ANALYSIS AND BIOREMEDIATION OF HEAVY METALS

By: - Vasanth Manohar

Enrolment number: - 091578

SUPERVISOR - Dr. Sudhir Kumar

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Bachelor of technology

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, WAKNAGHAT

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CERTIFICATE

This is to certify that the work entitled, **"Analysis and Bioremediation of Toxic Heavy Metals"** submitted by **Vasanth Manohar** in complete fulfilment for the award of degree of Bachelor of Technology in Biotechnology of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Signature of the Supervisor: 27/5/13

Name of the Supervisor: Dr. Sudhir Kumar

Designation: Associate Professor

Date: 27/5/13

ACKNOWLEDGEMENT

Any assignment puts to litmus test an individual's knowledge, credibility and experience and thus, sole efforts of an individual are not sufficient to accomplish the desired work. Successful completion of a project involves interest and efforts of many people and so this becomes obligatory on my part to record thanks to them.

Therefore, first of all I would like to thank my guide and mentor Dr. **Sudhir Kumar** for his guidance, help and constant encouragement throughout this project. Working under him was an enriching experience.

I express my heartfelt thanks to our Head of Department **Dr. R.S. Chauhan** for providing us the opportunity for doing this final year project.

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Vasanth Manohar

Date: 27/5/13

Summary:-

Soil is home for diverse bacteria, fungi, virus, etc., organisms. There are evidences which showed soil polluted with heavy metals can make the fungi to gain resistance for different heavy metals because of increased industrialization and also increased use of pesticides insecticides and other chemicals in Agriculture. This suggested in setting a hypothesis for isolation of heavy metal-resistant fungi to be used in the bioremediation of the contaminated soils. The aim of this study was to investigate the impact of increasing zinc concentrations on soil fungi that are cultivable under laboratory conditions in order to extract fungal strains resistant to heavy metals which can later be used for bioremediation purposes.

Signature of the Student: -

Name of Student: - Vasantha Manohar

Date: - 27/5/13

Signature of the Supervisor: - 27/5/13

Name of Supervisor: - Dr. Sudhir Kumar

Date: - 27/5/13

List of Figures: -

List of Tables: -

List of graphs:-

Chapter 1

Introduction

Soil is home for diverse bacteria, fungi, virus, etc., organisms. Among these are pathogenic fungi which are frequently recorded in cultivated soil worldwide for being highly pathogenic. Many fungi genera like *Beuveria Vuill*, *Metarhizium Sorokin*, etc. are the most common pathogenic fungi among the insect-pathogenic fungi [1]. However, other fungal species, including opportunistic pathogens as well as secondary colonizers, can also greatly affect the dynamics of insect population thriving in soil habitat [2]. There are evidences which showed soil polluted with heavy metals can make the fungi to gain resistance for different heavy metals or kill fungi. This suggested in setting a hypothesis for isolation of heavy metal-resistant fungi to be used in the bioremediation of the contaminated soils. The aim of this study was to investigate the impact of increasing zinc concentrations on soil fungi that are cultivable under laboratory conditions in order to extract fungal strains resistant to heavy metals which can later be used for bioremediation purposes.

Heavy metal is a member of a loosely defined subset of elements that exhibit metallic properties. It mainly includes the transition metals, some metalloids, lanthanides, and actinides.

Living organisms required metals like Iron, cobalt, copper, manganese, zinc, etc. for their proper daily activities. Other metals like Mercury, Chromium, and Lead are toxic metals that have no known vital or beneficial effect on the organisms, and their accumulation over time in the bodies of living organisms can cause serious illness [3].

Using these heavy metals in pesticides lead to the contamination of soil and ground water and causes several health problems like cancer (breast, brain and skin), genetic disorders, reduction in crop yield. The metals that generally present in pesticides, herbicides, insecticides are Arsenic, Zinc, Lead, Chromium, etc.

In the mid-1990s, studies from China reported the effective use of arsenic trioxide in achieving complete remission in patients suffering with APL (promyelocyticleukemia). But Arsenic has been reported to be a human carcinogen associated with malignancies of the lung, bladder, skin, liver, and prostate. Although the mechanism is not yet understood, chronic exposure to high concentrations of arsenic may lead to carcinogenic effects owing to hypo-methylation of DNA and the generation of deletion mutations [4].

Chapter 2:-

Review of literature:-

2.1 Description

Many microorganisms demonstrate resistance to metals in water, soil and industrial waste. Genes located on chromosomes, plasmids, or transposons encode specific resistance to a variety of metal ions. Some metals, such as cobalt, copper, nickel, serve as micronutrients and are used for redox processes, to stabilize molecules through electrostatic interactions, as components of various enzymes, and for regulation of osmotic pressure. Most metals are nonessential, have no nutrient value, and are potentially toxic to microorganisms. These toxic metals interact with essential cellular components through covalent and ionic bonding. At high levels, both essential and nonessential metals can damage cell membranes, alter enzyme specificity, disrupt cellular functions, and damage the structure of DNA. Microorganisms have adapted to the presence of both nutrient and nonessential metals by developing a variety of resistance mechanisms.

Metal resistance mechanisms exist are:

Exclusion by permeability barrier,

Intra- and extra-cellular sequestration,

Active transport efflux pumps,

Enzymatic detoxification,

Reduction in the sensitivity of cellular targets to metal ions

The understanding of how microorganisms resist metals can provide insight into strategies for their detoxification or removal from the environment. [5]

In a study, serpentine soils collected from Saddle Hills, Chidyatapu and Rutland of Andaman Islands, India were analyzed for physico-chemical and microbiological characteristics and compared with those from adjacent non-serpentine localities. The serpentine soils contained high levels of nickel (1740.0-8033.4 mg/kg dry soil), cobalt (93.2-533.4 mg/kg dry soil) and chromium (302.9-4437.0 mg/kg dry soil), in addition to 62-152 g of iron and 37-60 g of magnesium per kg dry soil.

Characteristically the serpentine soils showed low microbial density (6.2-11.3 x 10(6) colony forming unit/g soil) and activity (1.7-3.5 microg fluorescein/g dry soil/h) than non-serpentine outcrops. Serpentine microbial population was dominated by bacteria which represented 5.12 to 9.5 x 10(6) cfu/g of soil, while the fungal population ranged from 0.17 to 3.21 x 10(6) cfu/g of soil. A total of 342 isolates were compared for Ni, Co and Cr resistance.

These 25 serpentine strains also showed co-resistance to Cu, Zn and Mn but were sensitive to Hg and Cd. The selected bacterial isolates were resistant to ampicillin, penicillin G and polymyxin B, whereas fungal strains showed resistance to amphotericin B, nystatin and fusidic acid [6].

2.2 Fungal Characteristics:-

Aspergillus species are highly aerobic and are found in almost all oxygen-rich environments, where they commonly grow as molds on the surface of a substrate, as a result of the high oxygen tension. Commonly, fungi grow on carbon-rich substrates like monosaccharide's and polysaccharides. *Aspergillus* species are common contaminants of starchy foods, and grow in or on many plants and trees.

In addition to growth on carbon sources, many species of *Aspergillus* demonstrate oligotrophy where they are capable of growing in nutrient-depleted environments, or environments in which there is a complete lack of key nutrients. *A. niger* is a prime example of this; it can be found growing on damp walls, as a major component of mildew.

2.2.1 Commercial importance

Species of *Aspergillus* are important medically and commercially. Some species can cause infection in humans and other animals. Some infections found in animals have been studied for years. Some species found in animals have been described as new and specific to the investigated disease and others have been known as names already in use for organisms such as saprophytes. More than 60 *Aspergillus* species are medically relevant pathogens[7]. For humans there is a range of diseases such as infection to the external ear, skin lesions, and ulcers classed as mycetomas.

Other species are important in commercial microbial fermentations. For example, alcoholic beverages such as Japanese sake are often made from rice or other starchy ingredients like manioc, rather than from grapes or malted barley. Typical microorganisms used to make alcohol, such as yeasts of the genus *Saccharomyces*, cannot ferment these starches, and so *koji* mold such as *Aspergillus oryzae* is used to break down the starches into simpler sugars.

Members of the genus are also sources of natural products that can be used in the development of medications to treat human disease[8].

Perhaps the largest application of *Aspergillus niger* is as the major source of citric acid; this organism accounts for over 99% of global citric acid production, or more than 1.4 million tonnes per annum. *A. niger* is also commonly used for the production of native and foreign enzymes, including glucose oxidase and hen egg white lysozyme. In these instances, the culture is rarely grown on a solid substrate, although this is still common practice in Japan, but is more often grown as a submerged culture in a bioreactor. In this way, the most important parameters can be strictly controlled, and maximal productivity can be achieved. It also makes it far easier to separate the chemical or enzyme of importance from the medium, and is therefore far more cost-effective.

2.2.2 Pathogenesis

Some *Aspergillus* species cause serious disease in humans and animals. The most common pathogenic species are *A. fumigatus* and *A. flavus*, which produces aflatoxin which is both a toxin and a carcinogen, and which can contaminate foods such as nuts. The most common species causing allergic disease are *A. fumigatus* and *Aspergillus clavatus*. Other species are important as agricultural pathogens. *Aspergillus* spp. cause disease on many grain crops, especially maize, and synthesize mycotoxins, including aflatoxin

Aspergillosis is the group of diseases caused by *Aspergillus*. The most common subtype among paranasal sinus infections associated with aspergillosis is *A. Fumigates[9]*. The symptoms include fever, cough, chest pain, or breathlessness, which also occur in many other illnesses, so diagnosis can be difficult. Usually, only patients with already weakened immune systems or who suffer other lung conditions are susceptible.

In humans, the major forms of disease are:

- Allergic bronchopulmonary aspergillosis, which affects patients with respiratory diseases such as asthma, cystic fibrosis, and sinusitis
- Acute invasive aspergillosis, a form that grows into surrounding tissue, more common in those with weakened immune systems such as AIDS or chemotherapy patients
- Disseminated invasive aspergillosis, an infection spread widely through the body
- Aspergilloma, a "fungus ball" that can form within cavities such as the lung

Aspergillosis of the air passages is also frequently reported in birds, and certain species of *Aspergillus* have been known to infect insects [10].

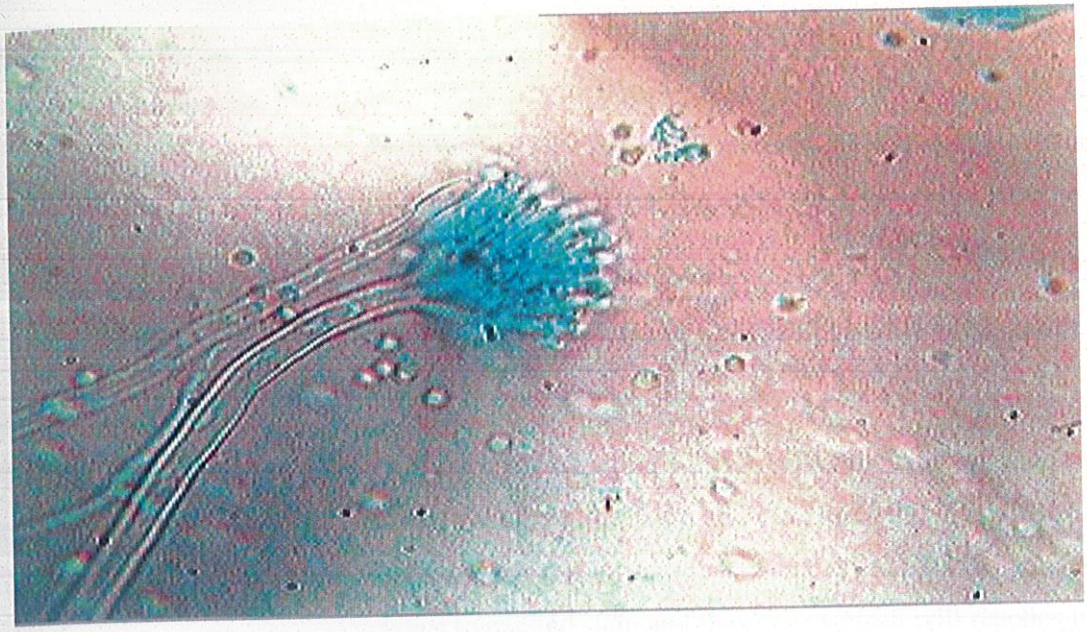


Figure 1:- Showing stained fungus at 100X

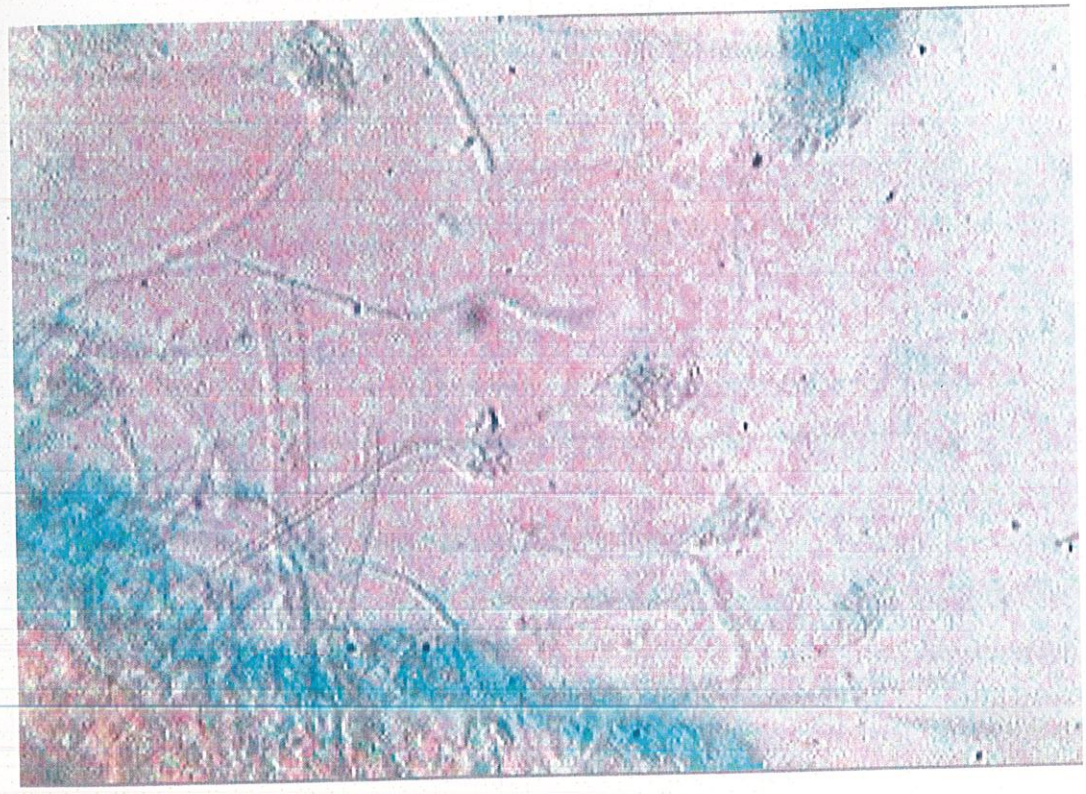


Figure 2:- Showing stained fungus at 40X

2.3 ATOMIC ABSORPTION SPECTROSCOPY

PRINCIPLE:- When the sample introduced through inlet into Atomic spectroscopy then the sample will get spread on the burner and due to high energy level given by the flame (acetylene + air=2300^{0}c) the ground state electron will excited and after it will reach to ground state by releasing energy. The detector will catch the energy released and gives the value of the metal present in that sample.

After sampling and digestion of it than we will go for analyzing the metal content in the sample by using appropriate lamp which is having a suitable frequency

For example for analyzing Zinc contain in a sample than first we will select the lamp of Zinc which is having wavelength of 213.80nm and then switch on the lamp and then open the Winlab software and then switch on the flame by giving Acetylene and air as fuels which gives 2300 ° Celsius and this makes the ground state electron reaches to excited state and then the electron will reaches to the ground state by emitting energy.

Table showing different metals and their respective wavelengths for analysis

S. No.	Metal	Wave length
1	Arsenic	193.70
2	Chromium	357.87
3	Zinc	213.80
4	Nickelc	232.00
5	Iron	248.33

Table 1: Showing wavelength of different metals

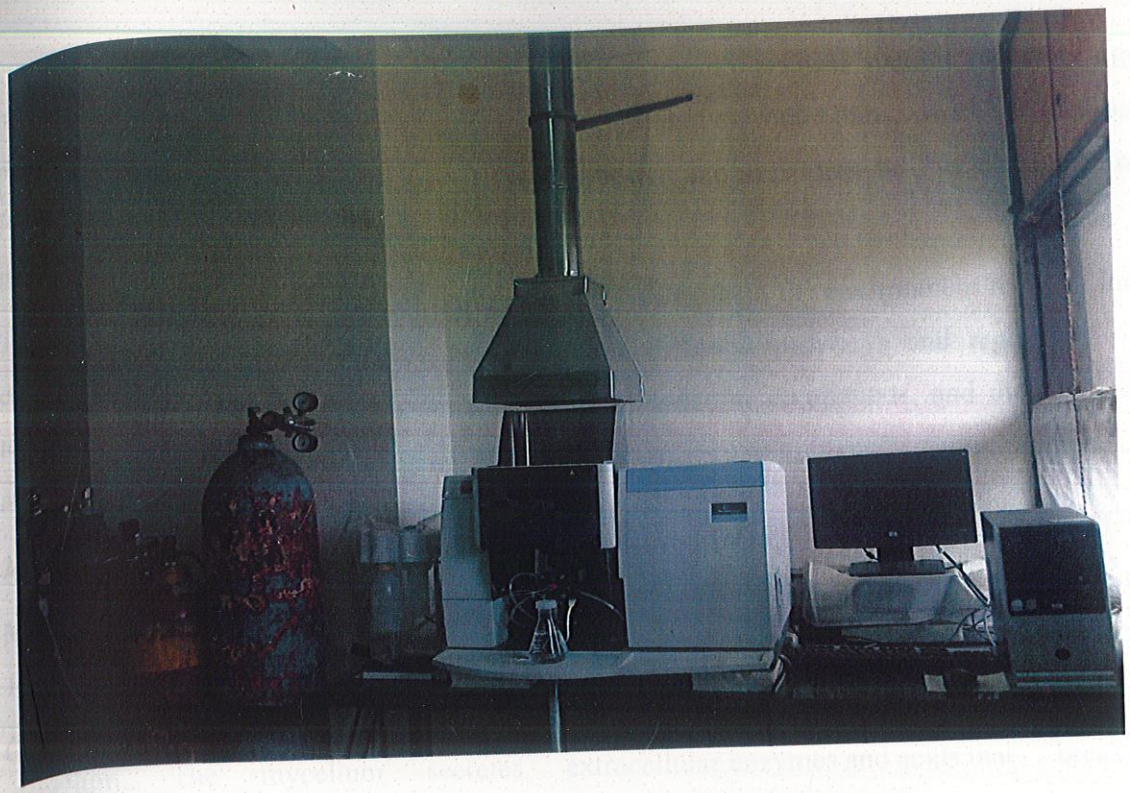


Figure 3:- Atomic Absorption Spectrophotometer

2.4 Bio-remediation

Bioremediation is the use of micro-organism metabolism to remove pollutants. Technologies can be generally classified as *in situ* or *ex situ*. *In situ* bioremediation involves treating the contaminated material at the site, while *ex situ* involves the removal of the contaminated material to be treated elsewhere. Some examples of bioremediation related technologies are phytoremediation, bioventing, bioleaching, landfarming, bioreactor, composting, bioaugmentati on, rhizofiltration, and biostimulation.

Bioremediation can occur on its own (natural attenuation or intrinsic bioremediation) or can be spurred on via the addition of fertilizers to increase the bioavailability within the medium (biostimulation). Recent advancements have also proven successful via the addition of matched microbe strains to the medium to enhance the resident microbe population's ability to break down contaminants. Microorganisms used to perform the function of bioremediation are known as **bioremediators**. [11]

Not all contaminants, however, are easily treated by bioremediation using microorganisms. For example, heavy metals such as cadmium and lead are not readily absorbed or captured by microorganisms. The assimilation of metals such as mercury into the food chain may worsen

matters. Phytoremediation is useful in these circumstances because natural plants or transgenic plants are able to bio-accumulate these toxins in their above-ground parts, which are then harvested for removal. [12] The heavy metals in the harvested biomass may be further concentrated by incineration or even recycled for industrial use.

The elimination of a wide range of pollutants and wastes from the environment requires increasing our understanding of the relative importance of different pathways and regulatory networks to carbon flux in particular environments and for particular compounds, and they will certainly accelerate the development of bioremediation technologies and biotransformation processes.

2.4.1 Mycoremediation:-

Mycoremediation is a form of bioremediation in which fungi are used to decontaminate the area. The term *mycoremediation* refers specifically to the use of fungal mycelia in bioremediation.

One of the primary roles of fungi in the ecosystem is decomposition, which is performed by the mycelium. The mycelium secretes extracellular enzymes and acids that break down lignin and cellulose, the two main building blocks of plant fiber. These are organic compounds composed of long chains of carbon and hydrogen, structurally similar to many organic pollutants. The key to mycoremediation is determining the right fungal species to target a specific pollutant. Certain strains have been reported to successfully degrade the nerve gases VX and sarin.

2.4.2 Advantages

There are a number of cost/efficiency advantages to bioremediation, which can be employed in areas that are inaccessible without excavation. For example, hydrocarbon spills (specifically, petrol spills) or certain chlorinated solvents may contaminate groundwater, and introducing the appropriate electron acceptor or electron donor amendment, as appropriate, may significantly reduce contaminant concentrations after a long time allowing for acclimation. This is typically much less expensive than excavation followed by disposal elsewhere, incineration or other *ex situ* treatment strategies, and reduces or eliminates the need for "pump and treat", a practice common at sites where hydrocarbons have contaminated clean groundwater.

2.5 Uses of heavy metals

Nickel:-

- Nickel is a metal, commonly used to make coins, magnets, jewelry, stainless steel, electronics, and components of industrial machines.
- The attractive mirror-finish that can be achieved by nickel plating. [13]

Zinc:-

- Zinc is a trace element that is essential for human health.
- It is used for the negative plates in some electric batteries and for roofing and gutters in building constructions. [14]

Iron:-

- Most common metal used in automobiles, machine tools, the hulls of large ships, building parts and machine parts are made out of iron.
- Iron (III) chloride is used in the treatment of sewage, as a dye for cloth, as a colouring agent for paint, an additive in animal feed, an in the manufacture of printed circuit boards.[15]

Chromium

- Potassium dichromate was found to be an excellent mordant in textile dyeing.
- Chromic sulphate was introduced in a process for tanning leather. [16]

2.6 Disadvantages of heavy metals:-

Problems with Nickel:-

Some people may experience an asthma-like reaction when they come in contact with nickel.

Those individuals who work in nickel processing plants or refineries have shown signs of bronchitis on a chronic level as well as poor lung function.

Breathing large amounts of dust containing nickel compounds, like that found in these refineries, has been shown to cause cancers of the nasal sinus and the lungs.

It has been discovered that rats and dogs exhibit problems with their blood, liver, stomach, kidneys, even the immune system after drinking or eating large quantities of nickel. It has also proved to cause lung disease and caused issues with their development and reproduction.[17]

Problems with Chromium

If it is inhaled, chromium can cause an irritated nose, throat and lungs. If inhaled long enough, it can result in damage to the mucous membranes, nosebleeds, cause ulcers and even perforate the septum.

In Russia, exposure to chromium is blamed for a wide number of premature senility cases. Studies have also proven that inhalation of chromium can increase the chances of developing lung cancer.

Interestingly enough, chromium is extremely reactive with vitamin C. When exposure is coupled with vitamin C in the body, it can result in severe damage to the individual's DNA inside the lung's cells. However, outside of the cells, vitamin C actually serves to protect against the damage to the cells. [18]

Problems with Zinc

- High levels of zinc can damage the pancreas and disturb the protein metabolism, and cause arteriosclerosis and respiratory disorders.
- The Humans can handle proportionally large concentrations of zinc, too much zinc can still cause eminent health problems, such as stomach cramps, skin irritations, vomiting, nausea and anaemia. [19]

Problems with Iron

- Iron may cause conjunctivitis, choroiditis, and retinitis if it contacts and remains in the tissues.
- Chronic inhalation of excessive concentrations of iron oxide fumes or dusts may result in development of a benign pneumoconiosis, called siderosis, which is observable as an x-ray change.
- No physical impairment of lung function has been associated with siderosis. [20]

Main concern to carry out our study is to reduce the effect of heavy metals for the health of human system. The usage of heavy metals in an uncontrolled and miss handled methods lead to the decomposition and distribution of the heavy metals into the natural environments (Soil, Water system, Air, Food, etc.). Our objective is to analyse the amount of heavy metals present in soil and water at a particular locality or in a living system in order to suggest bioremediation mechanisms.

Many studies [21] had shown that due to heavy industrialization and agriculture which led to the contamination of the soil and ground water which lead the bacteria and fungi and other living organisms to resist themselves to heavy metals and too many other many changes in their living form as we can see the above table showing the utilization of different metals and their reducing levels in mines and industries but as India is a agricultural country utilization of agricultural pesticides, herbicides, and other chemicals are used in high quantity which lead to increase in the concentration of different micro element metals like Zinc, Copper, Manganese etc; this lead to changes in the biological organisms and made them resistant for micro elements in large quantity (40mg or >100mg) which may cause many sever problems as soil is reservoir for organisms like bacteria, fungi, viruses which can be pathogenic to humans which may causes many health problems and problem to environment. For this study samples of soil were collected from paddy, corn, sugar cane and tomato fields; and water samples were collected from ground water and sea water. Concentration of metals such as Chromium, nickel, iron and zinc were determined through atomic spectroscopy in the collected environmental samples in order to study their over accumulation crossing the permissible limits due to human interventions and the consequences of their accumulation.

Chapter 3

Materials and Methodology

3.1 Materials

3.1.1 Sampling:-

Samples are collected from different areas as listed below

- Solan

  1. Agriculture Fields (Tomato and Corn fields) (1420 meters above sea level)

  2. Area near Jaypee University of Information Technology

- Vishakapatman (30 meters above sea level)

  1. Paddy fields

  2. Sugarcane fields

  3. Corn fields

  4. Soil collected from home

Figure 4: Showing Corn fields

Figure 5: Showing Paddy fields

Overview of the project

- Isolation of heavy metal and Antibiotic resistant bacteria
- Capability to tolerate Concentration of metals And antibiotics
- Finding for reasons and Reporting that this group is resistant.

Analysis of metals by ASS

3.1.2 Soil chemical analysis:-

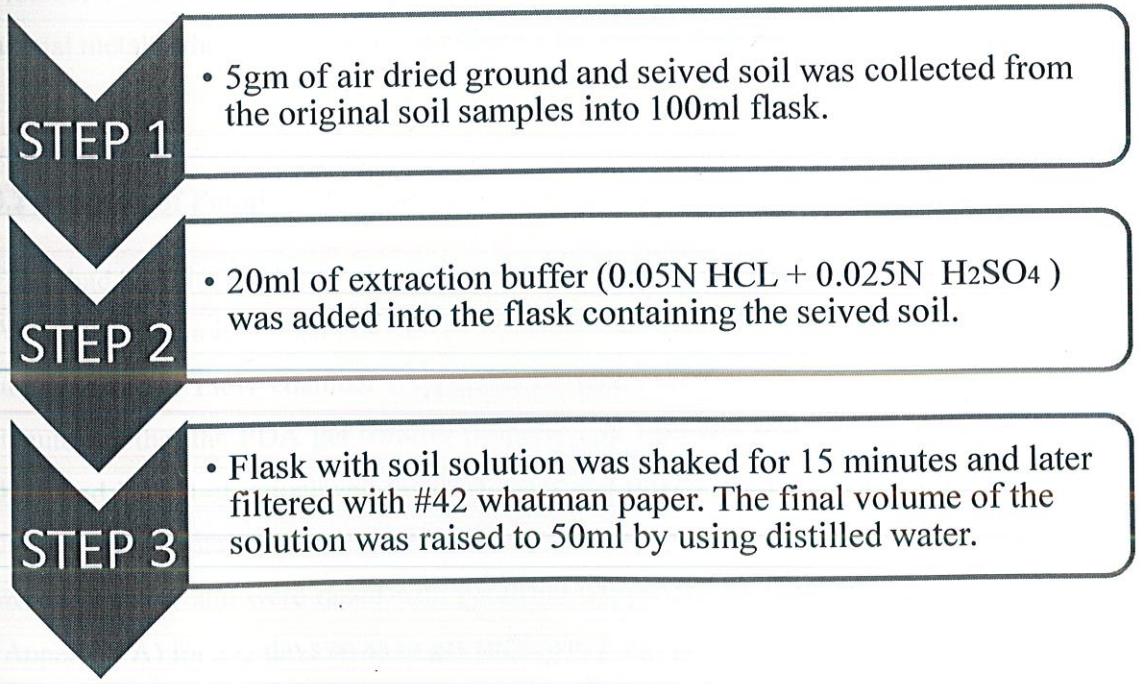
Ph of the had been checked by mixing the soil in distilled water and vortex the test tube containing the soil samples for 1 minute and pH rod containing KCl is inserted into the test tube and they are as fallows

Sample 1	6.42
Sample 2	7.2
Sample 3	7.03
Sample 4	6.08
Sample 5	6.02
Sample 6	6.1
Sample 7	6.81
Sample 8	7.71

3.1.3 Soil Digestion Protocols

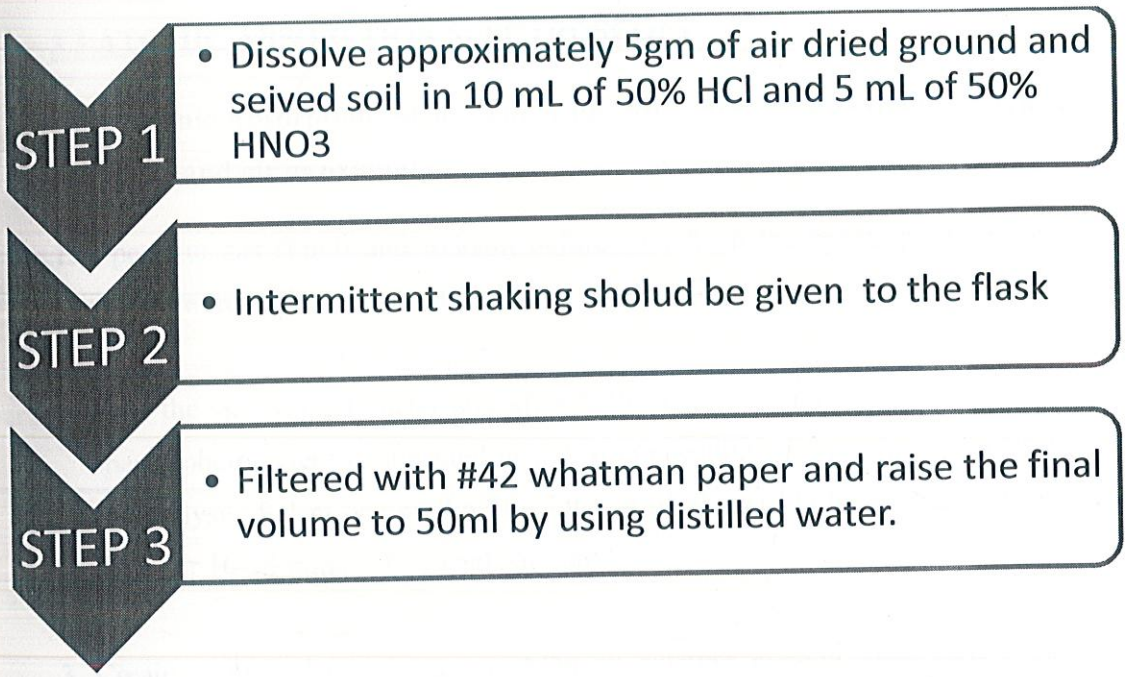
Protocol 1:

Place 5.0 g of an air-dried, ground and sieved sample in an Erlenmeyer flask. Add 20 ml of extracting solution (0.05N HCl + 0.025N H2SO4). Place in a mechanical shaker for 15 minutes. Filter through Whatman #42 filter paper into a 50-mL volumetric flask and dilute to 50 ml with extracting solution.



Protocol 2:

Dissolve approximately 5gm of air dried ground and seived soil in 10 mL of 50% HCl and 5 mL of 50% HNO3 and give Intermittent shaking for 5 minutes then to the flask and then filter the sample with #42 whatman paper and raise the final volume to 30ml by using distilled water.



Protocol 1 was followed in the present study since most of the metals considered for analysis are normal metals whereas protocol 2 is effective for metals with high bonding affinity.

3.2 Isolation of Fungi:-

Fungi had been isolated from the soil samples by making media of Potato Dextrose Agar (Appendix A) and the media had been autoclaved by using Autoclave (Appendix B) and the plating was done in Laminar Air Flow chamber (Appendix B) under sterile conditions. The plates were left for 5 minutes so that the PDA get solidify properly and 1gm soil from each sample has been taken and dissolved in 5ml of autoclaved distilled water and mixed gently, and 10 ul had taken and spreaded on the plates containing solidified media by rotating the plate and moving the spreader. The plates were left to dry and were raped with parafilim (Appendix B) and kept the plates at 37 °C incubator (Appendix A) for 2-3 days so as to get sufficient fungi growth.

A plate is made which contain Zinc metal of concentration of 10mg/ml of which the stock solution is of 0.1M and than the fungi which is grown on the plates are streaked on the plate which had contain Zinc of 10mg/ml and the plate was rapped with parafilm and the kept in incubator for 2-3 days

3.3 ATOMIC ABSORPTION SPECTROSCOPY

3.3.1 Atomic Absorption: Steps to operate: Air – Acetylene System (Acetylene as fuel and air as oxidant)

1. Open the gas (Fuel) and oxidant before switch on the spectrophotometer. Drain out the moisture from air pump by lifting it up from one side

2. Start the spectrophotometer and after 5-10 minute switch on computer and make sure the spectrophotometer is connected or not. If connection present, then open Winlab32 software for analysis. Select one method for interest of element to be analyzed. Then switch on the lamp for 10-15 mins before analysis start.

3. Create a method or we can work in an existing method. (this step is initial step after switching on the instrument)
4. Optimize the burner position with respect to light path and burner height.
5. Open continuous graphics and set "auto zero" without igniting the flame. Rotate the burner base anticlockwise to get maximum absorption and then slowly rotate it clockwise to get zero position and then give an extra ¼ clockwise turn.
6. Ignite the flame from software for nebulizer optimization. Put the sampling tube of nebulizer in one of the standards (e.g. Cu 1mg/ml) to set the max. absorption. Rotate the rear O-ring anticlockwise to set it at maximum absorption and tighten the assembly with the rear O-ring.
7. Optimization of the gas flow to get maximum absorption.
8. Sample analysis protocol as following steps.
9. Analyze blank (e.g. we took 1% HNO_3) twice to get it at zero.
10. Analyze standards one by one. The display will have three readings for the same standards with mean and standard deviation. The same is for unknown samples.
    Note down the readings.

11. Switch off the lamp and put sampling tube in blank for 5 minutes before switching off the flame. Then close the acetylene fuel and bleed gases.
12. Switch off the spectrophotometer.
13. Switch off the exhaust after 5 minutes. This is to make sure the exclusion of all harmful vapours.

3.3.2 Atomic Absorption: Steps to operate: N_2O – Acetylene System (Acetylene as fuel and air as oxidant)

1. Switch on the main power supply connected to N_2O cylinder and let the thermal regulator attached to N_2O to heat up. This is required because high temperature in the thermal regulator will force the gas supply to system. Then, open the gâs supply of the cylinder to a pressure close to 44 psi. Pressure should be within the prescribed safety limits.
2. Open the acetylene gas supply to a max. pressure of $1kg/cm^2$.
3. Make sure that burner with small burner head is in place which is meant for N_2O – Acetylene System.
4. Switch on the instrument and then software.
5. Switch on the lamp required for the element of interest. Wait for 5 min. for stabilization and optimize burner head as explained earlier for Air – Acetylene System. (Note: Path of beam should be uninterrupted passing close to 1 cm above the burner head and orientation of burner needs to be corrected).
6. Go to work space, Switch on the flame and exhaust. Wait for 5 minutes to stabilize the system.

3.3.3 Points to remember:

1. We do not need air in N_2O-Acetylene system.

2. Always see that path of light should be 1 cm up from burner height.

3. Do not forget to start exhaust.

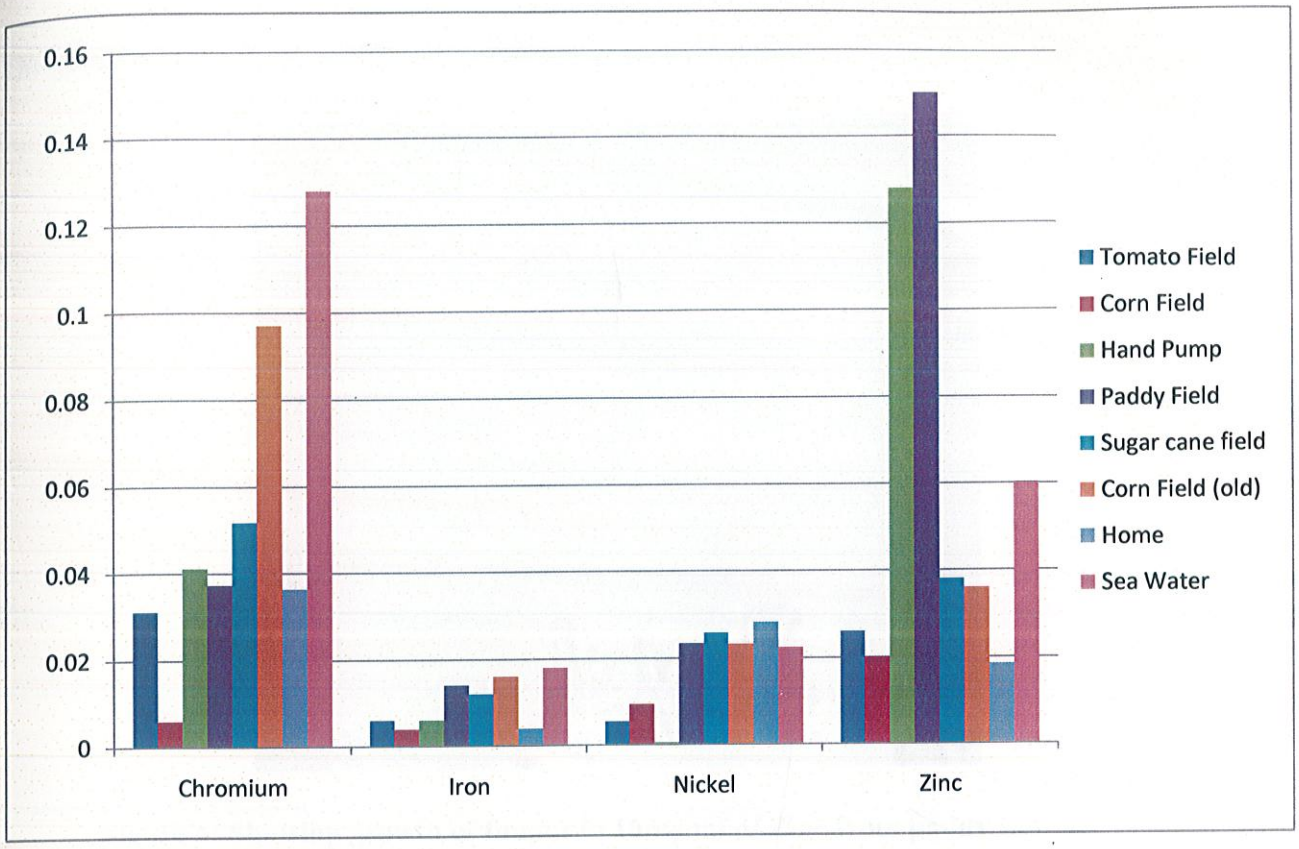
4. Do not touch the burner immediately after the analysis. Wait for few minutes. See any deposition on the burner's slit and scrap it off.

RESULTS

Table 2: Concentration of metals in different samples obtained after atomic spectroscopy analysis

S. No.	Element	Permissible amount (mg/lit)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
1	Chromium	0.05	0.0312	0.006	0.0412	0.0374	0.0518	0.0972	0.0364	0.128
2	Iron	0.30	0.006	0.004	0.006	0.014	0.012	0.016	0.004	0.018
3	Nickel	0.02	0.0054	0.0094	0.0006	0.0234	0.0258	0.0232	0.0282	0.0224
4	Zinc	5	0.026	0.2	0.128	0.15	0.038	0.036	0.184	0.06

Sample 1	Tomato fields
Sample 2	corn fields
Sample 3	Hand pump
Sample 4	paddy fields
Sample 5	sugarcane fields
Sample 6	corn (old) fields
Sample 7	Home
Sample 8	sea water



Graph showing different metal concentration in the samples in mg/ml

The above graph represents concentration of heavy metals (chromium, nickel, iron, zinc) in different samples. It can be inferred from the results that the concentration of chromium and iron heavy metals is more in sea water, nickel concentration is more in soil collected from home and zinc concentration is more in paddy fields.

The fungi from paddy fields are resistant up to 10mg/ml of Zinc

The fungi from soil collected from home are resistant up to 1mg/ml of Nickel

The fungi from Sea water of Vishakhapatnam are resistant up to 1mg/ml of Chromium

The fungi from Sea water are resistant up to 3mg/ml of Iron

The growths of the fungi in different metals are as shown below:-

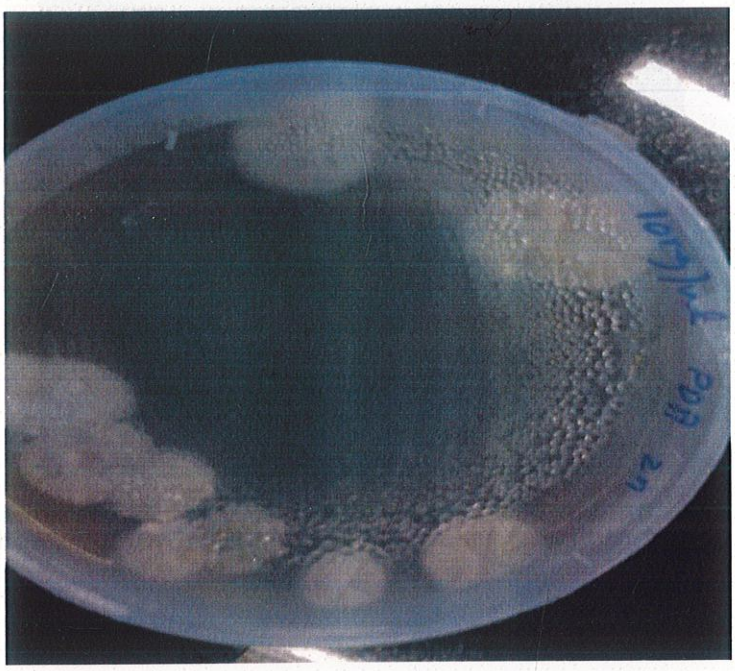


Figure 6: Showing growth of fungus in 10mg/ml of Zinc from paddy sample

Figure 7: Showing growth of fungus in 1mg/ml of Nickel from corn (old)

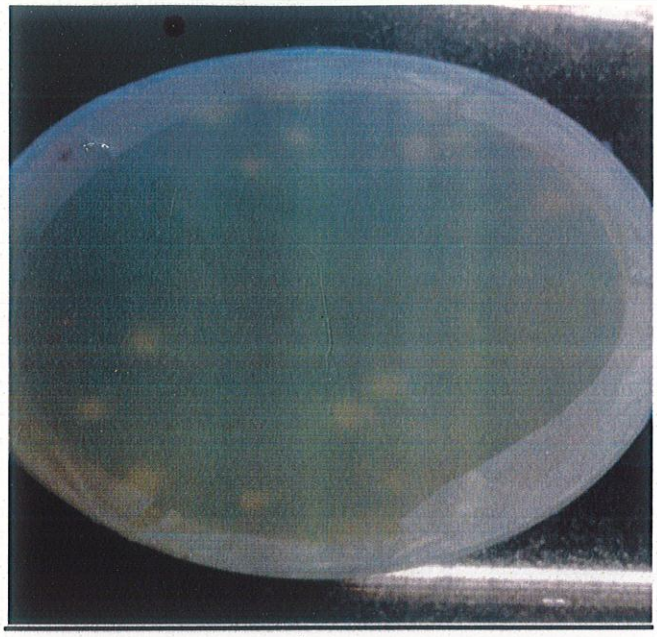


Figure 8: Showing growth of fungus in 1mg/ml of Zinc of sugar cane sample

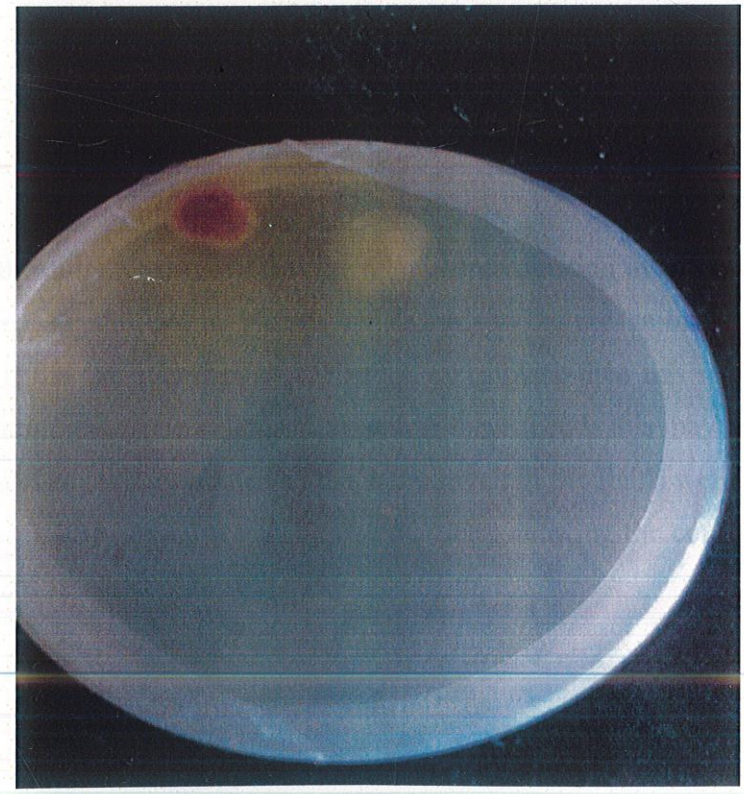


Figure 9: - Showing growth of fungus on cobalt plate (1mg/ml) of tomato sample

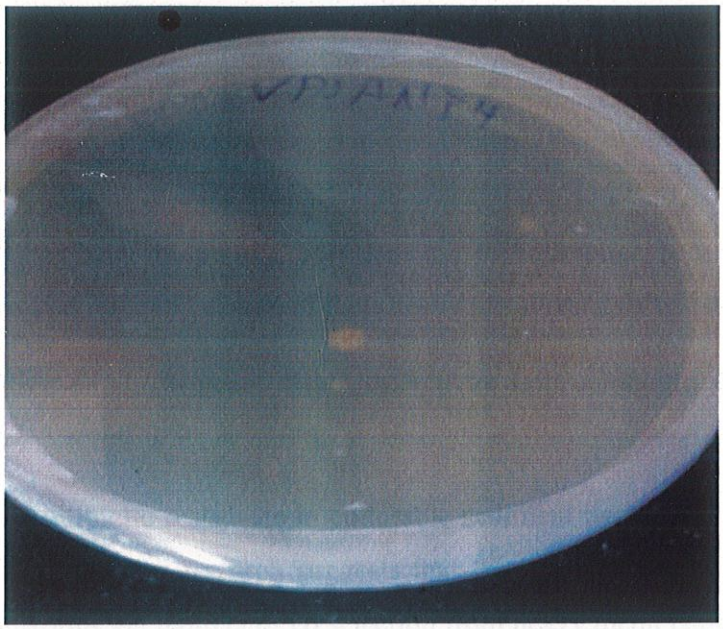


Figure 10: - Showing growth of fungus on cobalt plate (1mg/ml) of tomato sample

DISCUSSION

As the above stated results we can say that the metal contamination in the soil is increasing due to use of pesticides, insecticides and herbicides which leads to the contamination of the soil and also the ground water. If the same amount of utilization takes place then this may lead to many sever problems and the fungi resistance will also increase to these metals this may cause health effects as the ground water is also getting polluted as the water given in the fields were absorbed by soil and cause contamination in the ground water. They are articles showing the contamination of Zinc and Nickel to **50, 100, 200, 300 and 500 µg/g** [22]

3.4 Antibiotic resistant check

- Studies had shown that bacteria are not only resisted to antibiotics because of using them continuously they are also resisted because of their heavy metal resistant nature.
- The changes take place on single gene of the plasmid for both antibiotic resistant and heavy metal resistant.
- The spatial pattern of antibiotic resistance in culturable sediment bacteria from four freshwater streams was examined. Previous research suggests that the prevalence of antibiotic resistance may increase in populations via indirect or co selection from heavy metal contamination. Sample bacteria from each stream were grown in media containing one of four antibiotics-tetracycline, chloramphenicol, kanamycin, and streptomycin-at concentrations greater than the minimum inhibitory concentration, plus a control. Bacteria showed high susceptibilities to the former two antibiotics. [23]

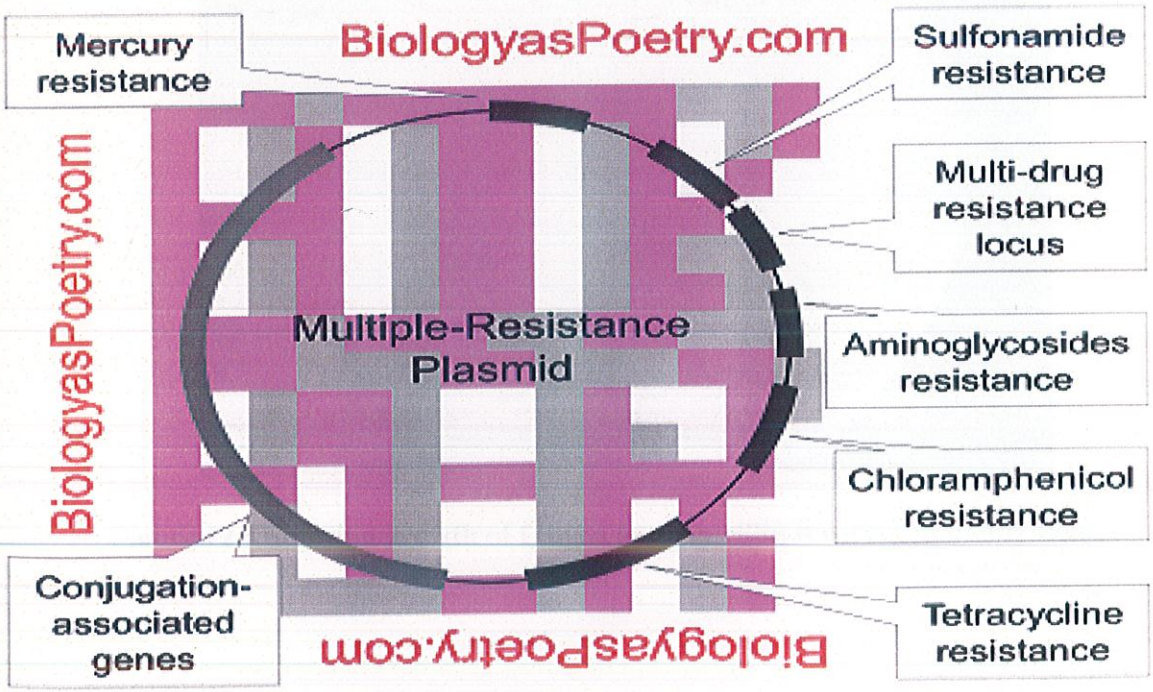


Figure 11: Bacterial Plasmid showing resistance toHeavymetal and antibiotis

[23]

The media (PDA, PDB) are taken and have been autoclaved by using autoclave (Appendix B) at 121 ° Celsius for 15 minutes at 15lds pressure (appendix A)

When the temperature of the media had reached 40 ° Celsius then antibiotic which were sterilized by filter sterilization by using 0.2micron size filter was used had added to the media and poring was done inside Laminar Air Flow chamber (S.M instruments, gurgoun) (appendix A) in patteri plates (genyx co lit) of 25ml are taken and then after pouring the plates were left for solidification and then the soil sample of 1mg had taken and dissolved in 5ml of autoclaved distilled water and mixed gently later 10ul of sample had taken and spreaded on the plate when it get solidified and then it was covered with parafilm and then plates were kept at 37 ° Celsius incubator and then the growth was checked.

The growth is as shown

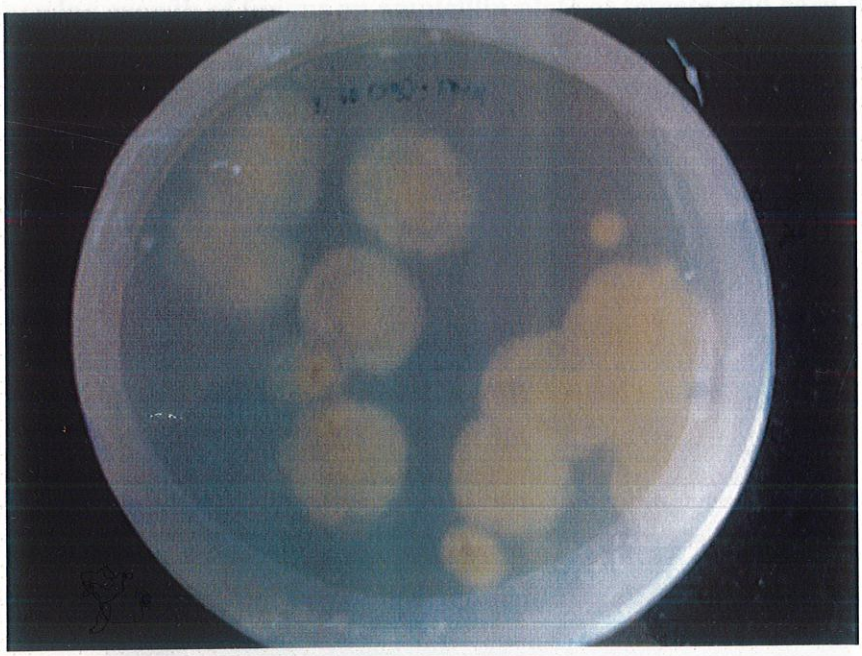


Figure 12:- Showing growth of fungus on ampicillin if corn and paddy fields

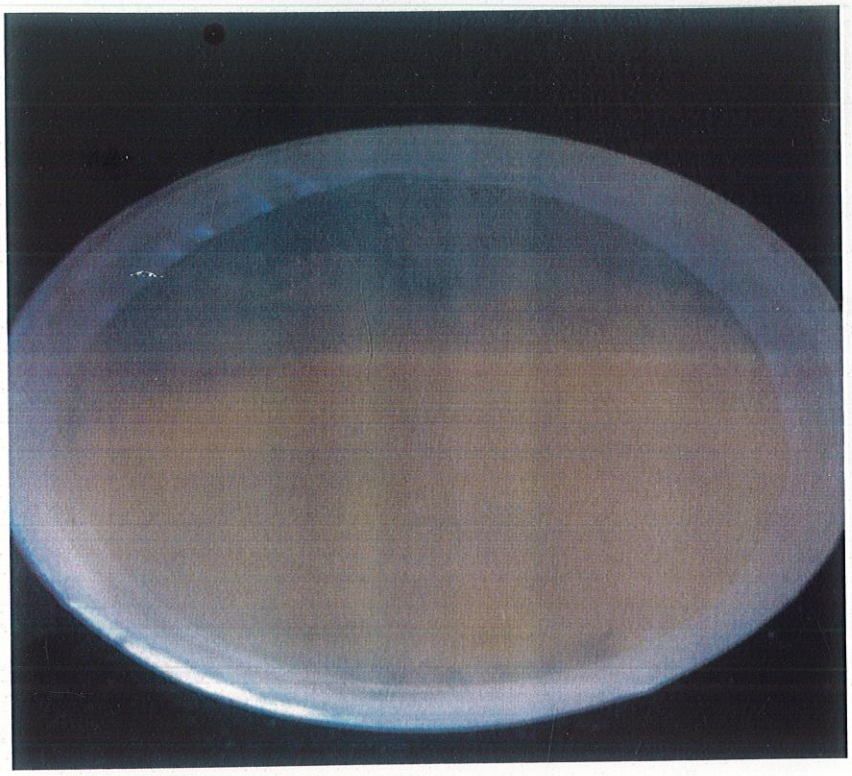


Figure 13:- Showing growth of fungus on ampicillin plate of tomato and corn fields

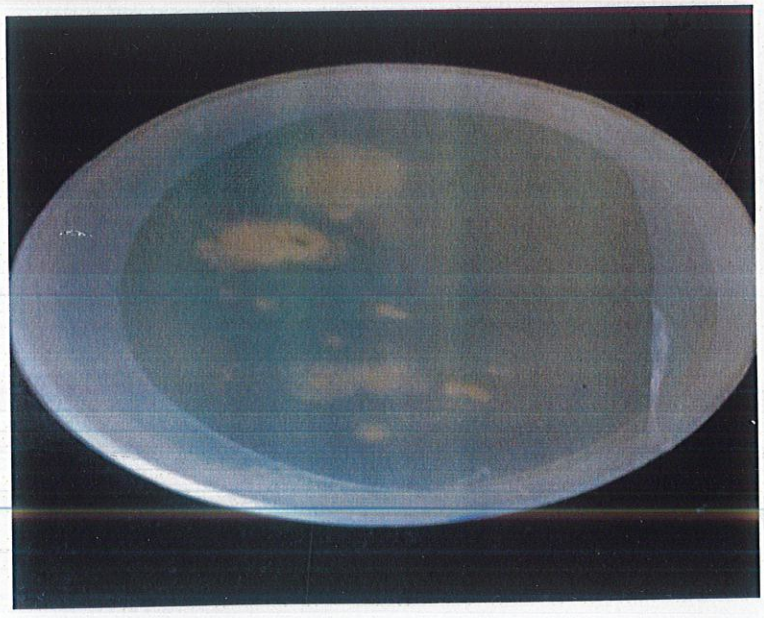


Figure 14:- Showing growth of fungus on ampicillin of Sugar cane and Home fields

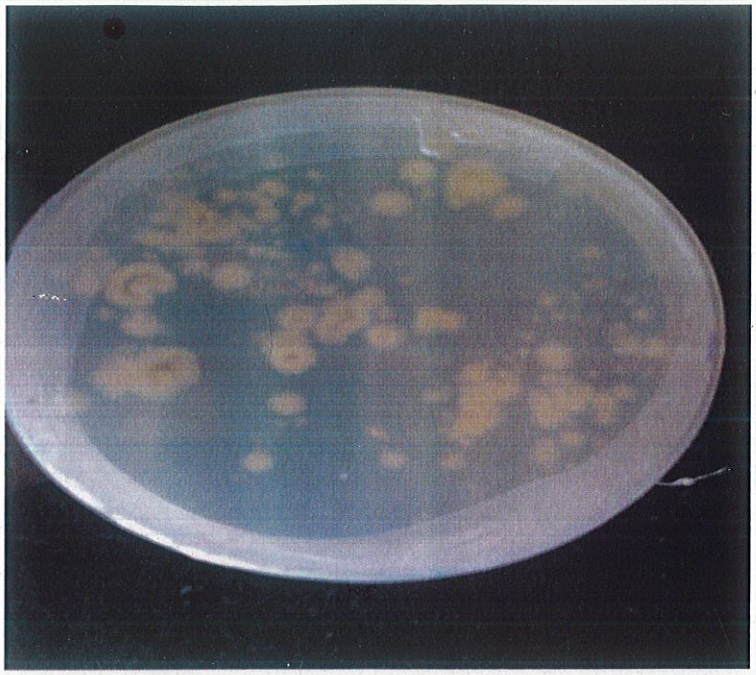


Figure 15:- Showing growth of fungus in kanamycin of Corn and Paddy fields

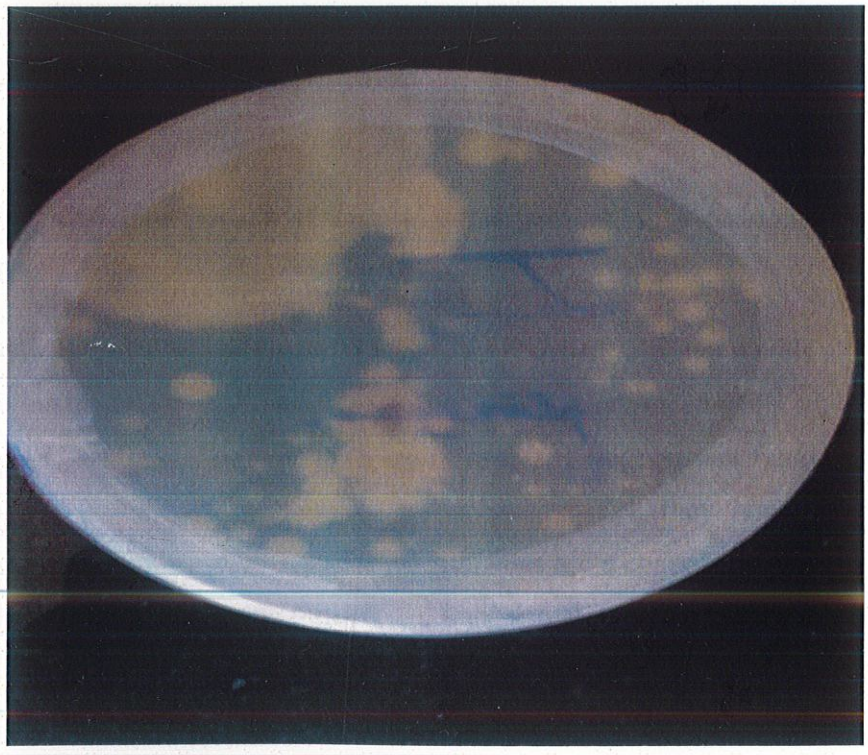


Figure 16:- Showing growth of fungus in kanamycin of tomato and corn fields

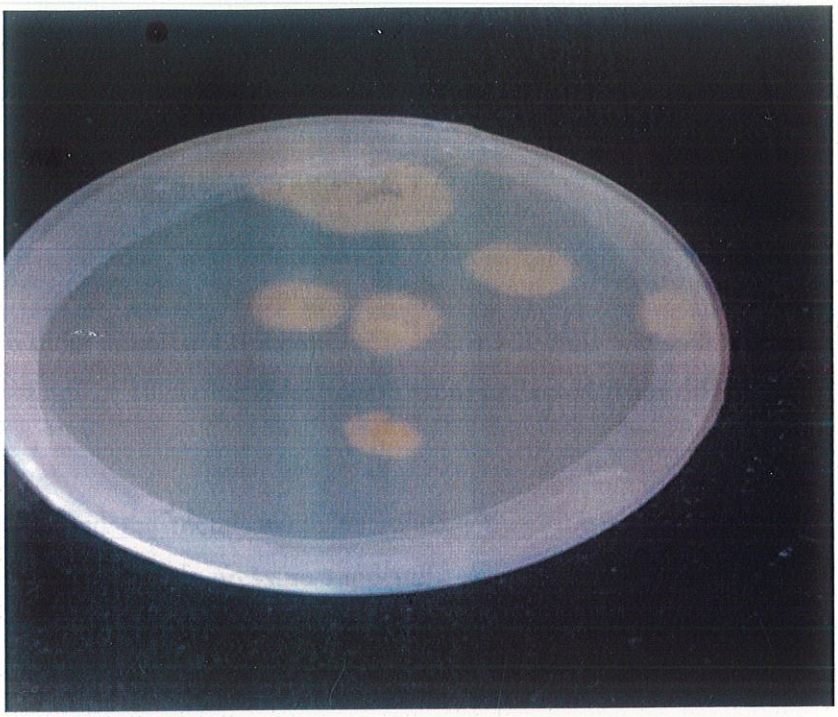


Figure 17:- Showing growth of fungus in kanamycin of Sugar Cane and Home

DISCUSSION

The above figures 12-17 shows the growth of bacteria which are antibiotic resistant (ampicillin, kanamycin of 150ug/ml)

By this we can assume that there may be bacteria which are resistant to heavy metals at different concentrations and this happen due to the growth of fungi on the contaminated soil which lead to make fungi resistant to the antibiotics as well as to heavy metals. Drug resistant fungi cause both acute and chronic infections with high morbidity and mortality. Yet, they remain an under-recognized threat to human health. Most serious fungal infections are a consequence of underlying health problems such as AIDS, cancer, cystic fibrosis and transplantation [24]

3.5 Isolation of fungi resistant to antibiotics and heavy metals

We had also checked the fungi which are resistant for multiple antibiotics and as well as metal and multiple metal resistant fungi in all the samples which were collected from different geographical locations.

The Potato Dextrose Agar and the metals were autoclaved by using autoclave (Appendix A) at 121 ° Celsius for 15 minutes at 15lds pressure (appendix A). Antibiotic were sterilized by filter sterilization by using 0.2micron size filter was used.

When the temperature of the media had reached 40 ° Celsius then antibiotic had added to the media along with that metals were added and then poring was done inside Laminar Air Flow chamber (S.M instruments, gurgoun) (appendix A) in patteri plates (Genaxy co lit) of 25ml are taken and then after pouring the plates were left for solidification and then the soil sample of 1mg had taken and dissolved in 5ml of autoclaved distilled water and mixed gently later 10ul of sample had taken and spreaded on the plate when it get solidified and then it was covered with parafilm and then plates were kept at 37 ° Celsius incubator and then the growth was checked.

Results

There was no growth observed in the plates after 2-3 days so, by this we can conclude that there were no metals which are resistant for both heavy metals and antibiotics

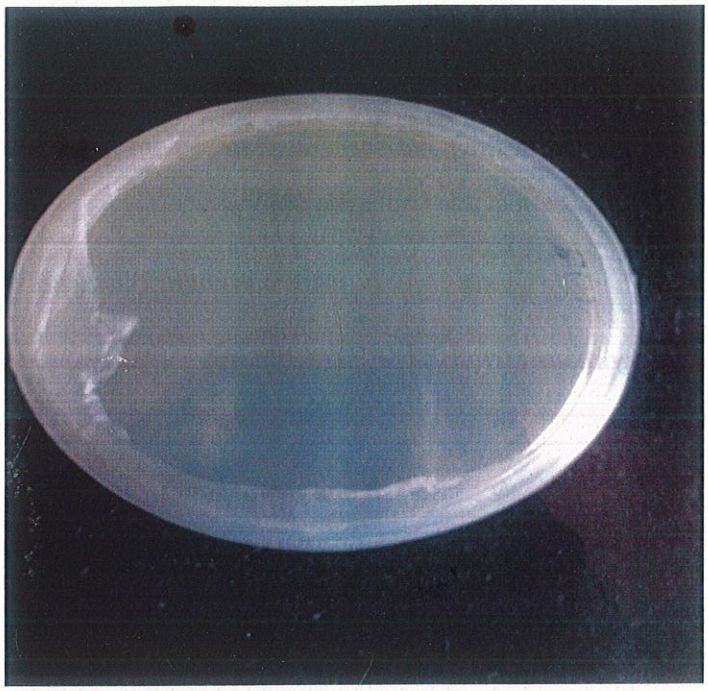


Figure 18:- showing no growth of fungus resistant for metals and antibiotics

Discussion:-

The results of multiple metal resistant along with antibiotics are negative but there is a chance of getting fungi which are resistant to multi metals and antibiotics if the same amount of utilization take place.

3.6 Analysing Zinc resistant growth

Zinc and its uses:

Various applications of zinc are there in now a day's some of them are Zinc Coatings on Steel, Other Zinc Coatings, Building and Construction Industries, Zinc Castings, Zinc Recycling [25]. In pesticides industries metals like Zinc chloride is used in pesticides like.

Zinc metal is a odourless bluish-white lustrous solid with melting point of 419 degree Celsius pure zinc have less stability in environment so it react with other metals and form zinc oxide and other forms.[26]

Zinc chloride: - This is a clear very deliquescent salt with a boiling point of 260 ° Celsius and completely soluble at room temperature. Zinc chloride uses in herbicides and preservatives.[27]

- Used in target of fungus and moss

Zinc oxide: - This odourless white or yellowish powder with melting point 2010 ° Celsius. Zinc oxide is used in Fungicide, Herbicides, bacteriostat. [27]

- Used in target of fungus, moss and bacteria

Zinc sulphate: - This odourless white solid with melting point 600 ° Celsius. Zinc sulphate monohydrate used in herbicides. [27]

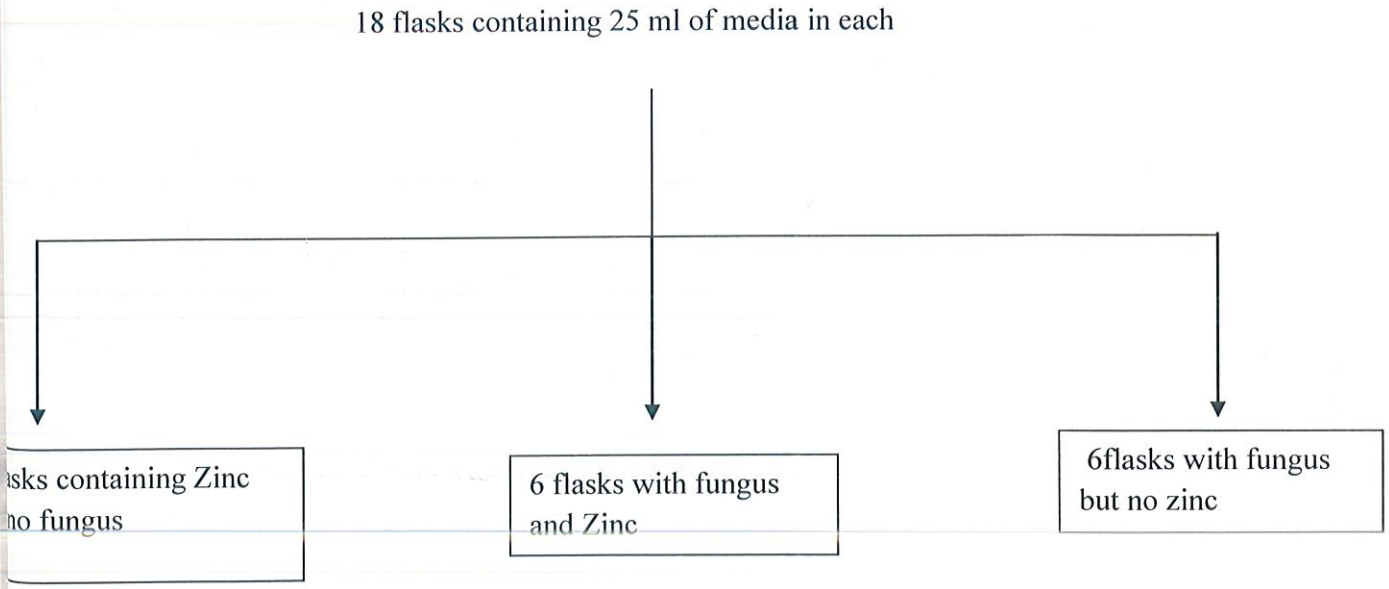
- Target is moss

3.6.1 Cause for Zinc resistant:-

OmZnT1 belongs to the Zn-Type subfamily of the cation diffusion facilitators, whereas OmFET belongs to the family of iron permeases. Their properties were investigated in yeast by functional complementation of mutants affected in metal uptake and metal tolerance. Heterologous expression of *OmZnT1* and *OmFET* in a Zn-sensitive yeast mutant restored the wild-type phenotype. Additionally, *OmZnT1* expression also restored cobalt tolerance in a Co-sensitive mutant. A GFP fusion protein revealed that OmZnT1 was targeted to the endoplasmic reticulum membrane, a result consistent with a function for OmZnT1 in metal sequestration. Similarly to other iron permeases, OmFET-GFP was localized on the plasma membrane. *OmFET* restored the growth of uptake-defective strains for iron and zinc. Zinc-sensitive yeast mutants expressing OmFET specifically accumulated magnesium, as compared to cells transformed with the empty vector. [28]

3.6.2 Work on zinc tolerance

Autoclave the media (pda, pdb) are taken and have been autoclaved by using autoclave (Appendix B) at 121 ° Celsius for 15 minutes at 15lds pressure (appendix A) Poring had done inside Laminar Air Flow chamber (S.M internationals, gurgoun)(appendix A) in plates of 25ml made by (genyx co lit) (appendix B) and the plate containing 10mg/ml concentration of zinc by adding 5ml zinc from stock solution of 0.1M zinc which is autoclaved in 50ml of autoclaved media and mixed the media slowly to avoid air bubbles and pour the media in the plates and then after left the plates for solidification and then the fungus which have resistance to zinc at 10mg/ml concentration had taken and streaked on the new plate and then left for 3days for enough growth and in between this 25ml of PDB media has been prepared 18 flask in which 6 flasks are with zinc (10mg/ml) in which fungus and 6 flask were with fungus in which zinc is absent and 6 flasks are with both zinc and fungus.

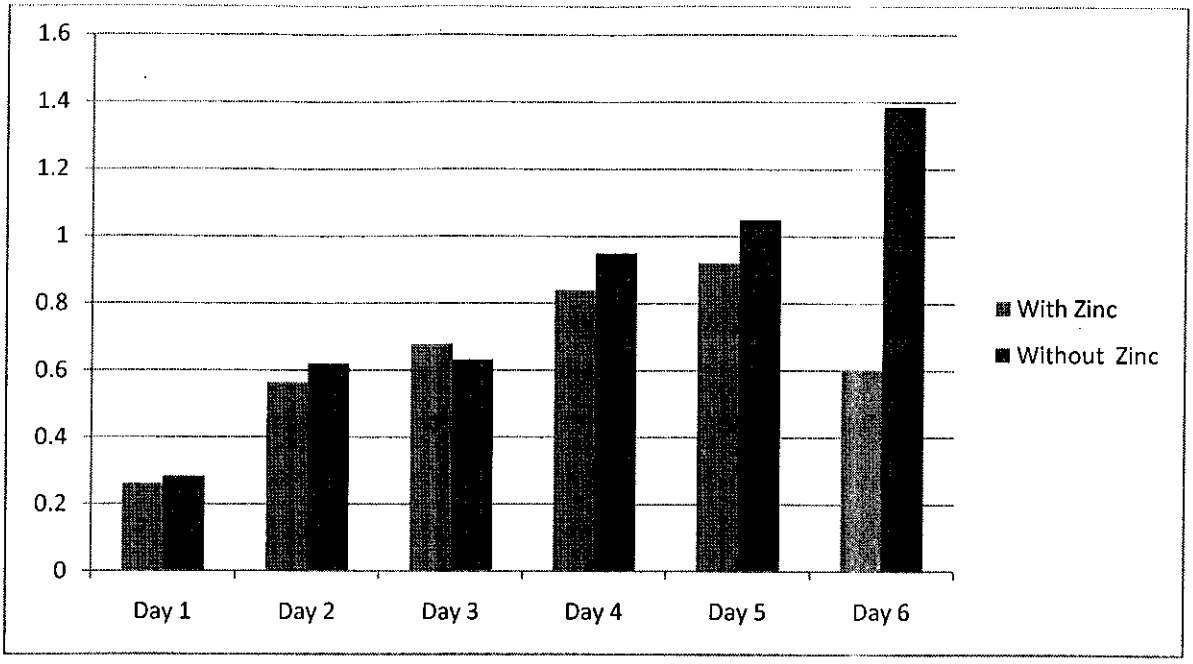


The inoculation was done by a straw of diameter 3mm the straws were sterilized by using 70% ethanol and then left overnight at 50 ° Celsius and then the straw was taken and then inoculation had took place by putting the straw in the plate and cutting the media with the help of the straw and inoculation had been done in the flask, similarly in the same way rest of the flasks had been inoculated in the same way as stated above.

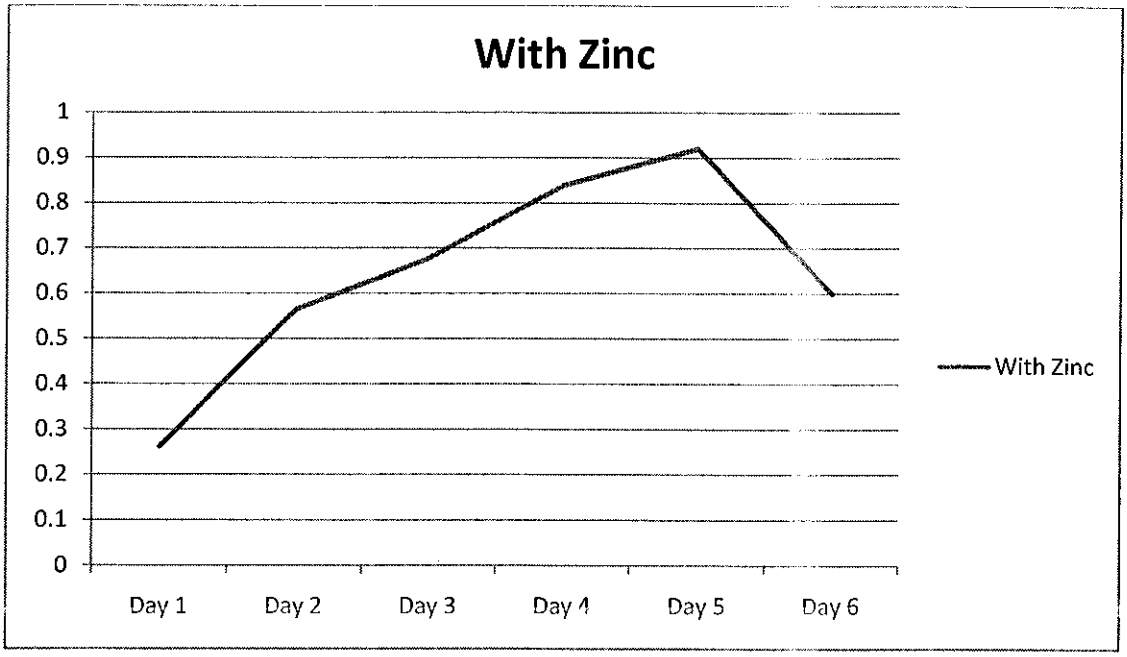
The growth of the fungus was checked every day by finding the dry weight by transferring the fungus into a 50ml tarson vial and centrifuges the tarson vial by using centrifuge of (Appendix B) and then the media was collected into another tarson vial and then the fungus was washed with autoclaved distilled water 2-3 times and that the fungus was kept in oven for 5 hours at 80 ° Celsius and then left the vials under sun by covering it with parafilm to prevent the enter of the dust into the vial till the fungus get deride completely. The weight of the fungus was taken by using weighing balance and the weights are listed in below table.

S.no	Day	Avg wt of vial (empty) (gm)	Weight of fungus **with zinc** (gm)	Weight of fungus **without zinc** (gm)	Wt of dried fungus **with zinc** (gm)	Wt of dried fungus **without zinc** (gm)
1	Day 1	12.06	12.321	12.343	0.261	0.283
2	Day 2	12.06	12.623	12.679	0.563	0.619
3	Day 3	12.06	12.737	12.691	0.677	0.631
4	Day 4	12.06	12.899	13.009	0.839	0.949
5	Day 5	12.06	12.980	13.109	0.920	1.049
6	Day 6	12.06	12.659	13.444	0.599	1.384

Table3:- Growth fo fungus from day 1 to day 6



Graph2:- Showing growth of fungus in both presence and absence of Zinc



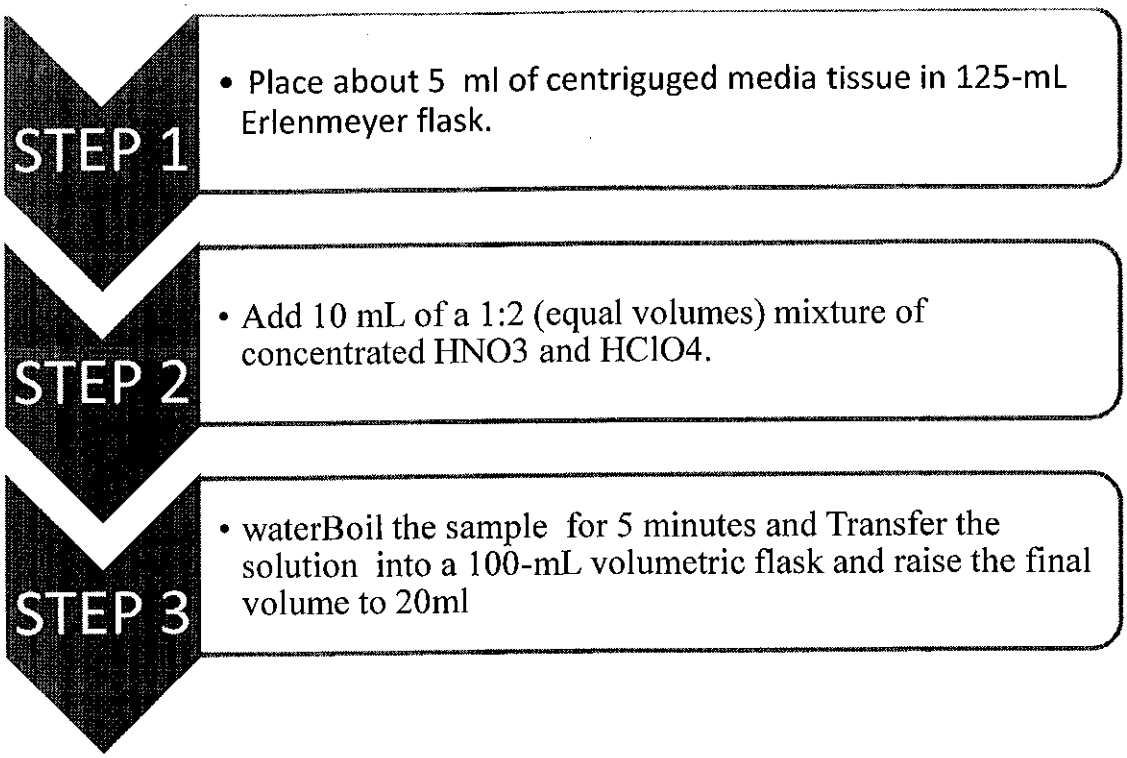
Graph3:- Showing the growth of fungus within 6 days in the presences of zinc

Figure 19: Showing growth of fungus in PDB, containing Zinc

3.6.3 Digestion of media

Digestion of media in which the fungus is growing along with zinc was digested by using Nitric acid and Perchloric acid in 1:2 ratio as fallows

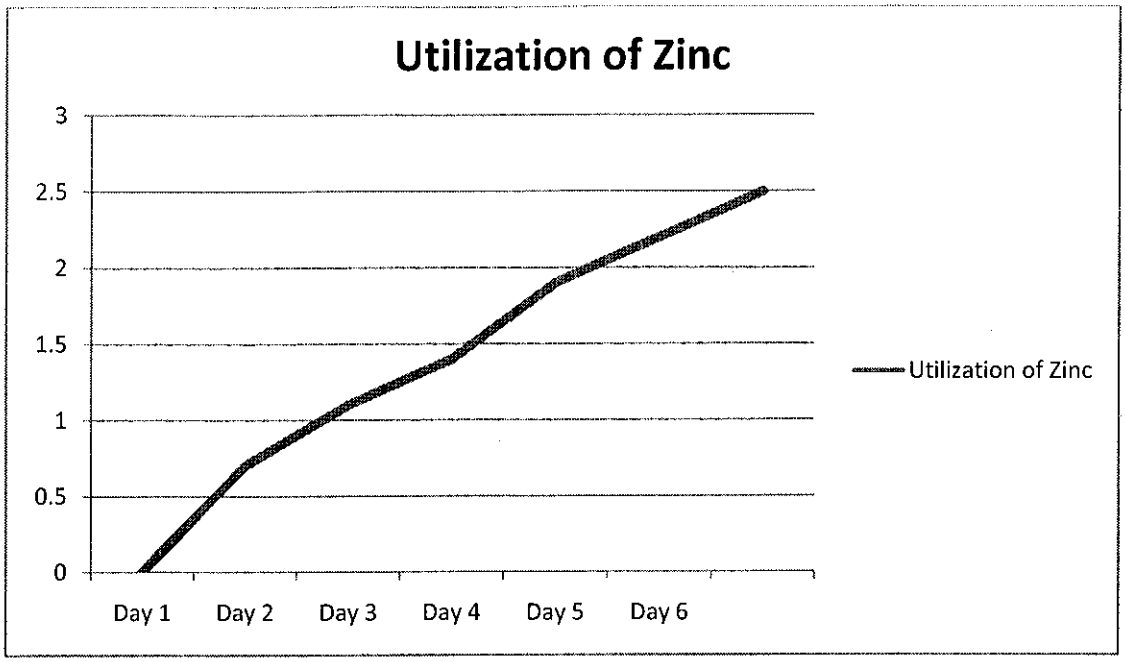
The media was taken and centrifuged by using eltek centrifuge (Appendix A) at 6000 Rpm for 10min and then the 5ml of clear media had taken in a flask and 10ml of HN03 and HCl04 in 1:2 ratio was added and then the flasks were kept in water bath at 100 ° Celsius for 5 min and then the media was transferred to another flask and then the media was diluted in 2ml in 18ml of 1% HN03 for finding the concentration of the zinc left in the media by using AAS of (Appendix B).



Results:-

S.no	Day	Original concentration	After growth of fungus	Utilizations
0	Day 0	10 mg/ml	10 mg/ml	0
1	Day1	10mg/ml	9.3 mg/ml	0.7
2	Day 2	10mg/ml	9.1 mg/ml	1.1
3	Day 3	10mg/ml	8.6 mg/ml	1.4
4	Day 4	10mg/ml	8.1 mg/ml	1.9
5	Day 5	10mg/ml	7.8 mg/ml	2.2
6	Day 6	10mg/ml	7.7 mg/ml	2.3

Table 4:- Showing utilization of Zinc by fungus



Graph4:- Sowing utilization of Zinc by fungus

Chapter 4

Conclusions:-

In summary, changes in zinc sulphate from 1mg-10mg concentrations in the nutrient media had an effect on the abundance and diversity of cultivable soil fungi in the laboratory experiment. It was shown that zinc influenced fungal populations differently, apparently due to the fact that each of them exerts selection pressure for soil fungi with specific properties. Intermediate concentrations of these metals seem to enhance abundance and diversity of fungal populations. At elevated metal concentrations in the medium the abundance of cultivable fungi decreased with marked elimination of some fungus species from populations. The reactions of the fungal communities from different soil types suggested that there is no general resistance of the total community but rather resistance of particular species. Furthermore, fungi distinguished by their tolerance to Zn even at high concentrations of metals in the medium. *Aspergillus Spp* which recently were proved as insect pathogens were also metal-resistant. Such metal-resistance peculiarities of entomopathogenic fungi may be explored for the search of virulent strains to be applied as biopesticides. We presume that these results can also reflect tolerance of the fungal communities and populations in the soil.

Bioremediation methods going to be under taken for standardization and analysed for the better treatment:

1. By growing plants (metallophites) in the area where heavy metals are used.
2. Controlling the use of heavy metals by using alternative biological materials/ living systems.
3. Development of physical methods in combination with biological systems to clean the contaminated environments.
4. Suggestion of alternative food materials to reduce the development of heavy metals associated resistant bacteria inside the human system.

APPENDIX - A

PDA – Media was made by HIMEDIA (Mumbai)

Contents: - Infusion from potatoes 200grm/lit

Dextrose- 20grm/lit

Agar- 15mg/lit

Making - 39grm in 1000ml

PDB – was made by HIMEDIA (Mumbai)

Contents: - Infusion from potatoes 200 grm/lit

Dextrose- 20grm/lit

Making – 24grm in 1000ml

Zinc Sulphite: - $ZnSO_4.7H_2O$

Qualigens Fine Chemicals

Division of GLAXO India Limited

Mumbai

Maximum assay is 99%

Nickel Sulphite: - $NiSO_4.6H_2O$

S.D fine chemicals limited

Mumbai

Maximum assay is 98%

Cobaltus Chloride: - $CoCl_2. 6H_2O$

Qualigens Fine Chemicals

Division of GLAXO India Limited

Mumbai

Maximum assay is 97%

Mercuric Chloride: - $HgCl_2$

Merck limited

Mumbai

Maximum assay is 99%

APPENDIX - B

List of instruments

Atomic Spectroscopy (prekin elmer)

Vortex (Banglore Genel)

Serological Water bath NSW-125

Laminar Flow Bench (S.M internationals)

Autoclave (NSW, Eqitron, India)

Micropipette (Eppendorf and Axygen)

Microtips (Axygen)

Glassware (Borosil)

Centrifuge (Elteck)

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