc (Zn) is higher than abandoned soil (US) sample. But in US sample calcium (Ca) was higher than thermal power plant soil (TS) and agricultural soil (AS) sample and arsenic (As), manganese (Mn) and magnesium (Mg) was higher than thermal power plant soil (TS).

Table 4.1: - Physiochemical analysis of soil samples

Sample	pH (6.5-8.7)	EC (<0.8S)	Organic Carbon (OC) (0.4 -0.75)	Phosphorus (5-9Kg/Acre)	Potassium (55Kg/Acre)	Texture
Abandoned Soil	8.7	0.180	0.540	5.3	>300	Sandy loam
Agricultural Soil	7.8	1.620	0.990	30.8	>300	Sandy loam
Thermal Power Plant Soil	8.6	0.130	0.420	3.0	>300	Sandy loam

Table 4.2:- Heavy metals analysis of soil samples

Element	Abandoned Soil (mg/kg)	Agricultural Soil (mg/kg)	Thermal Power Plant Soil (mg/kg)
Aluminum	4958	5750	5795
Arsenic	3.95	4.63*	0.20
Boron	243	207	351
Barium	149	140	298
Calcium	22253	8058	9330
Cadmium	0.925	1.35*	1.225
Cobalt	6.48	10.1*	9.0
Chromium	12.18	40.2	44.63*
Copper	7.05	17.63	22.83*
Iron	14835	21643*	19340
Potassium	3760	5157.5	2680
Magnesium	5130	6720	2652.5
Manganese	256	378*	239
Sodium	704	750	1800
Nickel	12.3	22.45*	21.98
Phosphorus	535	593	1483
Lead	9.95	11.4	12.48*
Sulphur	553	261	1924
Zinc	91	71	120*

(*Concentrations of heavy metals in Agriculture and Industrial soil samples more compared to Abandoned soil sample)

All soil samples tested on ICAP-AES from Department of Soil Sciences, Punjab Agricultural University, Ludhiana.

4.2 Genomic DNA Isolation

Microbial DNA was extracted from all three soils in triplicate by CTAB method (Ellis et al., 2003). Quantity of DNA was checked on spectrophotometer and quality of DNA was checked on 0.8% agarose gel (Fig.-4.1).

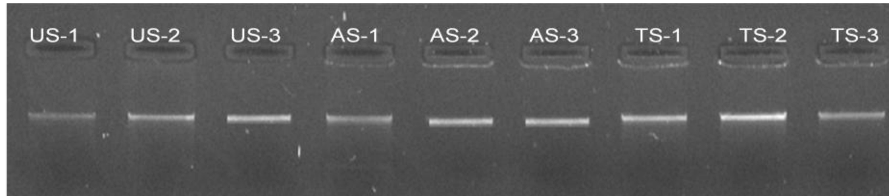

Fig 4.1: Extracted DNA of three different soils in triplicate.
US- abandoned soil, AS-agricultural soil and TS-thermal power plant soil.

4.3 Amplification of 16S rRNA gene

Full gene and internal primers of 16S rRNA gene of prokaryotes were check by PCR amplification. 16S rRNA full gene length (primer 27F-1492R) approximately 1500 bp and internal fragment (primer 27F-519R) ~500bp, (primer 515F-907R) ~400 bp and (primers 895F-1492R) ~600bp were amplified shown in fig 4.2, 4.3 and 4.4. We could able to amplify the required product in the predefined size in all three samples.

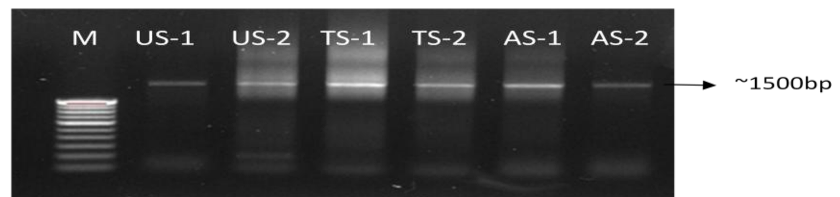

Fig 4.2:- Amplified 16S rRNA gene (~1500bp) of bacteria from all three soil samples (27F-1492R).

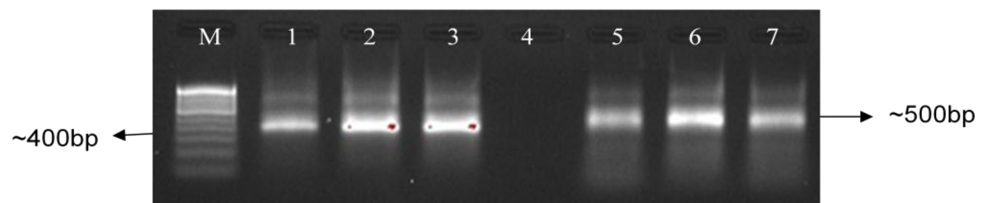

Fig 4.3:- Amplified fragments of 16S rRNA gene 515F-907R (~400bp) and 27F-519R (~500bp)

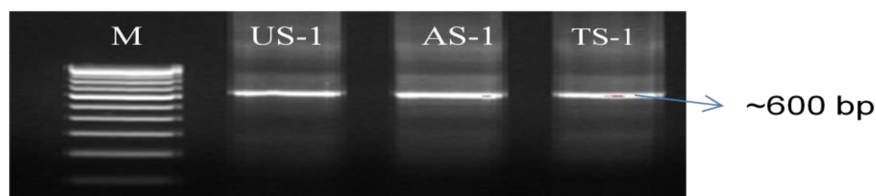

Fig 4.4:- Amplified fragments of 16S rRNA gene 895F-1492R (~600bp)

4.4 Cloning, Colony PCR and Sequencing

Amplified 16S rRNA gene (Primer 27F-1492R) fragment was ligated with vector TOPO TA 2.1(Invitrogen, USA) and transformed into competent cells E. coli strain Top 10 (Invitrogen, USA). 366 white colonies were picked from of all three soil samples and sub-cultured on LB agar (Luria Bertani agar) plates. The colony PCR with sub-cultured clones were performed with M13F and M13R primers and ~1700 bp product marked as positive clones (200 bp of vector sequence + 1500 bp of 16S rRNA gene) shown in fig. 4.5. 128 clones marked as positive and plasmid was isolated for sequencing of inserted fragment. M13F, 515F and 895F were used for genome walking to cover the whole ~1500 bp gene. The sequencing information was generated for all the 128 clones, 54 samples around 1500 bp and 72 samples around 925 bp.

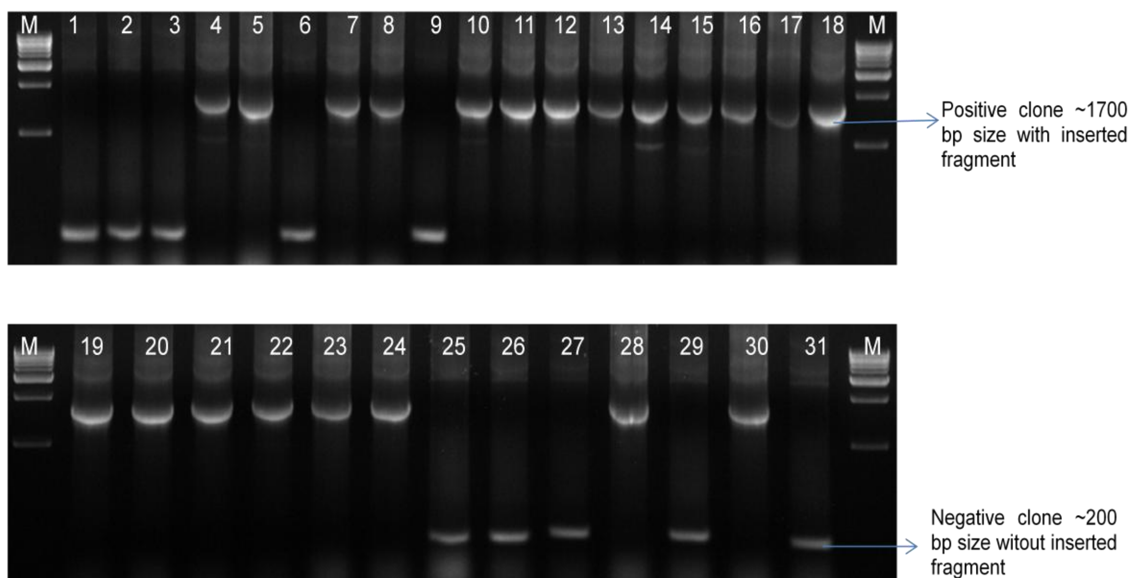

Fig 4.5: Colony PCR showing positive and negative clones amplification

All the sequenced fragments were clustered (to walk the genome) and chimeras were removed by SeqMan Pro Lasergene v10.0 (DNA STAR). BLAST analysis was carried out at NCBI to group the bacteria. All 126 clones aligned for phylogenetic analysis by MEGA 5 (Neighbor Joining method)(Tamura et al., 2011). Three different Phylogenetics tree of every soil type and one combined Phylogenetic tree were predicted showed in fig. 4.7, 4.8, 4.9, 4.10. Microbial profile of thermal power plant, abandoned and agricultural soil showed in fig. 4.6.

Microbial profile of Abandoned, Thermal Power plant and Agricultural Soil

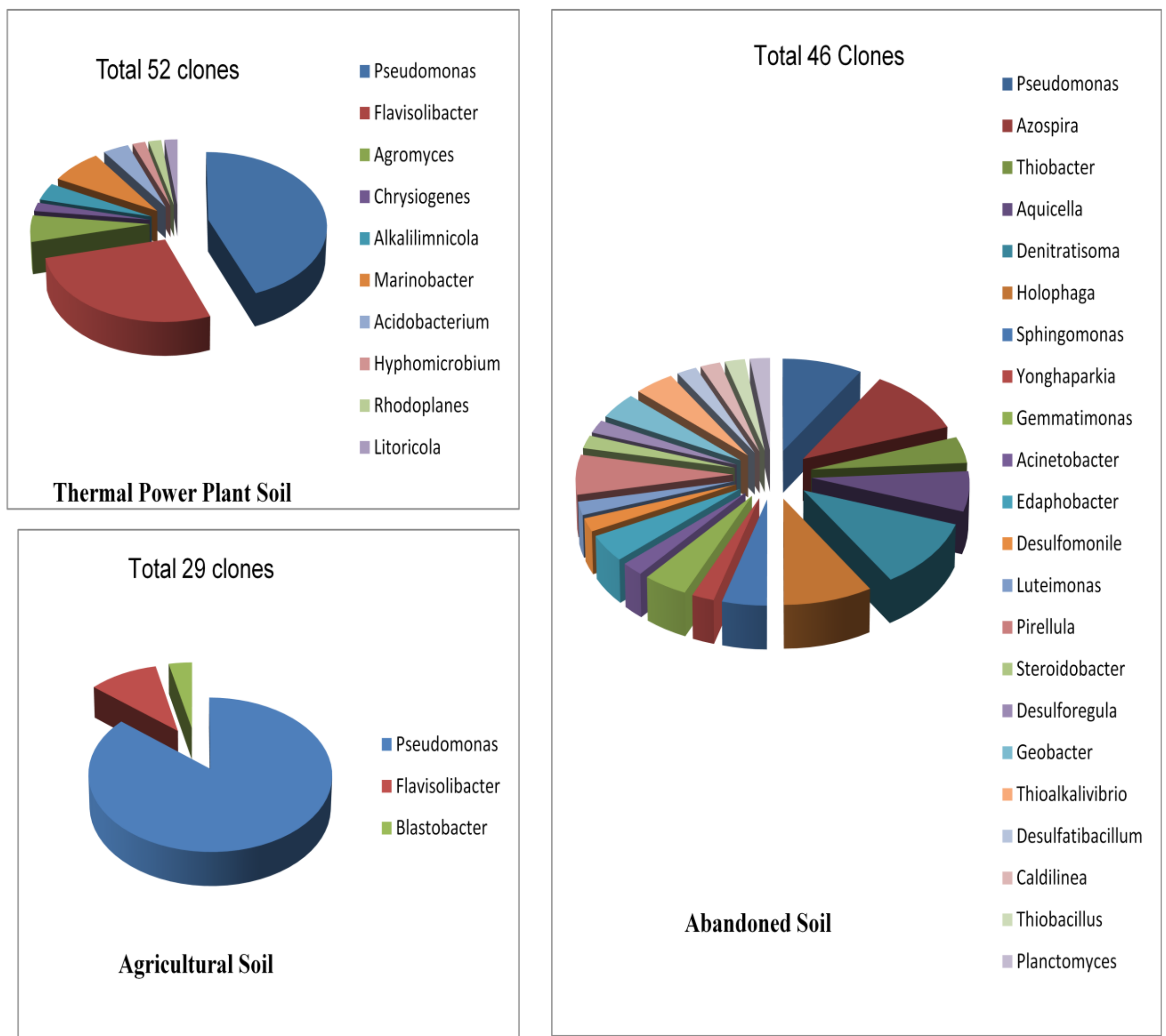

Fig 4.6: Microbial profile of Thermal Power Plant, Abandoned and Agricultural soil

4.5 BLAST and Phylogenetic analysis

BLAST analysis result was according to taxonomic group shown in table 3. All bacterial clones fell into 8 bacteria phyla, majority of clones belong to phylum Proteobacteria (21 clone species) (70.7%), 3 species with Acidobacteria (6.4%), 2 species each with Actinobacteria (3.3%) and Planctomycetes (3.3%) and 1 species each with Chrysiogenetes (0.8%), Bacteroidetes (13.6%), Chloroflexi, Gemmatimonadetes (1.7%). Proteobacteria has 4 sub-phylum Alphaproteobacteria (5.6%), Betaproteobacteria (14.7%), Gammaproteobacteria (74.2%) and Deltaproteobacteria (5.6%).

Gene clusters shows extent of similarity and diversity in a gene of different species. This study showed that 3 major clusters are Proteobacteria, Bacteriodates, Acidobacteria, 2 minor clusters are Planctomycetes, Gemmatimonadetes and three minor clusters are showed <85% similarity than Actinobacteria, Chloroflexi, Chrysiogenetes in soil samples, so these cluster may be considered as novel phyla. Proteobacteria phylum is dominant and it has 4 sub-phylum (Alpha, Beta, Gamma and Delta-proteobacteria) in soil samples followed by Bacteriodates, and Acidobacteria. In agricultural soil only 2 clusters Proteobacteria (Gamma and Alpha-proteobacteria) and Bacteriodate are present. Thermal power plant soil has 5 clusters Proteobacteria (Gamma and Alpha-proteobacteria), Bacteriodates, Acidobacteria, Actinobacteria, Chrysiogenetes and dominant Proteobacteria. Abandoned soil has 6 clusters Proteobacteria (Gamma, Beta, Delta and Alpha-proteobacteria), Acidobacteria, Chloroflexi, Planctomycetes, Gemmatimonadetes Actinobacteria and dominant Proteobacteria. This study revealed that maximum bacterial diversity present in abandoned soil and Proteobacteria is dominant in all.

Based on a similarity of >97%, indicating identical clones (Ellis et al., 2003) and similarity between clones and NCBI data <85% could not be assigned to any existing bacterial phyla and thus may represent novel phyla (Rappe & Giovannoni, 2003). 45 clones with similarity (99-100%) to Pseudomonas stutzeri and 3 each clones Pseudomonas balearica and Pseudomonas plecoglossicida. 17 clones

with similarity (92-96%) to Flavisolibacter ginsengisoli, 4 clones with similarity Marinobacter lutaoensis, 5 clones each with similarity Denitratisoma oestradiolicum (91%), Azospira restricta (90-92%), 4 clones with similarity Holophaga foetida (83-84%). 3 each clones with similarity to Pirellula staleyi (93-94%), Agromyces ulmi (76%) and Aquicella siphonis (93%). 2 each clones with similarity to Alkalilimnicola ehrlichii (93%), Acidobacterium capsulatum (84%), Geobacter metallireducens (86%), Gemmatimonas aurantiaca (88%), Thioalkalivibrio denitrificans (90%), Edaphobacter aggregans (80-86%), Sphingomonas kaistensis (95-96%) and Thiobacter subterraneus (91%). 1 each clones with similarity to Chrysiogenes arsenates (80%), Hyphomicrobium sulfonivorans (97%), Rhodoplanes serenus (97%), Litoricola lipolytica (85%), Thiobacillus denitrificans (93%), Caldilinea aerophila (81%), Desulfatibacillum alkenivorans (83%), Desulforegula conservatrix (82%), Steroidobacter denitrificans (91%), Luteimonas composti (90%), Desulfomonile limimaris (85%), Planctomyces maris (86%), Acinetobacter haemolyticus (98%), Yonghaparkia alkaliphila (79%) and Blastobacter denitrificans (98%). 18 clones showed <85% similarity to NCBI data so these clones may belong to novel phyla. identification of which is a thriving area of future research.

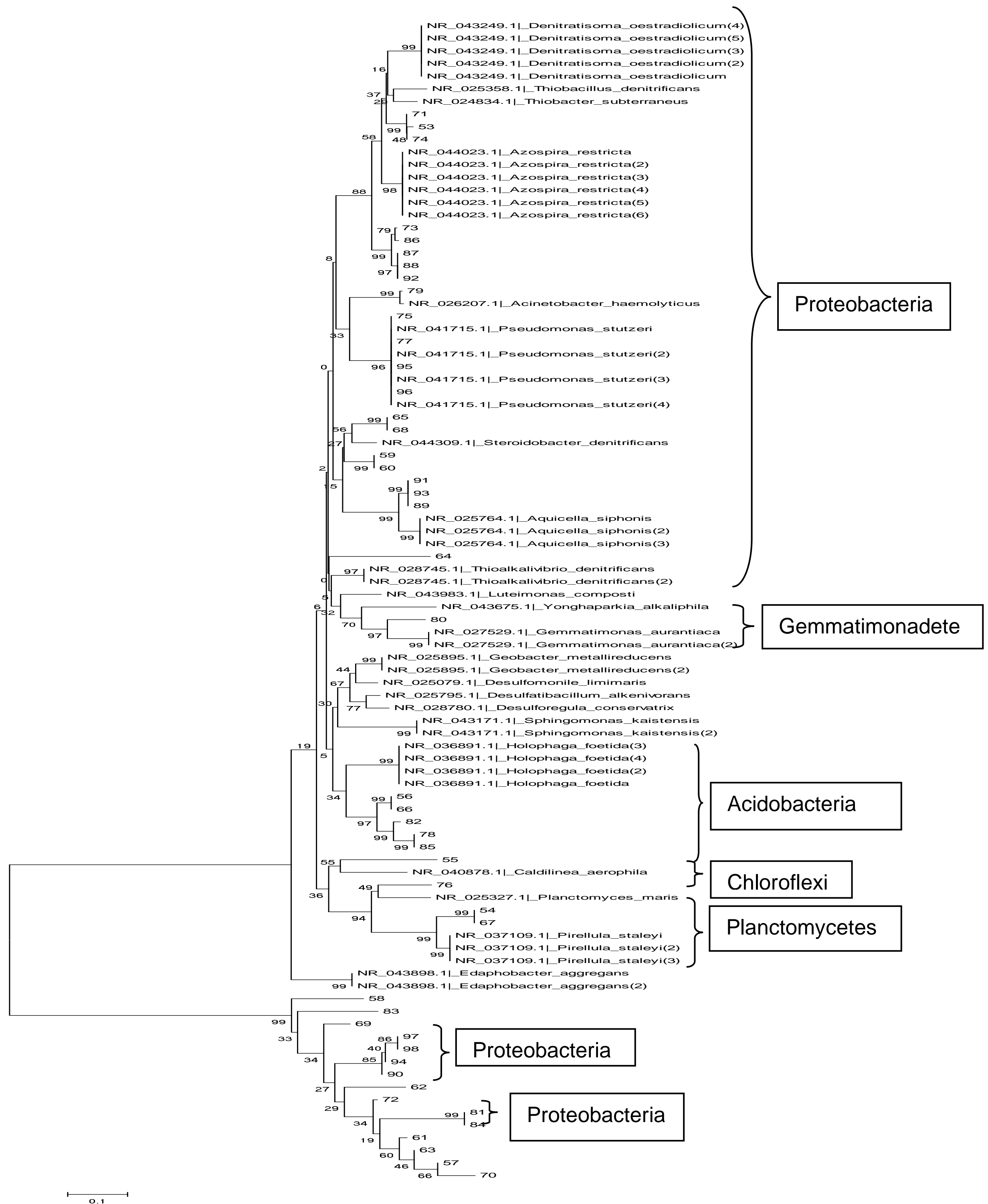

Fig.4.7: Evolutionary relationships of taxa of abandoned soil : The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 4.92584810 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 92 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 224 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

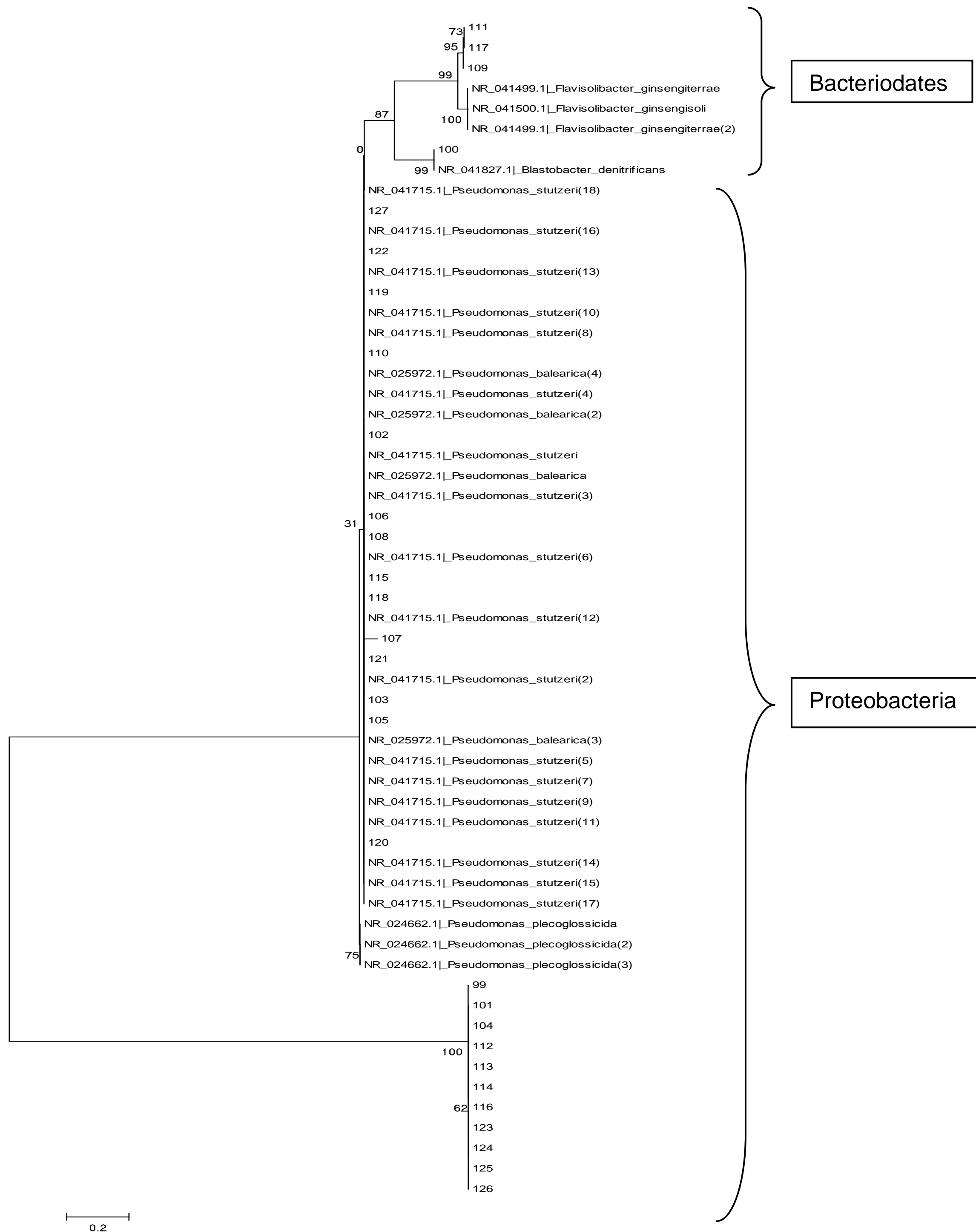

Fig. 4.8: Evolutionary relationships of taxa of agricultural soil: The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 3.13341310 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 58 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 614 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

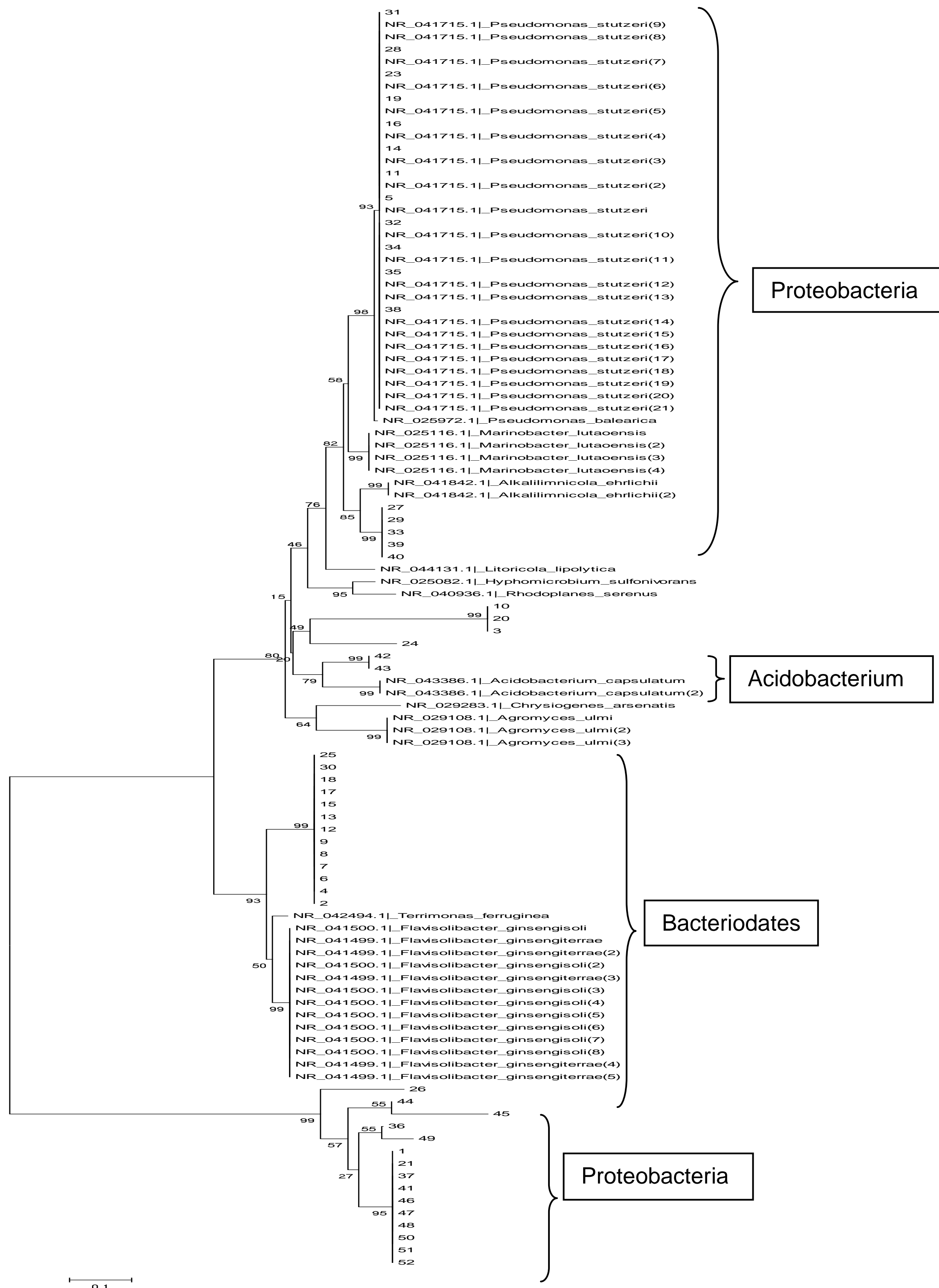

Fig. 4.9: Evolutionary relationships of taxa of thermal power plant soil: The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 3.21045858 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood metho and are in the units of the number of base substitutions per site. The analysis involved 102 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 238 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

Table 3:- Result of BLAST [NCBI] analyses of the bacterial clone sequences of all three soil samples in groups

S. No.	Clone ID	Length (bp)	% age Similarity	Closest Bacteria (Accession no)	Taxonomic affiliation of Clone	References (Related to close match)
1.	TS-1	919	100	Pseudomonas stutzeri ATCC 17588 (NR_041715)	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae ; Pseudomonas	**Unpublished.** Comparison of Phenetic and Phylogenetic Classification Systems for the Fluorescent Pseudomonads
2.	TS-5	1495	100			
3.	TS-11	947	99			
4.	TS-14	948	99			
5.	TS-16	970	99			
6.	TS-19	970	99			
7.	TS-21	948	99			
8.	TS-23	1496	99			
9.	TS-28	1496	96			
10.	TS-31	938	99			
11.	TS-32	945	99			
12.	TS-34	1497	99			
13.	TS-35	1499	100			
14.	TS-37	968	99			
15.	TS-38	1495	99			
16.	TS-41	951	99			
17.	TS-46	948	99			
18.	TS-47	959	100			
19.	TS-48	980	99			
20.	TS-50	942	100			
21.	TS-51	978	99			
22.	TS-52	970	100			
23.	US-75	1496	99			
24.	US-77	1495	99			
25.	US-95	938	99			
26.	US-96	945	99			
27.	AS-99	915	99			
28.	AS-101	949	99			
29.	AS-102	790	100			
30.	AS-104	979	99			
31.	AS-105	1496	99			
32.	AS-108	1495	99			
33.	AS-110	1495	100			
34.	AS-113	947	99			
35.	AS-114	945	99			
36.	AS-115	891	100			
37.	AS-116	948	99			
38.	AS-118	1495	99			
39.	AS-119	952	99			
40.	AS-120	1495	100			
41.	AS-121	1495	100			

42.	AS-122	912	99			
43.	AS-125	944	99			
44.	AS-126	937	100			
45.	AS-127	1495	99			
46.	AS-103	858	100	Pseudomonas balearica (NR_025972)		
47.	AS-106	858	100			
48.	AS-107	784	94			
49.	AS-112	983	96	Pseudomonas plecoglossicida (NR_024662**)**		
50.	AS-123	940	96			
51.	AS-124	940	96			
52.	TS-2	930	94	Flavisolibacter ginsengisoli (NR_041500) (NR_041499)	Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Chitinophagaceae; Flavisolibacter.	(Yoon & Im 2007)
53.	TS-4	1493	94			
54.	TS-6	1494	94			
55.	TS-7	931	94			
56.	TS-8	1495	94			
57.	TS-9	891	94			
58.	TS-12	969	94			
59.	TS-13	966	94			
60.	TS-15	960	94			
61.	TS-17	869	92			
62.	TS-18	951	94			
63.	TS-25	926	93			
64.	TS-26	929	92			
65.	TS-30	1494	93			
66.	AS-109	1492	94			
67.	AS-111	928	94			
68.	AS-117	1490	94			
69.	TS-3	1428	76	Agromyces ulmi (NR_029108)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Agromyces.	(Rivas et al., 2004)
70.	TS-10	1428	76			
71.	TS-20	1425	76			
72.	TS-24	1502	80	Chrysiogenes arsenatis DSM 11915 (NR_029283)	Bacteria; Chrysiogenetes; Chrysiogenales; Chrysiogenaceae; Chrysiogenes.	(Macy, et al., 1996)
73.	TS-27	1505	91	Marinobacter lutaoensis (NR_025116)	Bacteria; Proteobacteria; Gammaproteobacteria; lteromonadales; Alteromonadaceae; Marinobacter.	(Yang et al., 2003)
74.	TS-33	1507	91			
75.	TS-39	1504	91			
76.	TS-40	1503	91			

77.	TS-29	958	92	Alkalilimnicola ehrlichii MLHE-1 (NR_041842)	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Ectothiorhodospiraceae; Alkalilimnicola.	(Oremland et al., 2002)
78.	TS-36	958	93			
79.	TS-42	1495	84	Acidobacterium capsulatum ATCC 51196 (NR_043386)	Bacteria; Acidobacteria; Acidobacteriales; Acidobacteriaceae; Acidobacterium.	(Ward et al., 2009)
80.	TS-43	1497	84			
81.	TS-44	968	97	Hyphomicrobium sulfonivorans (NR_025082**)**	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae; Hyphomicrobium.	(Borodina et al., 2002)
82.	TS-45	944	97	Rhodoplanes serenus (NR_040936**)**	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae; Rhodoplanes.	(Okamura et al., 2009)
83.	TS-49	962	85	Litoricola lipolytica (NR_044131**)**	Bacteria; Proteobacteria; Gammaproteobacteria; Oceanospirillales; Litoricolaceae; Litoricola.	(Kim et al., 2007)
84.	US-53	884	93	Thiobacillus denitrificans (NR_025358)	Bacteria; Proteobacteria; Betaproteobacteria; Hydrogenophilales; Hydrogenophilaceae; Thiobacillus.	(Kelly & Wood, 2000)
85.	US-54	936	93	Pirellula staleyi (NR_037109)	Bacteria; Planctomycetes; Planctomycetia; Planctomycetales; Planctomycetaceae; Pirellula.	(Griepenburg et al., 1999)
86.	US-67	941	93			
87.	US-62	933	94	(NR_037109)		
88.	US-55	1463	81	Caldilinea aerophila DSM 14535 (NR_040878)	Bacteria; Chloroflexi; Caldilineae; Caldilineales; Caldilineaceae; Caldilinea.	(Sekiguchi et al., 2003)
89.	US-56	899	83	Desulfatibacillum alkenivorans (NR_025795)	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfobacterales; Desulfobacteraceae; Desulfatibacillum.	(Cravo-Laureau et al., 2004)
90.	US-57	965	86	Geobacter	Bacteria; Proteobacteria;	(Lovley, et al., 1993)

91.	US-63	929	86	metallireducens (NR_025895)	Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter.	
92.	US-58	957	88	Gemmatimonas aurantiaca T-27 (NR_027529)	Bacteria; Gemmatimonadetes; Gemmatimonadales; Gemmatimonadaceae; Gemmatimonas.	(Zhang et al., 2003)
93.	US-80	1496	87			
94.	US-59	1497	90	Thioalkalivibrio denitrificans (NR_028745)	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Ectothiorhodospiraceae; Thioalkalivibrio.	(Sorokin et al., 2001)
95.	US-60	1495	90			
96.	US-61	960	86	Edaphobacter aggregans (NR_043898)	Bacteria; Acidobacteria; Acidobacteriales; Acidobacteriaceae; Edaphobacter.	(Koch et al., 2008)
97.	US-72	930	80			
98.	US-64	1509	82	Desulforegula conservatrix (NR_028780)	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfobacterales; Desulfobacteraceae; Desulforegula.	(Rees & Patel, 2001)
99.	US-65	1496	91	Steroidobacter denitrificans (NR_044309)	Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Sinobacteraceae; Steroidobacter.	(Fahrbach et al., 2008)
100.	US-66	1513	83	Holophaga foetida (NR_036891)	Bacteria; Acidobacteria; Holophagae; Holophagales; Holophagaceae; Holophaga.	(Liesack et al., 1994)
101.	US-78	1512	84			
102.	US-82	1508	83			
103.	US-85	1515	84			
104.	US-68	924	90	Luteimonas composti (NR_043983)	Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Luteimonas.	(Young et al., 2007)
105.	US-69	952	90	Azospira restricta (NR_044023)	Bacteria; Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae; Azospira.	(Bae et al., 2007)
106.	US-86	948	92			
107.	US-90	937	92			
108.	US-97	950	91			
109.	US-98	952	91			

110.	US-70	941	85	Desulfomonile limimaris (NR_025079)	Bacteria; Proteobacteria; Deltaproteobacteria; Syntrophobacterales; Syntrophaceae; Desulfomonile.	(Sun et al., 2001)
111.	US-71	1496	90	Denitratisoma oestradiolicum (NR_043249)	Bacteria; Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae; Denitratisoma.	(Fahrbach et al., 2006)
112.	US-74	1495	90			
113.	US-87	1496	91			
114.	US-88	1496	91			
115.	US-92	1497	91			
116.	US-73	1497	91	Thiobacter subterraneus (NR_024834)	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Thiobacter.	(Hirayama et al., 2005)
117.	US-94	937	92			
118.	US-76	1480	86	Planctomyces maris (NR_025327)	Bacteria; Planctomycetes; Planctomycetia; Planctomycetales; Planctomycetaceae; Planctomyces	(Griepenburg , et al., 1999)
119.	US-79	1497	98	Acinetobacter haemolyticus (NR_026207)	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter.	(Rainey et al., 1994)
120.	US-81	970	96	Sphingomonas kaistensis (NR_043171)	Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingomonas	(Kim et al., 2007)
121.	US-84	924	95			
122.	US-83	936	79	Yonghaparkia alkaliphila (NR_043675)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Yonghaparkia.	(Yoon et al., 2006)
123.	US-89	1500	93	Aquicella siphonis (NR_025764)	Bacteria; Proteobacteria; Gammaproteobacteria; Legionellales; Coxiellaceae; Aquicella.	(Santos et al., 2003)
124.	US-91	1499	93			
125.	US-93	1499	93			
126.	AS-100	1446	98	Blastobacter denitrificans (NR_041827)	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; Blastobacter.	(Berkum et al., 2003)

Chapter V
Discussion

Earth has many metals and nonmetals and some of these are in trace amount essential for living organisms for their cellular mechanisms. Heavy metals and metalloids when present in high concentrations are toxic for living beings. These can be toxic in many different ways like DNA damage, oxidative damage via the production of reactive oxygen species, through binding to essential respiratory chain proteins etc. It can be expected that soil organisms are adapted to bear toxic metals with their environment (Canovas et al., 2003). Many microorganisms have evolved different mechanism to protect them from the toxicity of heavy metals and metalloids. Some of the mechanisms are resistant and some are to detoxify these heavy metals by reducing them. Microbes use many mechanisms like chelation, extrusion and uptake of heavy metals and plasmid encoded efflux mechanisms (Canovas et al., 2003; Wang et al., 1997).

In this study abandoned soil is used as a control to compare with agriculture and thermal power plant soil. Physiochemical analysis of all three soil samples shows that pH of all soil samples is alkaline. Electrical conductivity of all the three soils were higher than normal which should be <0.8 S. Organic carbon and phosphorus concentration of abandoned soil and thermal power plant soil is less than agricultural soil. But concentration of potassium of all samples is too much higher as showed in table 1.

Abandoned soil has less concentration of heavy metals as compared to agriculture soil and thermal power plant soil. Previous study showed that in thermal power plant fly ash has high amount of heavy metals as compared to garden soil (Ansari et al., 2011; Gupta et al., 2002; Rai et al., 2000; Scotti et al., 1999; Vajpayee et al., 2000). Our studies are in accordance that heavy metals in thermal power plant soil have high concentration of heavy metals and metalloids as compared to abandoned soil. But calcium, potassium, magnesium and manganese concentration in abandoned soil is higher than thermal power plant soil. Agriculture soil also has high concentration of heavy metals than abandoned soil, but calcium concentration is lower than abandoned soil. Agriculture land is contaminated with higher concentration of heavy metals due to excessive use of fertilizers, pesticides and insecticides to increase yields of crops. Thermal power

plant produces fly ash which is settled at soil of adjoining area of thermal power plant. Coal ash is known to contain significant quantities of heavy metals such as arsenic, lead and selenium, which can cause cancer and neurological problems and is thus disastrous (Ansari et al., 2011). Fly ash containing soil has water holding capacity, due to very small size of fly ash particle 0.1 to 100 µm (Ansari et al., 2011; Gupta et al., 2002). Resultant of which is leaching of heavy metals in the soil thereby increases the content.

Hundal et. al., (2006) studied the heavy metal analysis of soil of Punjab, North West India. According to our study heavy metals in Bathinda soil are too much higher concentration than previous study. Abandoned soil-agricultural soil-thermal power plant soil has heavy metals As 3.04-3.6-0.15, B 104.3-88.8-150.6, Cd 9.25-13.5-12.25, Co 18.5-28.9-25.7, Cr 71.6-236.5-262.5, Cu 1.9-4.8-6.2, Fe 240.2-350.4-313.1, Mg 28.6-37.5-14.8, Mn 31.3-46.3-29.3, Ni 35.1-64.1-62.8, Pb 3.7-4.2-4.6 and Zn 10.1-7.8-13.3 fold higher concentration than previous study's maximum concentration in alluvial soil with sand dunes .

Soil microbial biomass is a sound indicator of soil health since it regulates nutrient cycling and acts as a highly labile source of plant available nutrients. Due to proper moister content and metals microbial diversity increases. But only those bacteria dominate which have capacity to resist, accumulate and can reduce heavy metals and metalloids. But high concentration of heavy metals can hinder the microbial metabolic processes (Kohli & Goyal, 2010). The abandoned soil microbial diversity when compare with agricultural soil, it is directly or indirectly related to by anthropogenic activities (Navarrete et al., 2010).

Phylogenetic study of microbial diversity of three soil samples shows that all three soils contain different microbial profile. All soil samples have 32 different species of bacterial clones and these 32 different species belong to 8 different phyla i.e. 21 clone species Proteobacteria, 3 clone species with Acidobacteria, 2 clone species each with Actinobacteria and Planctomycetes and 1 clone species each with Chrysiogenetes, Bacteroidetes, Chloroflexi and Gemmatimonadetes. All phyla's bacterial species are Gram negative except Actinobacteria and Chloroflexi. The predominance of gram-negative bacteria at higher concentration of metal is

probably due to their higher level of intrinsic metal resistance than majority of the grampositive bacteria. The basis of this difference might be due to the differences in the chemical composition of cell wall of gram-negative bacteria and gram-positive bacteria (Anyanwu et al., 2011).

Abandoned soil has maximum bacterial diversity, contains 22 bacterial species followed by thermal power plant soil 10 bacterial species and least bacterial diversity observed in agricultural soil only 5 bacterial species. Due to low vegetative diversity and more xenbiotic input in agricultural soil, its overall species diversification may be reduced to bottleneck (Roesch et al., 2007). Agriculture soil and thermal power plant soil is dominant by Pseudomonas stutzeri followed by Flavisolibacter ginsengisoli but in abandoned soil has mixed bacterial diversity. Pseudomonas stutzeri, Pseudomonas balearica, Pseudomonas plecoglossicida, Hyphomicrobium sulfonivorans, Rhodoplanes serenus, Acinetobacter haemolyticus and Blastobacter denitrificans belong to phylum Proteobacteria which are closely related with sequences of clones. These similarity match of these clones is >97%. But all other clone's sequences belonging to 8 phyla are distant from their belonging genera. These shows similarity match between 79-96%. Most common species are present in all three different soil samples are Psedomonas stutzeri and Flavisolibacter ginsengisoli.

Previously many 16S rRNA based soil microbial diversity study has been published. Most of the clone library of soil microbial diversity belongs to 7-10 different phyla. The dominant phyla in the libraries are Proteobacteria, *Acidobacteria, Actinobacteria, Verrucomicrobia, Bacteroidetes, Chloroflexi,* Planctomycetes, Gemmatimonadetes, and Firmicutes. Members of these nine phyla make up an average of 92% of soil libraries (Janssen, 2006). These phyla are Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, Deinococcus-Thermus, Chloroflexi, Firmicutes, Gemmatimonadetes, Planctomycetes, Nitrospira, Chlamydia and Verrucomicrobia. Proteobacteria, Acidobacteria and Actinobacteria are preponderance than other phyla in all soil bacterial diversity. Different soil bacterial diversity studies performed like from grassland, Victoria (Australia) (Joseph et al., 2003), Uranium mine, North Cave Hills, South Dakota

(USA) (Rastogi et al., 2010), cultivated soil (Taiwan) (Yu et al., 2008), forest soil (USA) (Roesch et al., 2007), Antarctica soil (Shivaji et al., 2004), sandy soil Scotland (UK) (Ellis et al., 2003), all above mentioned phyla present in these soils.

In this study most of the bacterial species found in this region's soil are belong to phylum Proteobacteria. All three bacterial species P. stutzeri, P. balearica and P. plecoglossicida belonging to class Gammaproteobacteria. These Pseudomonas sp. has capability of denitrification, nitrogen fixation, biosorption, resistance, degradation, accumulation, bioremediation and reduction of heavy metals and metalloids (Boricha & Fulekar, 2009; Canovas et al., 2003; Lalucat et al., 2006; Mullen et al., 1989; Nievas et al., 2006; Strandberg et al., 1981; Wang et al., 1997). P. stutzeri has high resistance and high biosorption potential to many heavy metals like Al, Cr, Co, Cu, Ni, Pb, Zn, Mn, Pu, U, Se, Ag, Ti and Th and also has capability to degrade aromatic compound like [polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), benzo(A)pyrene, and benzo-(B)fluoranthene] are major environmental pollutants. This can also degrade the organophosphate insecticide parathion and hazarardous compound and petroleum hydrocarbons. The genus Pseudomonas is dominant or predominant in association with wheat, barley, and wetland rice (Lalucat et al., 2006; Nievas et al., 2006).

P. plecoglossicida was isolated from ayu cultured pond in Japan and also in cow dung in India that has property for bioremediation of pesticide (Cypermethrin) (Boricha & Fulekar, 2009; Nishimori et al., 2000). Many members of Pseudomonas spp. were found in the highly polluted with Uranium mining waste in Bulgaria and have high potential to accumulate and/or to reduce U (VI) (Geibler et al., 2004; Radeva et al., 2008).

Proteobacteria is dominant phylum of this study also has 20 more bacterial species. Rhodoplanes serenus (Okamura et al., 2009), Thiobacillus denitrificans (Beller, 2005; Beller et al., 2006), Thioalkalivibrio denitrificans (Sorokin et al., 2001), Steroidobacter denitrificans (Fahrbach et al., 2006) are denitrifying bacteria. Blastobacter denitrificans (Berkum & Eardly, 2002) and Azospira restricta (Bae et al., 2007) are nitrogen fixing bacteria. Thiobacillus denitrificans has ability

to couple denitrification to sulfur-compound oxidation, to catalyze anaerobic, nitrate-dependent oxidation of Fe (II) and U (IV) (Beller et al., 2006), Thiobacter subterraneus (Hirayama, et al., 2005) and Thioalkalivibrio denitrificans are sulfur-oxidizing bacteria (Sorokin et al., 2001). Marinobacter lutaoensis (Yang et al., 2003), Thiobacter subterraneus (Hirayama et al., 2005) and Luteimonas composti (Young et al., 2007) are thermohilic bacteria. Thiobacillus denitrificans (Beller, et al., 2006), Acinetobacter haemolyticus (Pei et al., 2009) Alkalilimnicola ehrlichii (Hoeft et al., 2007) and Geobacter metallireducens (Beller et al., 2006; Lovley et al., 1993; Ortiz-Bernad et al., 2004) has resistance and capability to reduce heavy metals (Ni, Pb, Cu, Hg, As, Cr (IV), Mn (IV), U (VI), V (V), Fe). Geobacter metallireducens (Ortiz-Bernad et al., 2004), Desulfatibacillum alkenivorans (Cravo-Laureau et al., 2004), Desulforegula conservatrix (Rees & Patel, 2001) Desulfomonile limimaris (Sun et al., 2001) are sulfur reducing bacteria alkene-degrading and can oxidize alkanes, amino acids and aromatic compounds. Hyphomicrobium sulfonivorans was previously isolated from garden soil. This has capability to oxidize dimethyl sulfide (Boden et al., 2011). Litoricola lipolytica was isolated from coastel seawater of east sea of Korea and it has fat dissolving capability (Kim et al., 2007). Luteimonas composti is a moderate thermophilic bacterium isolated from food waste in Taiwan (Young et al., 2007). Sphingomonas kaistensis was isolated from soil in Daejeon, South Korea (Kim et al., 2007). Aquicella siphonis was isolated from water at the thermal spa at Sao Gemil, Portugal (Santos et al., 2003).

Bacteroidetes phylum (Flavisolibacter ginsengisoli species) is second dominant in thermal power plant soil and agricultural soil, but it is absent in abandoned soil. Flavisolibacter ginsengisoli has been previously reported in ginseng cultivated soil of South Korea (Yoon & Im, 2007).

Actinobacteria phylum has 2 bacterial species Agromyces ulmi in thermal power plant soil and Yonghaparkia alkaliphila found in abandoned soil samples. Agromyces ulmi xylan-degrading bacterial strains were isolated from a decayed Ulmus nigra tree in Spain (Rivas et al., 2004). The genus Agromyces comprises 22 recognized species, most of the species isolated from soil and in rhizosphere

of plants (Lee et al., 2011). Yonghaparkia alkaliphila isolated from alkaline soil of Kwangchun, Korea and this is resistant to copper and cadmium (Dhal et al., 2011; Yoon et al., 2006).

The phylum Acidobacteria is one of the most abundantly distributed bacterial groups in the environment. We have reported three different species of this phylum. Acidobacteria phylum has Acidobacterium capsulatum, Edaphobacter aggregans and Holophaga foetida bacterial species. Acidobacterium capsulatum was isolated from sediments in acidic drainage from the Yanahara pyrite mine in Japan. It plays important role in iron reduction and in nitrate, nitride reduction but not in nitrogen fixation or denitrification and does nitrogen cycling of soil and sediments (Ward et al., 2009). Edaphobacter aggregans was isolated from deciduous forest northern Bavaria, Germany (Koch et al., 2008). Holophaga foetida is a strictly anaerobic, demethylating homoacetogen that degrades aromatic compounds to acetate and is capable of transferring methyl groups from phenylmethylethers to sulfide, thus forming methanethiol and dimethyl sulfide (Koch et al., 2008). Acidobacteria the most predominant group found in several German uranium wastes polluted with uranium (Radeva et al., 2008).

Pirellula staleyi and Planctomyces maris belong to phylum Planctomycetes. Pirellula staleyi was isolated from freshwater Camus lake Baton Rouge, LA, USA (Butler et al., 2002). Planctomyces maris was isolated from Neritic waters, Puget Sound, Washington, USA and is heterotrophic, obligately aerobic and mesophilic. It shows significant growth under saline conditions (Bauld & Staley, 1976).

Chrysiogenes arsenates belong to phylum Chrysiogenetes. Chrysiogenes arsenates were isolated from a reed bed at Ballarat Goldfields in Australia. It is strictly anaerobic bacteria having arsenate As (V) to arsinite As (III) reducing capacity. It can also use nitrate or nitrite as the terminal electron acceptor (Macy et al., 1996).

Chloroflexi phylum has Caldilinea aerophila bacterial species. It was isolated from a hot spring sulfur-turf in Japan. It is a thermophilic bacterium and can grow in aerobic and anaerobic conditions (Sekiguchi et al., 2003).

Gemmatimonadetes phylum has Gemmatimonas aurantiaca bacterial species. It was isolated from an anaerobic–aerobic sequential batch reactor operated under enhanced biological phosphorus removal conditions for wastewater treatment. It can accumulate polyphosphate (Zhang et al., 2003).

This study shows that all bacteria reported in present study in soil sample are culturable. Most of the soil bacteria are heavy metals and metalloid resistant and are capable to reduce heavy metals. Abandoned soil and thermal power plant soil has maximum diversity as compared to agricultural soil. Most of the authors suggest fly ash can be used in agricultural soil to increase microbial diversity (Ansari et al., 2011; Gupta et al., 2002; Kohli & Goyal, 2010; Siddiqui & Singh, 2005). Abundance of bacterial species in abandoned soil can be correlated with lesser heavy metals content and less anthropogenic activities. On the other hand agricultural soil is the most affected one with high metal content and least bacterial diversity. But the species available here are more evolved either highly resistant or capable to detoxify these metals. Heavy metal reducing bacteria can be culture in laboratory and their bioremediation capability can be tested.

Conclusion

Bacterial diversity was assessed for all three soil samples (Abandoned, agricultural and Thermal power plant soil) collected from Bathinda region. Based on 16S rRNA gene sequence analysis, the abandoned soil has maximum bacterial diversity followed by thermal power plant soil, but agricultural soil has minimum bacterial diversity. Phylum Proteobacteria is dominant in this study followed by Bacteroidetes. Most of the bacteria belonging to clone sequences are heavy metal and metalloid reducers or tolerant. Metal reducer bacteria can be cultured in laboratory and that can be tested for bioremediation of heavy metals and metalloids that are toxic to soil ecosystem, plants and animals.

The continued use of sequence based phylogenetic approaches will yield more information, providing insight into the effectiveness of validity of current phylogenetic classification strategies and whether they reflect fundamental biological properties. In closing, we suggest that additional studies are needed to explore the extent of diversity within and between phylogenetic group to provide additional ecological and biological context.

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