MYCOREMEDIATION OF HEAVY METAL CONTAMINATED SOIL USING MUSHROOMS

Thesis

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by

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DECLARATION

I, **Dilna Damodaran P.V**., declare that the Research thesis entitled "**Mycoremediation of heavy metal contaminated soil using mushrooms**" which is being submitted to the **National Institute of Technology Karnataka, Surathkal** in partial fulfillment of requirements for the award of the degree of **Doctor of Philosophy** in **Chemical Engineering** is a bonafide report of the research work carried out by me. The material contained in this Research thesis has not been submitted to any University or Institution for the award of any degree.

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ABSTRACT

Remediation of soil contaminated with heavy metals has received considerable attention in recent years even though contamination of soil is not considered as pollution compared to air and water pollution. Low cost mitigation measures like phytoremediation and mycoremediation are commonly employed. Mycoremediation using macro fungi (mushroom) have proven to be efficient in removing heavy metals from soil through bioaccumulation. In the present study, *Galerina vittiformis*, a wild mushroom belonging to Strophariacea family was identified to effectively remove the heavy metals namely, Cu(II), Cd(II), Cr(VI), Pb(II) and Zn(II) at both 50 and 100 mg/kg from the soil under study (*in-vitro*). *G. vittiformis* was found to be effective in removing the metals from soil within 30 days. The bioaccumulation factor (BAF) for both mycelia and fruiting bodies with respect to these heavy metals at 50 mg/kg concentrations were found to be greater than one, which indicates hyper accumulating mechanism by this mushroom species. Bioaccumulation by both mycelial and fruiting body stage of the mushroom is found to follow the order; Pb(II) > Cd(II) > Zn(II) > Cu(II) > Cr(VI). The metal removal rates by the mushroom was analyzed using different kinetic rate constants and found to follow the second order kinetic rate equation for Pb(II), Cr(VI), Zn(II) and Cu(II) and first order rate kinetics for Cd(II). The metal uptake mechanism studies of *G.vittiformis* revealed the production of two types of Phytochelatins, namely PC_1 and PC_3 in response to heavy metal stress. These Phytochelatins are known to transfer the excess metal ions into the vacuoles of the cell and thereby reducing the metal toxicity in the cell. Both chemical and biological chelaters up to 10 mmol/ kg concentrations did not have any significant role in increasing the bioaccumulation potential of the *G.vittiformis*. Thus *G.vittiformis* exhibited high potential for mycoremediation of heavy metals without the help of chelaters. The maximum activity of *G. vittiformis* in terms of metal bioaccumulation from soil was found to occur at metal concentrations around 150 mg/kg for each of the metals under study in a multi-metal interaction system. *G. vittiformis* was found to be more effective in the removal of Pb(II), Cd(II), Cu(II) and Zn(II) from multi metal contaminated soil when compared to Cr(VI). The soil pH of around 6.5 was found to

be favorable for metal removal. Thus it may be suggested that *G. vittiformis* was identified as an effective bio-remediating agent in both single and multi-metal contaminated conditions in comparison to any other mushrooms reported in the literature.

Key words: *Bioaccumulation factor, Galerina vittiformis, Fruiting body, Heavy metals, Mycelia, Multi-metal interaction, Mushrooms, Mycoremediation, Phytochelatins.*

DEDICATED TO
MY PARENTS
AND
TEACHERS

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NOMENCLATURE

Description	Symbol
Base pairs	bp
Centimeter	cm
Degree Centigrade	^{o}C
Dollar	$
Equal	=
Gram per litre	g/L
Grams	g
Greater than	<
Hour	h
Kilo grams	kg
Kilovolt	kV
Lesser than	>
Litre	L
Mass per chagre	m/z
Meter	m
Micro litre	µl
Milli grams	mg
Milli meter	mm
Millimoles	mM
Northern hemisphere	N

Percentage	%
pH	pH
Time	t
Voltage	V

LIST OF ABBRIVATIONS

Abbreviation	Description
AAS	Atomic absorbtion spectroscopy
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
CA	Citric acid
CCD	Central composite design
cDNA	Complementary DNA
CPCB	Central Pollution Control Board
CPCB	Central pollution control board
DDT	Dichlorodiphenyltrichloroethane
DOE	Design of experiment
DTPA	Diethylene Triamino Penta Acetic acid
EDDS	[S,S]-ethylenediaminedisuccinic acid
EDTA	Ethylene diamine triaceticacid
EPA	Environmental protection agency
ESI	Electro spray ionization
FLAA	Flame atomic absorption spectrophotometry
FTIR	Fourier transform infrared spectroscopy
GA	Gallic acid
GFAA	Graphite furnace atomic absorption spectrophotometry

GSH	Glutathione
GSSG	Glutathione disulfide
HCL	Hydrochloric acid
HEDTA	N-(2- hydroxyethyle) ethyl diamine triacetic acid
HNO_3	Nitric acid
HR-ICP-MS	High resolution Inductively Coupled Plasma Mass Spectrometry
HSP	Heat shock proteins
ICP	Inductive coupled plasma mass spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
ITS	Internal transcribed spacer sequence analysis
MG	Methylglyoxal
MIC	Minimum inhibitory concentration
MRA	Multiple regression analysis
MRM	Multiple reaction monitoring
PC	Phytochelatins
ROS	Reactive oxygen species
RSD	Relative standard deviation
RSM	Response surface methodology
SDA	Sabrouds dextrose agar
SEC	Size exclusion chromatography
SEM-EDX	Scanning electron microscopy with energy

	dispersive X-ray spectroscopy
TBP	Tris- bufferd phenol
US-EPA	United states Environmental protection agency
WHO	World Health Organization
XRF	X-ray fluorescence

CHAPTER – 1

INTRODUCTION

Pollution can be defined as an undesirable change in the physical, chemical, or biological characteristics of the natural environment. The introduction of harmful substances or energy into environment by man's activities, cause ecological damage or interfere with legitimate use of the environment. Pollution may affect the soil, aquatic systems, or the atmosphere. Other forms of pollution in the environment include noise (e.g. from jet aircraft, traffic, and industrial processes) and thermal pollution (e.g. the release of excessive waste heat into lakes or rivers causing harm to wildlife). After the Industrial Revolution, due to increasing industrial processes such as fossil fuel combustion and mining activity, many pollutants have been emitted into the environment. This has resulted in an increase in the level of pollutants in the environment leading to high level of contamination of the environmental components such as air, soil and water. There are two main classes of pollutants: those that are biodegradable and non biodegradable. Biodegradable ones (e.g. sewage), can be rendered harmless by natural processes and therefore cause no permanent harm if adequately dispersed or treated; and those that are non biodegradable (e.g. heavy metals and poly chlorinated components like DDT), which eventually accumulate in the environment and may be concentrated in food chains (Allen 1988).

Among these pollutants, trace elements, especially heavy metals, are very important in relation to the toxicological and nutritional aspects. In recent years, heavy metal pollution has gained increasing attention due to its possible toxic effects. Many elements, for example arsenic, cadmium, mercury, etc., are toxic to living organisms at trace levels. The soil and ground water quality data reported by CPCB (Central Pollution Control Board), India in December 2009 has revealed that heavy metals like Cadmium, Lead, Mercury, Chromium, Cobalt, Zinc, Nickel and Manganese are the major pollutants and immediate mitigation measures are in need to bring down their levels in the environment.

Soil pollution induces a threat to ecological environment, food security and sustainable agricultural development. As per Environmental Protection Agency report

(EPA) published in July 2006 direct economic losses due to heavy metal contaminated soil in US exceed 2.5 billion U.S. dollars. Soil pollution has worsened and today there are more than 2,00,000 sites awaiting EPA (EPA/540/N-03/001) soil cleanup, which is very expensive and labor-intensive work. Even a small cleanup project can cost $10,000, while larger areas require millions of dollars to clean it up for future use. According to 2006 statistical report of Evironmental Protection Agency, about 12 million tons of grains are polluted each year by heavy metals that have found their way into the soil. A brief investigation of about 1.5 million acre of contaminated lands across India, revealed that out of 3250 acre of cultivated land irrigated with sewage water and solid waste 200 hectares of land has been destroyed due to dumping. The total area of arable land accounts for approximately 1/10, most of them concentrated in the economically developed areas. It was estimated that, each year due to heavy metal pollution in United States, 12 million tons of food has been wasted causing direct economic losses of more than 20 billion dollars (Dermirbas 2000). Soil pollution caused by harmful substances lead to accumulation of these in crops and enters the human body through the food chain, leading to various diseases in man. Soil pollution affects soil ecosystem's structure and functions that eventually poses a threat to ecological security.

Metal contamination of soil is considered to be hazardous to mankind as accumulation of metals in the cells, results in various disease conditions even at low concentration. The metals are classified as "heavy metals" if they have a specific gravity of more than 5 g/cm^3 in their standard state. There are 35 metals that are commonly found in our environment leading to occupational or residential exposure to men; 23 of these are the heavy elements or "heavy metals" such as antimony, arsenic, bismuth, cadmium, cerium, chromium, cobalt, copper, gallium, gold, iron, lead, manganese, mercury, nickel, platinum, silver, tellurium, thallium, tin, uranium, vanadium, and zinc. Heavy metals have the tendency to get accumulated in soils and plants. In plants they are found to create a negative influence on physiological activities like photosynthesis, gaseous exchange, and nutrient absorption causing

reductions in plant growth and yield (Devkota et al. 1981; Baker et al. 2007). In small concentrations, the traces of heavy metals in plants or animals are found to be nontoxic (Vries et al. 2007). Lead, cadmium and mercury are exceptions; they are toxic even at very low concentrations (Gorchev et al. 1991). Heavy metals may be retained in the soil as exchangeable metals, carbonates, hydroxides and oxides. In most cases, heavy metals are retained in the upper horizon of the soils (< 0.5 m) depending on local environmental conditions. Several applications of heavy metals largely in industrial sectors, through agricultural sources, road systems and sewage disposal lead to their wide distribution in soil, silt and water. The progressive accumulation of metals may inhibit the growth of indigenous organisms leading to lower degradation of organic pollutants or humus substances in the environment.

The concentrations of heavy metals need to be reduced to meet ever-changing legislative standards. According to the World Health Organization (WHO 1984), the metals of most immediate concern are cadmium, chromium, cobalt, copper, lead, nickel, mercury and zinc. Paper industries, mining and smelting industries, basic metal industries, chemical industries, petroleum refineries, food industry etc. are reported to release heavy metals in large concentrations in both sewage and industrial exhaust (EPA report 2012). These metals contaminate the soil and ground water, thus leading to exposure of human beings to these metals. Heavy metal toxicity can result in damaged or reduced central nervous function, lower energy levels, and damage to blood composition, lungs, kidneys, liver, and other vital organs. Long-term exposure may result in slowly progressing physical, muscular, and neurological degenerative processes that mimic Alzheimer's disease, Parkinson's disease and multiple sclerosis. Repeated long-term contact with some of these heavy metals or their compounds may even cause cancer (Hamman 2004; Mahavi 2005).

Metals cannot be destroyed, hence remediation of metal contaminated soil consists primarily of manipulating (i.e. exploiting, increasing, decreasing, or maintaining) the mobility of metal contaminant(s) to produce a treated soil that has an

acceptable total or leachable metal content. Metal mobility in soil-waste systems are determined by the type and quantity of soil surfaces, the concentration of metal of interest, the concentration and type of competing ions and complex ligands (both organic and inorganic). The pH, redox status and long term effects must also be considered for the mobility of metal ions in the soil. As organic constituents of the waste matrix degrade, its pH or redox condition changes, either through a natural process of weathering or human manipulation.Thus, the potential mobility of the metals is expected to change.

The scientific and technological challenges to be addressed are:

i) Understand the interactions between soil and heavy metals

ii) Establish a better scientific basis for deciding:

   a. What remediation approach to take at a given site?

   b. Which heavy metal forms in the soil are removable?

iii) Develop new cleanup technologies or enhance existing ones

iv) Develop a remediation process for heavy metals which is cost effective and environmentally friendly.

General scheme for soil remediation is presented in Fig.1.1.The only possible remediation method is based on concentration and subsequent removal. This concentrated end product can afterwards be dumped in a controlled way or recycled for metal recovery. Clean up (or remediation) technologies are available for reducing the harmful effects at heavy metal-contaminated sites. These include solidification / stabilization, soil washing, soil flushing, and bioremediation / phytoremediation.

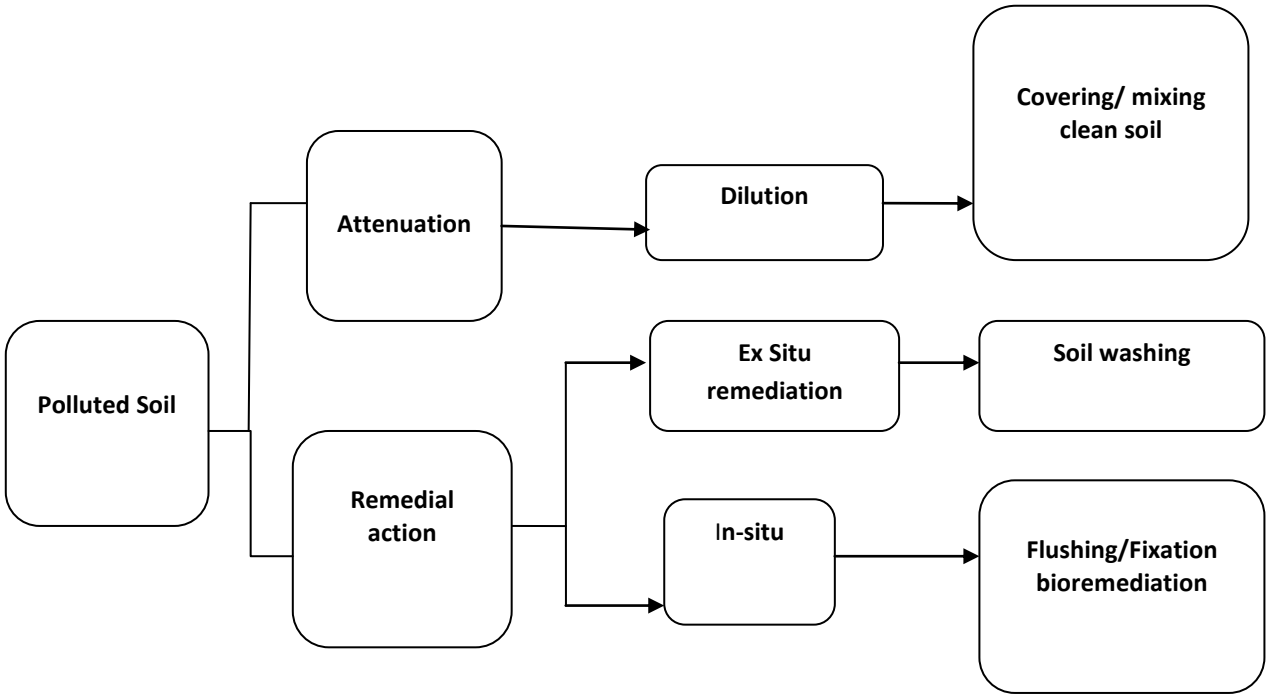


Fig. 1.1 General scheme for soil remediation procedures

1.1 Cleanup methods

Conventional technologies for clean-up or remediation involves the removal of metals from polluted soils by soil washing (Francis et al. 1999). This decontamination strategy is an *ex-situ* approach and found to be expensive, damages the soil structure and its ecology (Salt et al. 1995). The common methods to remediate metal-contaminated soil are soil flushing, solidification/stabilization, vitrification, thermal desorption and encapsulation (Bio-Wise 2003) However, these methods are applicable for smaller areas of soil sites with high metal contamination. These *in-situ* remediation methods require high energy input and expensive machinery as reported by Schnoor (1997) in his work on *in-situ* remediation process. *In-situ* remediation methods lead to the destruction of soil structure and decrease their productivity (Leumann et al. 1995).

The solubilization of metal from soil can be done by chemical leaching. The metal displaced in this way can be recovered via precipitation, adsorption, transformation and complexation processes. Immobilization of heavy metals through the addition of lime (Krebs et al. 1999), phosphate (Ebbs et al. 1998) and calcium carbonate ($CaCO_3$) (Chen et al. 2000) have been practiced as remediation techniques in common. These remediation technologies have the advantage of immediately reducing the risk factors arising from metal contamination, but may only be considered temporary alternatives because the metals will not be removed completely from the soil environment. In response to the growing needs of environmental issues due to soil contamination, many remediation technologies have been developed to treat soil leachate, by both *in-situ* and *ex-situ* methods (Aboulroos et al 2006; Fransis et al. 1999; Salt et al. 1995).

A particular contaminated site may require a combination of procedures to allow the optimum remediation for the prevailing conditions. Fig 1.2 shows the sequence of physicochemical methods for remediation of metal contaminated soil.

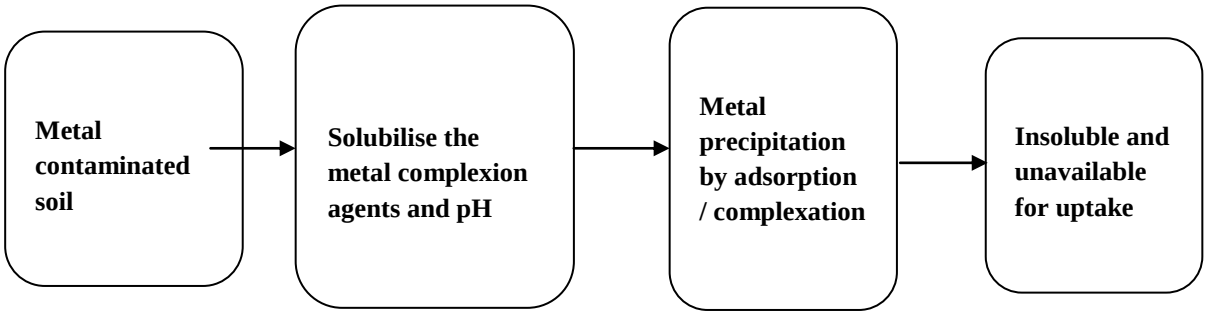


Fig 1.2 Sequence of physicochemical methods for remediation of metal contaminated Soil

Physical, chemical and biological cleanup technologies may be used in conjunction with one another to reduce the contamination to safer and acceptable levels. Bioremediation techniques are typically more economical than thermal and physico-chemical remediation methods (Selena et al. 2012;Cho et al. 2000;

Korda et al. 1997). Schnoor (1997) has also compared the cost of bioremediation with other soil remediation approaches like excavation and *in-situ* fixation. The comparative costs as reported by Schnoor (1997) are presented in Table 1.1.

Table 1.1 Cost involved in various kinds of soil heavy metal remediation (Schnoor 1997).

Type of Remediation	Cost/cubic meter	Time Required
Excavation and removal	$100-$400	6-9 months
In-situ fixation (including soil amendments)	$90-$200	6-9 months
Bioremediation	$15-$40	12-18 months

1.2 BIOREMEDIATION

In recent years, bioremediation methods have drawn the attention of the researchers as chemical detoxification methods failed to handle the issue of soil remediation economically. Bioremediation studies using microorganisms reveal that the microorganisms like, bacteria, algae and micro fungi are not very efficient in soil heavy metal removal. It is difficult to remove their biomass from the soil after remediation process (Atlas et al. 1981; Hamman 2004; Mahavi 2005). Soil bioremediation processes have been classified according to place and soil handling/conditioning as: *in-situ, ex-situ*/bioreactors. *In-situ* methods are useful to handle large amounts of pollutants and it is a most economical technique whereas the second class of processes are useful for remediation of sludge or sediments polluted with high concentration of recalcitrant contaminants (Zang et al. 2000), but their major drawback is that, they are not economical. Summary of various known bioremediation methods are shown in Table 1.2. Bioremediation using plants and

mushrooms are proven to fetch promising results by bioaccumulation of pollutants especially the heavy metals in their large biomass and ease of separation from the soil.

Table 1.2 Summary of Bioremediation strategies (Vidali 2001)

Technology	Examples	Benefits	Limitations	Factors to consider
In-situ	Biosparging Bioventing Bioaugmentation	Non invasive Relatively passive & natural attenuation Treats soil &water	Environmental constraints Extended treatment time Monitoring difficulties	Biodegradative property of indigenous microbes. Environmental parameters. Chemical solubility Distribution & geography
Ex-situ	Land farming Composting Biopiles **<u>Bioreactors</u>** Slurry reactors Aqueous reactors	Cost efficient Can be done on site Rapid degradation. Enhances mass transfer. Effective use of inoculants and surfactants.	Space requirements. Extended treatment time. Mass transfer problems. Soil require excavation High cost involved. High operation cost.	Same as *In- situ* Same as *In-situ* Bioaugmentation Toxicity of amendments Toxic concentrations of contaminants.

Plant mediated metal removal from polluted soil (Phytoremediation) is often used for soil remediation. However, plants take longer time to grow and recover these heavy metals from the soil. They store the heavy metals in parts like leaves, stems, roots and fruits and always have the possibility to render it back into the environment or getting in to the food chain which is still more dangers to mankind. Plants belonging Brassicaceae, Fabaceae, Euphorbiaceae, Asteraceae, Lamiaceae and Scrophulariaceae are reported to have hyperaccumulation properties however have major constraints like higher recovery time, adaptation to the local environment, selectivity in the heavy metals, higher rates of back contamination etc. Hence plant mediated metal removal is found to be laborious, uneconomical and technologically infavorable. It is evident that phytoremediation solely cannot solve these issues and we are in need for a robust methodology which can go hand in hand with all known techniques to remediate the contaminated sites more quickly, effectively and economically (Tangahu et al. 2011; Bestawy et al. 2013). Recently, the research pertaining to bioremediation of heavy metals from soil is focusing on the identification of advanced technology with advanced agronomical and engineering skills to reduce the action period and enhance the accumulation efficiency.

1.2.1 MYCOREMEDIATION

According to Dermirdas et al. (2002) some mushrooms accumulate Cd (II) and Hg (II) ions in high ranges, 1.45 to 2.65 mg/kg, compared to the tolerance level of plants employed for heavy metal remediation. Gast et al. (1988) have reported that, mushrooms can build up large concentrations of heavy metals, such as lead, cadmium and mercury than plants. Thus it can be inferred that, macro fungi such as mushrooms possess an effective mechanism that enables them to uptake some trace elements from the contaminated soil more efficiently than plants.

The bioconversion/bioremediation of environmental adulterants and maintenance of balanced ecosystems by mushrooms are called as "Mushroom mycorestoration or Mycoremediation". Mushrooms are macro in size, tough in texture and have other physical characteristics conducive for their development as biosorbents/bioaccumultors without the need for immobilization or deployment of sophisticated reactor configuration as in the case of microorganisms (Muraleedharan 1994; Jibran et al. 2011).

Advantages of Mycoremediation over Phytoremediation are as follows:

(i) Higher metal accumulation capacity (Isildak et al. 2007)

(ii) Both edible and Non-edible varieties can accumulate metals (Isildak et al. 2004)

(iii) Short lifespan (10-40 days)

(iv) Less chance of back contamination.

Literature reports on mushrooms for heavy metal removal from soil are very scarce. There is a need to isolate mushroom species of higher bioaccumulation efficiency in developing an efficient soil remediation technique and to study the relevant techniques by which the process efficiency be improved. Detailed survey of existing literature was carried out and is reported in Chapter 2. Key questions which arose after a detailed literature survey are given below:

(a) Whether some of the heavy metal tolerant mushroom species be used effectively for removal of metals from soil by bioaccumulation.

(b) Whether the mushroom species can uptake metals both in mycelia and fruiting body stages of their life cycle?

(c) Whether and how the conditions like soil pH and incubation time affect the metal uptake by mushrooms?

(d) Do these conditions depend on mushroom species and metals to be removed?

(e) Whether the metal bioaccumulation efficiency of the mushrooms varies from species to species?

(f) What is the site of bioaccumulation of metals in the mushrooms?

(g) What is the mechanism of bioaccumulation and kinetics of removal of metals by the mushrooms?

(h) Whether addition of chelating agents can enhance the metal bioaccumulation efficiency of the mushrooms?

(i) How does the metal removal efficiency of the mushrooms be affected by the presence of other metals and soil pH in multi-metal contaminated soil system?

Based on the key questions identified, the objectives of the currently reported research work were formulated. The present work reports the isolation and screening of several mushroom species and selection based on their heavy metal tolerance, bioaccumulation potential for removal of heavy metals from soil. A detailed study on selected macro fungus isolated from municipal waste dump yards for the removal of heavy metals like Cu(II), Cd(II), Cr(VI), Pb(II) and Zn(II) from the metal contaminated soils *in-vitro* is reported.

1.3 ORGANISATION OF THESIS

The thesis report was organized into 5 chapters and the details of each chapter are as follows.

Chapter - 1 presents the introduction about the harmful effects of heavy metal pollution in the environments, cause and effects of soil pollution by heavy metals, various remediation methodologies adapted till date to remove these heavy metals

from soil, advantages and disadvantage of the available methodologies, along with need for the current study on mycoremediation.

Chapter - 2 deals with extensive literature review on bioremediation and advantages of mycoremediation, isolation and screening of mushroom species, factors affecting their bioaccumulation efficiency, kinetics involved in removal process, effect of multi-metals on their metal removal efficiency, effect of chelaters in bioaccumulation and its applications. Objectives of the research work are presented at the end of this chapter.

Chapter - 3 presents the various materials and methodologies employed in the experimental work to achieve the stated objectives.

Chapter - 4 describe the results and the detailed discussion along with comparison to the literature reports.

Chapter - 5 presents the summary of the work along with conclusions followed by scope for future work.

CHAPTER – 2

REVIEW OF LITERATURE

The release of heavy metals into the environment (subsurface soil and groundwater) by antrhropogenic activities is a widespread and challenging problem. The contamination of the environment with toxic metals has become a worldwide problem, affecting crop yields, biodiversity and fertility, contributing to the bioaccumulation and biomagnifications in the food chain. In the last few decades, various research groups have recognized that certain chemical pollutants such as toxic metals may remain in the environment for a long period and can eventually accumulate to levels that could harm humans. The contamination of groundwater and subsurface soil in both developing and developed countries are a widespread and challenging problem. There are estimated to be in excess of 200,000 sites requiring some form of remediation as per USEPA/USACE 1991, 1998 reports, and many of these sites potentially threaten groundwater resources. In areas where the groundwater resources are not considered at risk, there are frequently impediments to the reuse of "brownfield" sites. Biological and engineering strategies designed to improve the use of phytoremediation to reduce the amount of heavy metals in contaminated soils has begun to emerge (Adriano et al 2004). Jointly, the U.S. Army Corps of Engineers (USACE) and the U.S. Environmental Protection Agency (USEPA) adopted a tiered system to evaluate this bioaccumulation potential (USEPA/USACE 1991, 1998).In addition, for many subsurface geological settings, conventional treatment methods, such as pump-and-treat technology were used, but found to be costly and inefficient. Emerging research in bioremediation along with biotechnological advances may provide effective, lower-cost alternatives, and it is important to fully understand all aspects of any new and innovative technology.

Recently, there has been a growing interest in studying the potential of mushrooms in metal uptake from soil and understanding the uptake mechanism and bioaccumulation potential. This chapter presents the relevant literature information with respect to studies carried out on bioaccumulation, factors influencing uptake and its mechanism. The review of literature on ill effects of soil heavy metal contamination, methods for remediation, various bioremediation agents known, mushroom as a potent bioaccumulating agent, factors affecting accumulation, effect

of chelaters, multi- metal interaction on metal uptake, mechanism of uptake and the techniques used for the study are also presented.

2.1 HEAVY METAL POLLUTION

Heavy metal pollution of soil is a current global problem with the development of industries and mining activities. The irrigation of waste water and application of sewage sludge to soil amplifies the problem. Heavy metals like chromium, lead, mercury and arsenic can cause serious carcinogenic, genotoxic and immune toxic effects on both humans and animals.

Mining and metal processing industries are mostly located in remote areas rich in ore deposition. Thus, the soil and groundwater have been poisoned and rivers are saturated with toxins. This makes it unusable for irrigation and drinking. Trace metals from soil may enter the human body through the use of contaminated ground water or through the consumption of plant and plant products grown in these environments. People working or residing in these areas are exposed to serious heavy metal contamination in soil, air and water.Heavy metal contamination can cause various health hazards like birth defects, asthma, mental retardation in children etc (Abechi et al. 2010; Bai et al. 2008; Li et al. 2001). Personnel involved in the activities like vehicle repairs, vulcanization, welding, battery charging and dealers in other facilitators of motor transportation are exposed to high levels of heavy metals released in to the environment through these activities. These activities account to major sources of metal contamination in developing countries like Nigeria (Adefolalu 1980; Mabogunje 1980). Sakagami et al. (1982) reported that there is a close relationship between trace metal concentration in roadside soil and those in the dust falls. Trace metals in the soil can also generate airborne particles and dusts, which may affect the air quality (Gray et al. 2003). Among the numerous environmental pollutants, an important role is ascribable to heavy metals whose concentration in soils, water and air are continuously increasing in consequence of anthropogenic activity. Efforts have been made to develop efficient remediation technologies to clean up such areas and

convert them to a better living space. Various physical and chemical methods are generally used to reduce the toxicity of heavy metals in the contaminated area.

2.2 DIFFERENT METHODS FOR HEAVY METAL REMOVAL FROM SOIL

Schnoor (1997) studied the advantages and disadvantages of various methods known for heavy metal remediation. Various researchers have adapted both chemical and biological methods for heavy metal removal from polluted water and soil. Diels et al. (2002) and Groudev et al. (2001) discussed about the various *in-situ* and *ex-situ* methods of heavy metal remediation from soil: *In-situ* methods like immobilization and precipitation treat large diffusely contaminated areas but alter the soil texture and other physiological properties. It also reduces the growth of numerous useful organisms in the soil. Similarly, Schiopu and Gavrilescu (2010) explored the progress in landfill leachate generation and the risk involved in heavy metal contamination of soil and water. The analysis addressed the opportunity and support for decision making concerning alternatives for leachate management and treatment. Advantages and limitations of treatment methods and processes involving leachate transfer, physicochemical methods, biodegradation, and combined methods are discussed in Chapter 1. In case of *ex-situ* methods, small areas are treated effectively and cost involved is very high compared to on site degradation (Dermirbas 2002). Hence an effective cost efficient method has to be formulated for better remediation. Table 1.1 gives an overview of the cost involved in various known soil remediation procedures.

2.3 BIOREMEDIATION

The metal removal potentials of micro organisms, fungi and plants were also studied as they serve as a cost efficient remediation technique. Bioremediation is a waste management technique that involves the use of organisms to remove or neutralize pollutants from a contaminated site (Mann et al. 1996). According to the United States environmental protection agency (US-EPA), bioremediation is a "treatment that uses naturally occurring organisms to remove hazardous substances

from the contaminated environment to reduce or remove their ill effects". 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 a process in which contaminated material is treated elsewhere. Some examples of bioremediation related technologies are phytoremediation, bioventing, bioleaching, landfarming,bioreactor, composting, bioaugmentation, rhizo-filtration, and biostimulation. Most of the organisms try to resist uptake of heavy metals, as it can disturb redox homeostasis by stimulating the formation of free radicals and reactive oxygen species (ROS) such as singlet oxygen (O_2), superoxide radicals (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$)) by avoidance/ resistance (Barconi et al. 2011, Romero et al. 2002, Saito et al. 2011). When any organism is able to restrict metal uptake, it will be able to survive in the presence of high internal metal concentration. Avoidance involves reducing the concentration of metal entering the cell by extracellular precipitation, biosorption to cell walls, reduced uptake, or increased efflux. In a second type of situation, heavy metals are intra-cellularly chelated through the synthesis of amino acids, organic acids, GSH, or HM-binding ligands such as metallothioneins (MTs), phytochelatins (PCs), compartmentation within vacuoles, and up regulation of the antioxidant defense and glyoxalase systems to counter the deleterious effects caused by reactive oxygen species(Anjum et al. 2012, Dubey et al. 2011, Sharma et al. 2009, Hossain et al.2011).

Lambert et al. (2007) and Chen et al. (2000) compared the efficiency of three known methods for remediation of heavy metals such as Pb(II), Cd(II), Zn(II) and Cu(II) from soil, namely excavation, stabilization and bioremediation. From their field study it was evident that bioremediation can be regarded as most economical and efficient remediation method.

From the literature studies it was observed that microorganisms are efficient in removing heavy metals from industrial effluents. Allen et al. (2002), studied on treatment of metallic residues or by-products of industries and other xenobiotic

organic compounds in a compost pit similar to a municipal solid waste treatment plant using a microbial consortium to reduce metal toxicity by converting them to organic combinations. Thus, the bioavailability of these heavy metals in organic combination was found to be less compared to that of mineral forms. Similar studies were conducted by Valls (2002), Kelly et al. (2004) Suranjana et al. (2009) and Rani et al. (2010). They used *Bacillus Sp., Micrococcus Sp., Escherichia Sp. and Pseudomonas Sp.* in bioremediation of Cu(II), Pb(II), Cd(II) and Cr(VI) contaminated soil and water by biosorption and bioaccumulation of Cu(II), Pb(II), Cd(II). The results showed 50-60% removal of most of the heavy metals using dead or live microbes but complete removal or detoxification was not possible by microbial treatment.

Bestawy et al. (2013) reported the heavy metal bioaccumulation and heavy metal tolerance profile of eight resistant indigenous bacteria isolated from acclimatized activated sludge. They were identified as Enterobacter *Sp.*,(Cu1), Enterobacter *Sp.*,(Cu2), Stenotrophomonas *Sp.*,(Cd1), Providencia *Sp.*, (Cd2), Chryseobacterium *Sp.*,(Co1), Comamonas *Sp.*, (Co2), Ochrobactrum *Sp.*,(Cr) and Delftia *Sp.*,(M1) according to their resistance pattern. The results showed that the strains Cu1, Cd1, Co2 and Cr were able to resist 275 mg Cu/L, 320 mg Cd/L, 140 mg Co/L and 29 mg Cr/L respectively. From the study results it was also observed that these were useful only in removing heavy metals from industrial effluents and not polluted soil.

Ahmad et al. (2005), Wuyep et al. (2007) and Adeyemi (2009) explored the possible bioremediation of Ar, Cr and Cd metals, by three filamentous fungi; *Aspergillus niger*, *Serpula himantioides* and *Trametes versicolor*. They found that these micro fungi were efficient in removing heavy metals from an initial concentration of 0.2, 0.4, 0.6 and 0.8% (w/v) arsenopyrite (FeAsS), Pottassium dichromate ($K_2Cr_2O_{7)}$ and cadmium sulphate ($CdSO_4$). Growth rates, dry weight (D.W) and metal accumulation were assessed. It was found that the efficiency of

accumulation of metals followed the order *P. squamosus >A. niger > S. himantioides > T. Versicolor.*

Shazia et al. (2013) conducted a study on various isolates of highly tolerant filamentous fungal species. *Aspergillus fumigatus* isolated from polluted soil collected from Kasur district, Pakistan had its bioaccumulation value: Pb(76.07 mg/kg), Cu (69.6 mg/kg) and Cr (40.0 mg/kg) at 800 mg/kg metal concentration. The purpose of this investigation was to determine adsorption behavior of different fungal isolates towards various heavy metals' toxicity and detrimental to flora and fauna. The objective of this study was to provide basis for further assessment and management of natural biosorbent (fungus) which could serve as an economical source of treating industrial effluents with toxic metallic ions.

Hrishikesh et al. (2010) found that Arbuscular Mycorrhiza (AM) is an obligate biotroph, which mainly improves phosphorus nutrition, ability to withstand water stress and offers a natural potential for biological control of root pathogen.AM fungi provide an attractive system to advance plant-based environmental clean-up. During symbiotic interaction the hyphal network functionally extends the root system of their hosts. Thus, plants in symbiosis with AM fungi have the potential to take up heavy metal (HM) from an enlarged soil volume. Here the recovery and complete degradation was not possible and hence not widely used for soil bioremediation.

Chaney et al. (1996) and Maiti et al. (2004) discussed about the phytoremediation studies in remediating heavy metals from soil. The hyper accumulating characteristics, physiological and biochemical mechanism for promoting metal tolerance, use of chelating agents etc. for certain plant species like *Arabidopsis Sp., Thalapsi caerulescens, Phaseolus vulgaris* and *Medicago sativa* L were discussed. They found that the hyper tolerance of metals is the key plant characteristic required for hyper accumulation and vacuole compartmentalization appeared to be the source of hyper tolerance of natural hyper accumulator plants. Alternatively, Pb(II) and Cr(VI) may be inactivated in the soil by plants and soil

amendments (phytostabilization). Little molecular understanding of plant activities critical to phytoremediation was achieved, but recent progress in characterizing Fe(II), Cd(II) and Zn(II) uptake by plant species studied indicates strategies for developing transgenic improved phytoremediation agents for commercial use. All plant parts have to be treated effectively to prevent further contamination as heavy metals gets accumulated in the plant parts like leafs, twigs, stem etc

Researchers have also studied about other potential heavy metal bioaccumulators. Among which macro fungi, mushrooms play a vital role. Vidali (2001) studied about various bioremediation methods using different organisms and found that mycoremediation (mushroom or macro fungi based bioremediation) and phytoremediation are efficient than bacterial remediation. They also reported that there is better ease of desorption of heavy metals from fungi than plants. Hence, mycoremediation for removing heavy metals from contaminated soil and water has been preferred over phytoremediation.

In spite of all the advances in the Phytoremediation, they could not answer all the cases of heavy metal contamination in riverbanks, agricultural fields, landfills across the globe (Millenkovic et al. 2005; Cheng 2002; Zhuang and Wang 2000). Phytoremediation solely cannot solve all these issues because of its limitations like selectivity of plant, climatic inhibitions, tolerance to heavy metals and back contamination by depuration or from ashes of fire woods; hence there is a need of a robust methodology which can go hand in hand with other techniques to remediate these areas more quickly, effectively and economically.

2.4 MYCOREMEDIATION

Mushrooms or macro fungi can act as effective bioaccumulation alternative to plants in removing toxic metals from soil and the process is referred as mycoremediation. Mushroom consist of haplodiplontic life cycle (*i.e* both haploid and diploid stage). During its haploid stage it exists as spores and during its diploid stage it exists as fruiting bodies. The mushroom fruiting body consists of a thin hollow stipe (stem) and a fleshy cap (pileus). The cap has thick outer covering and contains gills that bear spore (haploid). Fruiting bodies form from the mycelia only during the reproductive phase of mushrooms (Oei 1996; Oei 2000). A schematic representation of mushroom life cycle is shown in Fig. 2.1. Mushroom mycelia can serve as biological filters since their aerial structures consist of large biomass and have a tough texture which makes them potential sorbents (Volesky et al. 1995). Mushrooms are known to have high metal/ metalloid tolerance which helps them to thrive and accumulate metals from the contaminated environment. They also have shorter life cycle (30 days) and better adaptability compared to plants; hence mycoremediation can be regarded as an evolved remediation technique.

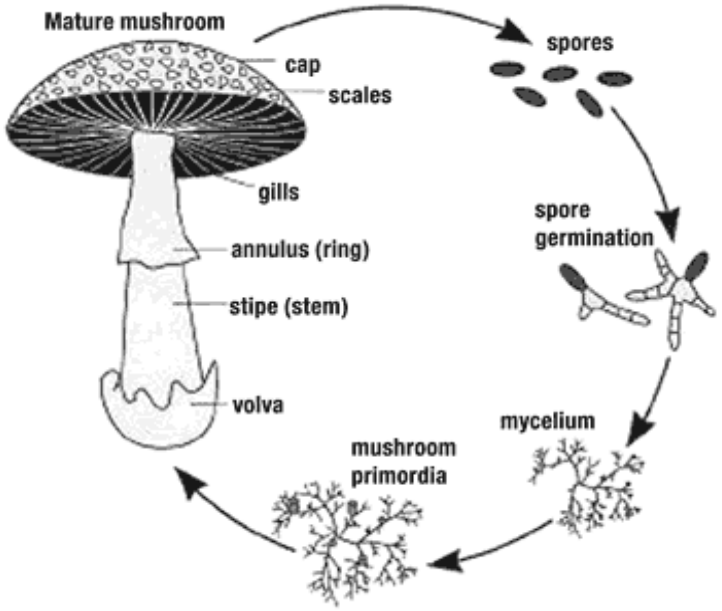


Fig. 2.1 Mushroom life cycle

Gabriel et al. (1997) reported the heavy metal content in some fruiting bodies collected from National park Sumava in Czech Republic. They also reported that the key role in metal accumulation was attached to cell wall polysaccharides, cystine-rich proteins and pigments like melanin. Similarly heavy metal bioremediation using mushrooms species both edible and non-edible like *Formes applanatus* and *Polyporus frondosis* (Gabriel et al. 1996), *Armillariella mellea* (*Siegel* et al. 1990; *Stijve* et al. 1990) for Cr(VI), Cu(II), As(II) and Cd(II),*Pleurotus sapidus, Polyporus sulphureus, Pleurotus ostreatus* and *Lactarius deliciosus* (Vetter 1994; Tyler 1982; Deils et al. 1999; Ita et al. 2006), *Russula delica* and *Rhizopogon roseolus* (Cayir et al. 2010; Isildak et al. 2010; Reider et al. 2011) for Fe(II), Zn(II), Mn(II), Ni(II), Co(II) and Pb(II) accumulation were reported.

Kulshreshtha et al. (2012) and Gaur et al. (2014) in their review article on recent advancements on bioremediation-based abolition of heavy metals comprehensively discussed toxicological manifestations of heavy metals along with the detailed description of bioremediation technologies employed such as phytoremediation and mycoremediation for the potential removal of these metals from various polluted environments. Mushrooms in both dead and live forms have been reported to have bioaccumulation potential.

2.5 *IN-VITRO* ESTABLISHMENT OF MUSHROOMS

Mushroom cultivation technology is friendly to the environment. The mushrooms can be produced on substrates like paddy straw, cotton wastes, coffee waste, water hyacinth, tree saw dust, sugar cane bagasse, wild grasses and various categories of refuse and ligno cellulosic wastes with minimum investment (Dermirbas 2009). Due to advances in basic knowledge and practical technology relevant to mushroom farming (mushroom cultivation), mushroom products (mushroom derivatives) and mushroom bioremediation (mushroom mycelia and fruiting bodies), these principles can be applied globally, but must be implemented according to locally available substrates, labour and climatic conditions. The mushrooms from the polluted sites are collected aseptically using sterilized forceps.

The totipotent regions of the mushroom are isolated and are grown on a nutrient medium (vegetative growth). The mycelium formed is then inoculated on to treated grains to prepare the grain spawn. These grain spawns are then cased with soil mixture rich in organic content for mushroom fruiting body formation (Ting Chang 2006). Various researchers have formulated substrates combinations for casing and spawning, its concentrations, humidity and other conditions required for *in-vitro* cultivation of mushrooms (Oei 2005; Sesli 2007; Primer 2009; Polat et al. 2011). As per the literature reports it is understood that the mushrooms grow well in an environment having high relative humidity, RH: 80-90% and atmospheric temperature between 20- 24ºC. They also reported a complete guide for isolating wild mushrooms; described about different ways to characterize the collected mushrooms, its edibility, important steps to be followed for wild mushroom collection *etc*. Literature reports say that the mushrooms species can be identified by their morphological characteristic like pileus shape, its colour, spore print and presence of crown or cap.

2.6 BIOACCUMULATION OF METALS BY MUSHROOMS

The consumption of wild edible mushrooms is increasing, even in the developed world, due to a good content of proteins as well as a higher content of trace minerals (Agrahar-Murugkar and Subbulakshmi 2005). Lead, cadmium, iron, copper, manganese, zinc, cobalt, chromium, nickel, magnesium, aluminum, tin, and arsenic were chosen as representative trace metals whose levels in the environment represent a reliable index of environmental pollution. Metals such as iron, copper, zinc, and manganese are essential metals since they play an important role in biological systems, whereas aluminum and lead are non-essential metals as they are toxic even in traces (Unak et al. 2007). The essential metals can also produce toxic effects when the metal intake is excessively elevated (Al-Khlaifat and Al-Khashman 2007; Gopalani et al. 2007). Heavy metal concentrations in mushroom are considerably higher than those in agricultural crop plants, vegetables, and fruit. This suggests that

mushrooms possess a very effective mechanism that enables them readily to take up some heavy metals from the ecosystem.

Table: 2.1 Heavy metal content in sporocarp of various tolerant mushrooms

Sl. No	Mushroom Species	Metal content in sporocarp, mg kg^{-1} of dry wt.	References
1	*Agaricus bisporous*[1] *Boletus edulis*[1] *Lepiota rhacodes*[2] *Paxillus rubicondulus*[1]	Pb (4), Cd (3.48), Cu (5.) Cu (66.4), Cd (6.58), Pb (3.03) Pb (66), Cd (3.7) Pb (0.69), Cd (0.78), Cu (51.0) Zn (16.8)	Srivastava et al. 2006
2	*Agaricus bisporous*[1]	Cu (107),Pb (1),Zn (57.)	Turkekuel et al. 2003
3	*Havlvella leucomelaena*[2] *Pleurotus sp.*[1]	Pb (4.8), Cd (.0) Pb (3.4), Cd (1.18), Cu (13.6), Zn (9.8)	Mitra et al. 1994
4	*Tricholoma terreum*[1] *Havlvella leucomelaena*[2]	Cu (5), Zn (179), Cd (0.56), Pb (4.4) Pb (3.1), Cd (1.1)	Dermirbas 2001
5	*Paxillus involutus*[2] *Rhizopogonaceae luteolus*[1] *Omphalotous olearius*[2] *Hygrophorous hedyricii*[2] *Ciocybe dealbata*[2] *Lepiota alba*[2]	Cu (57.0), Pb (1.6.0), Fe (991), Cd (0.84), Pb (3) Cu (13), Zn (30), Mn (13), Fe (620), Cd (0.26), Pb (2.8). Cu (21), Zn (27), Mn (36), Fe (95), Cd (1.3), Pb (5.2). Cu (37), Zn (97),Mn (11), Fe (395), Cd (1.2), Pb (2.7) Cu (41), Zn (115), Mn (30), Fe (386), Cd (0.86), Pb (3.2) Cu (29), Zn (86), Mn (22), Fe (779), Cd (0.8), Pb (5.8)	Yilmaz et al. 2003
6	*Tricholoma terreum*[2] *Agaricus bisporous*[1]	Pb (4), Cd (1.6), Cu (35.8), Zn (48.0) Pb(0.8), Cd(0.78)	Zhu et al. 2011
7	*Pseudevernia furfuraceae*[2] *Scorpiurum circintum*[2]	Al (12.51), As(0.23), Cd (0.19), Cu (2.5), Cr(0.11),Pb (5.1), Zn(17.9), Mn(12.9) Al(17.51), As (0.32), Cd(0.35), Cu (3.2), Cr (1.1), Pb(6.3), Zn (46.1), Mn (46.7)	Basile et al. 2007
8	*Aspergillus foeitidus*[2]	Al (32.5), Co (5.95), Cr (6.23), Mg (44.9), Zn (2.4), Ni (189.5)	Ge et al. 2011
9	*Poria Sp.*[2] *Nectria cinnabarina*[1] *Gonoderma lucidium*[1] *Paragyrodous sphaerosporous*[1] *Polyporous frondosis*[1]	Zn (90.3), Cu (30.8), Pb (1.0), Mn(31.3), Cd (0.1) Zn (30.1), Cu (29.3), Pb(1.9), Cd(0.2), Mn (19.3) Zn(60.1), Cu (43.8), Pb (0.7), Mn (30.4), Cd (0.31) Zn (115), Cu (34.4), Pb (0.4), Mn (37.3), Cd (0.2) Zn (120.1), Cu (34.4), Pb (0.4), Mn (37.3), Cd (0.2)	Ita et al. 2006

10	*Phellinus badius*[2] *Phellinus sanguineus*[2]	Cd (110), Cu (60), Hg (61), Ni (56) Cd (80), Cu (42), Hg (35), Ni (66)	Baldrian 2003
11	*Tricoloma terreum*[2] *Boletus badius*[1] *Russula delica*[1]	Pb (3.64), Cu (34.86), Cd (0.67), Zn (54.13), Cr (2.54) Cu (44.54), Pb (4.48), Cd (0.91), Zn (34.17), Fe (264.62), Cr (2.86) Cu(19.55), Pb (2.02), Cd (1.22), Zn (38.5), Cr (6.95)	Isildak et al. 2007
13	*Pleurotous platypus*[1] *Agaricus bisporous*[1]	Cd (34.9), Pb (27.10) Cd (33.7), Pb (29.67)	Vimala et al. 2009
14	*Lactarius delicious*[1] *Rhizopogon roseolous*[1] *Russula delica*[1]	Cd (0.26), Cr (0.12), Cu (6.15), Pb (0.73), Zn (76.7) Cd (0.18), Cr (0.10), Cu (21.2), Pb (2.03), Zn (36.7) Cd (0.42), Cr (0.27), Cu (52.2), Pb (0.77), Zn (58.2)	Cayır et al. 2010
15	*Sarcosphaeera crassa*[1] *Cantharellus cibarius*[1] *Suillus luteus*[1] *Morchella rigida*[1] *Agarocybe aegerita*[1]	Ag (0.044), As (8.03), Cd (0.016), Cr (0.98), Pb(0.02) Ag (0.022), As (0.03), Cd (0.036), Cr (0.69), Pb (0.04) Ag (0.015), As (0.15), Cd (0.034), Cr (0.15), Pb (0.06) Ag (0.087), As (0.24), Cd (0.007), Cr (0.44), Pb (0.02) Ag (0.074), As (0.44), Cd (0.010), Cr (0.25), Pb (0.018)	Konuk et al. 2007
16	*Agaricus arvensis*[2] *Agaricus silvicola*[1] *Macrolepiota procera*[1] *Lycoperdon perlatum*[1]	Cd (117) Cd (67.9) Pb (53.8) Pb(50)	Petkovsek and Pokorny 2013
17	*Pleurotus sajor-caju*[1]	Zn (30.0)	Jibran and Milsee 2011
18	*Pleurotus ostreatus*[1]	Cd (103)	Tay et al. 2011
19	*Pleurotus tuber-regium*[1]	Cd (0.16), Cr (5.6), Cu (21.2), Pb (2.03), Zn (46.7)	Oyetayo et al. 2012
20	*Agaricus bisporous*[1]	Pb (76.07),Cu (69.6),Cr (40.0), Ar (30.0)	Chauhan and Suhalka 2014

❖ **[1] :- Edible; [2] :- Non edible Mushrooms**

Many wild edible mushroom species have been known to accumulate higher concentrations of heavy metals (Kalac et al. 1991; Demirbas 2000, 2001; Svoboda et al. 2000; Kalac and Svoboda 2001; Falandysz et al. 2003; Dursun et al. 2006; Cocchia

et al. 2006; Chen et al. 2009; Zhu et al. 2010). Some mushrooms that accumulate significant amount of metals are given in Table 2.1.

The accumulation of heavy metals in macrofungi has been found to be affected by environmental and fungal factors. Environmental factors, such as organic matter amount, pH, and metal concentrations in soil, and fungal factors, such as species of mushroom, morphological part of fruiting body, development stages, age of mycelium, and biochemical composition, affect metal accumulation in macrofungi (Garcia et al. 1998; Kalac and Svoboda 2001).

As bioaccumulation of mushroom from soil results in removal of metals from soil, mushrooms were selected for mycoremediation based on their metal bioaccumulation potential. Though there are reports on metal bioaccumulation by mushrooms when consumed as food, literatures on bioaccumulation potential for account of mushrooms as mycoremediation agents are scare.

2.7 SOURCES AND ISOLATION OF HEAVY METAL TOLERANT MUSHROOMS

According to Kalac et al. (2000) and Iram et al. (2013) wild grown mushrooms have been a very popular delicacy in many countries and annual consumption may exceed 10 Kg for some individual. Fruiting bodies of mushrooms are appreciated not only for texture and flavor but also for their chemical and nutritional properties. Mushrooms have also been reported to be therapeutic foods, useful in preventing diseases such as hypertension, hypercholestromea and cancer. These functional characteristics are mainly due to their chemical composition Manzi et al (2001). Most of wild mushrooms are known to accumulate high levels of heavy metals like cadmium, mercury and lead. Many investigations have worked on the metal contents in wild and edible mushrooms (Gast et al.1988; Manzi et al. 1999; Kalac et al. 2000; Iram et al. 2013).

Wild grown edible and non edible mushrooms were isolated from both polluted and non polluted site by Gast et al. (1988) and were analyzed for their heavy metal contents (Cu(II), Pb(II), Cd(II), Zn(II) and Mn(II). Studies on metals in mushrooms have shown a correlation between fungal metal concentrations and point sources of metal pollution, such as smelters and road sides (McCreight and Scroeder, 1977; Bargali et al. 1984). Under natural condition, heavy metals concentrations of some species of wild grown edible mushrooms can be high even if the degree of pollution is low (Falandaysz et al. 2003).

Demirbas, (2002) studied the metal bioaccumulation levels in three mushrooms growing in the East Black Sea region and analyzed spectrometricaly for the presence of trace element (Pb, Cd, Hg, Cu, Mn, and Zn) levels. Heavy metal (Hg, Pb, Cd and Cu) bioaccumulation levels of samples of three mushrooms (*Armillaria mellea*, *Polyporus squamosus*, *Polyporus suiphureus*) obtained from the East Black Sea region were investigated. The Hg (II) level of *Armillaria mellea* samples increasesed sharply with increasing Hg (II) concentrations in the fortified soil samples.

Isildak et al. (2003), Das (2005) and Begum et al (2009) collected and analysed 10 different wild grown edible mushrooms collected from municipal waste dump yards in Tokat, Black Sea region Turkey. Concentrations of heavy metals like Cu, Cd, Zn, Fe were high in mushroom species like *Agaricus* Sp, *Marasmius* Sp and *Morchella* Sp etc. Edible, non-edible and poisonous macro fungi were collected around the Balykesir-Manisa highway from two different areas (roadside and background area) in 1998-1999 by Yilmaz et al. (2003). Cu, Zn, Fe, Mn, Co, Cd, Ni and Pb contents were determined by atomic absorption spectrophotometer in 24 macro fungi species. In their study highest Pb and Zn contents were found in *Lycoperdon perlatum* as 6.5 mg/kg and 274 mg/kg respectively.

Jerzy et al. (2007) and Dan et al. (2008) reported efficient heavy metal accumulating fungi from non- polluted areas. Parasol Mushroom (*Macrolepiota*

procera) was isolated from nineteen spatially distant sites across Poland, while 13 species of heavy metal accumulating mushrooms from five different sites of China. At each site well grown and roughly similar in the cap size specimens of mushroom were collected over a relatively large area of land to avoid excessive local sampling. Among the tested mushroom species, *Termitomyces microcarpus* showed highest Cu, Pb, Cd, and As content and all other species showed fairly good uptake efficiency.

Similarly Elekes et al. (2010) and Mleczek et al. (2013) isolated heavy metal tolerant mushroom species from the forest area of Bucegi Mountains and Europe. They were the staple food for the native population who were unaware of the risk of heavy metal toxicity by their consumption. They reported that the heavy metals concentrations in the fruiting body of mushrooms were different from one species to another and mean values of 11.94 mg/kg for Ti, 1.07 mg/kg for Sr, 1163.86 mg/kg for Bi and 17.49 mg/kg for Mn were shown. The bioconversion factor of heavy metals represents the level of metal concentration in the mushroom's body correlated with the metallic element in the soil on which the fungus grow. The bioconversion factor was reported to have the highest values in *Marasmius oreades* species for bismuth and titanium. Their study was to bring awareness about the dangers of heavy metal intake through the intake of wild mushroom species. They also described about the Hg(II) and Pb(II) accumulation in human organs leading to fatal toxicity to human race.

Joshi et al (2011) and Chauhan and Suhalka (2014) also have reported similar heavy metal tolerance in wide range of mushroom species like species of *Agaricus* from metal processing industries, tannery and combustion of wood and areas prone to coal and mineral oil spillage.

Researchers have isolated heavy metal tolerant mushroom species from both metal contaminated and non contaminated areas. The metal uptake ability of mushrooms mainly depends on the species of the mushroom and property of metal ions. Heavy metal tolerance and metal bioaccumulation efficiency was reported in

Volvariella. volvacea (Purkayastha et al. 1992), *Pleurotus species, Pycnoporus and Pholiota species* and one each of the species: *Agrocybe, Cryptoporus, Coriolus, Inonotus, Lampteromyces, Grifola, Flammulina, Lyophyllum, Agaricus, and Polyporus* (Sanglimsuwan et al. 1993), *Lactarius deliciosus, Russula delica, and Hizopogon roseolus* (Cayır et al. 2009; Isiloglu et al. 2001), *Macrolepiota crustosa, Russula virescens, Calvatia craniiformis* (Chen et al. 2009; Kalac 2010). The concentration of accumulated metal was higher in mushrooms picked from polluted areas. Moreover, some species have accumulating and even hyper accumulating ability for various elements. The possibility to evaluate toxicological risk or nutritional asset has been thus limited.

2.8 SITES OF BIOACCUMULATION IN MUSHROOMS

Compared to green plants, mushrooms can build up large concentrations of some heavy metals (Stijve and Roschnic, 1974; Kuusi et al., 1981). The uptake of pollutants like heavy metals by mushrooms involves a combination of two processes: (i) bioaccumulation i.e. active metabolism-dependent processes, which includes both transport into the cell and partitioning into intracellular components; and (ii) biosorption *i.e.* the binding of pollutants to the biomass without requiring metabolic energy. Several chemical processes may be involved in biosorption, including adsorption, ion exchange processes and covalent binding (Monachese et al. 2012). According to Martin et al. (1997) and Kulshreshtha et al. (2014), the polar groups of proteins, amino acids, lipids and structural polysaccharides (chitin, chitosan, glucans) may be involved in the process of biosorption. The metal after the uptake are distributed unevenly in the mushroom body. The highest levels were observed in the spore forming part (pileus) of the fruiting body (Thomet et al. 1999; Das 2005). The mushroom fruiting body consists of a stipe (stem) and a cap. The distribution of heavy metals after bioaccumulation in the fruiting bodies can be determined by subjecting each part of fruiting bodies to metal analysis. The uptake and distribution of Cd(II) and Zn(II) concentrations in both mycelia and different parts of mushrooms such as *Agaricus macrosporous, Agaricus silvicola* and *Stropharia rugosoannulata* were

studied by Thomet et al. (1999). They also studied about the distribution of heavy metals in base, gills, stem and the cap of the mushrooms. Similarly Dermirdas (2001) and Isiladak et al. (2003) reported that the cap of fruiting bodies have higher potential to accumulate heavy metals like Cu(II), Cd(II), Pb(II), Zn(II), Mn(II),Fe(II), Cr(VI) and Ni(II) from the contaminated areas. Similar studies were reported by various researchers like Miersch et al. (2005), Campos et al. (2009) and Elekes et al. (2010).

2.9 FACTORS INFLUENCING BIOACCUMULATION

As bioaccumulation is growth associated, factors affecting the growth also favour bioaccumulation of heavy metals. Metals usually in their sulphate or phosphate form are soluble and can be easily taken up by plants, animals and microbes. Ideal growth conditions are the temperatures ranging between 20 and 30^{o}C for most of the cultivable mushrooms (Chen et al. 2009).The process of heavy metal accumulation of mushroom is species-specific. Elevated concentration of heavy metals has been observed in the fruiting bodies of mushrooms collected from the areas adjacent to heavy metal smelters (Kalac et al. 1991). Studies by Gast et al. (1988) proved that heavy metal levels in the mushrooms are mainly affected by pH and organic matter content of the soil. Similar studies were also carried out by Yahaya et al. (2009), Adeyemi et al. (2009), Demirbas (2001), Schmitt and Meisch (1985), Vimala and Das (2009), Juna et al. (2009) and Sharma et al. (2010). From these literature study reports it is understood that soil pH between pH 7 and pH 4.5(acidic range) is favored for the *in-vitro* growth of mushrooms. The soil pH plays an important role in growth of the mushroom species. It also helps in determining the formation of fruiting bodies *in-vivo* conditions. Hence selection and maintenance of suitable pH plays a significant role in producing fruiting bodies at *in-vitro* conditions. The factors that influence bioaccumulation of metals by mushrooms play an important role in mycoremediation process.

2.10 METAL UPTAKE MECHANISM IN MUSHROOMS

The process of heavy metal accumulation of mushrooms is species-specific. Elevated concentrations of heavy metal have been observed in the fruiting bodies of mushrooms collected from the areas adjacent to heavy metal smelters and road sides, as they are more prone to heavy metal concentration (Isiloglu et al. 2001; Kalac et al. 1996 and Kalac et al. 1991). Mushrooms have the capacity to accumulate heavy metals readily when they are in phosphate or sulfate salt forms Eg. $PbSO_4$, $CdSO_4$ etc. according to Demirdas (2002). Heavy metals usually accumulate in mushrooms as organic or inorganic compounds or associated with proteins and lipids. The study on the concentrations of Cd (II), Zn (II), Cu (II) and Hg (II), as well as cytosolic Cd-binding capacity (CCBC), glutathione (GSH) and free proline (Pro) were quantified in fruiting bodies of *B. edulis* by Hansen et al. (2007). They used size exclusion chromatography (SEC), followed by metal determinations with atomic absorption chromatography (AAS) and inductive coupled plasma analyser coupled with mass spectral analyzer (ICP-MS) for proper understanding of metal distribution among cytosolic compounds. In their study, the presence of phytochelatins (PCs), a family of cystein-rich oligopeptides, was confirmed in Cd-containing SEC fractions by HPLC-MS.

The role of phytochelatins in metal tolerance in both plants and fungi have been reported. Clemens et al. (1999) reported the molecular biotechnological studies (cDNA) on plant species and showed that a wheat cDNA, *TaPCS1* can increase the Cd (II) tolerance in *Saccharomyces cerevisiae.* They used HPLC-MS techniques to detect the presence of the PCS genes, encode phytochelatin syntheses and mediate metal detoxification in eukaryotes.

The significance of components such as amino acids, organic acids, glutathione (GSH), or metal-binding ligands, which control the antioxidant defence system to scavenge reactive oxygen species (ROS) and methylglyoxal (MG) were discussed in the study reports of Hossain et al. (2012). In their article, they described

that the heavy metals that enter the cell may get sequestered by amino acids, organic acids, glutathione (GSH), or by specific metal-binding ligands. Their study report helps to integrate a recent understanding of physiological and biochemical mechanisms of HM-induced plant stress response and tolerance based on the findings of current plant molecular biology research.

Cuptapun et al. (2010) reported that the heavy metals such as Cu and Zn are essential for normal plant growth, although elevated concentrations of both essential and non-essential metals can result in growth inhibition and toxicity symptoms. Plants possess a range of potential cellular mechanisms which are involved in detoxification and metal stress tolerance. These include roles for the following, for mycorrhize and for binding to cell wall and extracellular exudates, for reduced uptake or efflux pumping of metals at plasma membrane, for chelation of metals in cytosol by peptides such as phtochelatins for the repair of stress damaged proteins and for compartmentalization of metals in vacuoles by transporters located tonoplast. Similar studies were also conducted in various other known phytoremediation agents, by Hentschel et al. (1993), Purkayastha et al. (1992), Goldani et al. (1994), Hall, (2002), Hansen et al. (2003), Julian et al. (2004), Smith (2007), Cuptapun et al. (2010).

2.10.1 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

A classical method for analysis of molecules or compounds produced by the organisms is FTIR analysis. Irradiation of molecules with IR light induces an oscillation of chemical bonds at characteristic frequencies and, thus energy is absorbed. The resulting transmission of radiation is measured over a frequency spectrum from about 400- 4,000 cm^{-1}. The so called finger print areas between 400-1,500 cm^{-1} shows deformation bands which are characteristic of every molecule and allow for the chemical substances to be identified from spectrum files. Partial structures are analysed by dilation oscillations in the area from 1,500 - 4,000 cm^{-1}, as chemical bonds generate distinct valency oscillation bands (Heyd et al. 2008). This technique has been frequently used by researchers as the spectrum gives information

of various functional groups present in both the mushroom surface and its components.

Kong and Yu (2007) studied the use of oldest and well established experimental techniques for the analysis of secondary structure of polypeptides and proteins *i.e* Infrared spectroscopy. FTIR is convenient, non-destructive, requires less sample preparation, and can be used under a wide variety of conditions. They explained about the recent developments in Fourier transform infrared (FTIR) spectroscopy technique and its applications to protein structural studies. The experimental skills, data analysis, and correlations between the FTIR spectroscopic bands and protein secondary structure components were discussed. The applications of FTIR to the secondary structure analysis, conformational changes, and structural dynamics and stability studies of proteins were also discussed.

Gurdeniz et al. (2009) used FTIR for fatty acid analysis of oil samples. They also describe FT-IR spectra as a tool for determining fatty acid from extracts.

Remenarova et al. (2011) identified the functional groups that are involved in metal accumulation using FT-IR. The IR spectrum of metal-free and metal –loaded fungal biomass showed differences in functional groups of –CO,-OH/-NH, -CN,-CH_2 and –C_6H_5, especially the presence of higher concentration. Proteins produced during the stress by the fungi were analysed. The difference in the absorbtion peak between the control and metal accumulated biomass help to estimate the effect of proteins. Similar observations were also reported by Lin et al. (2012) and Gabr et al. (2008) in their studies on heavy metal accumulation in *pleurotus* Sp.

Morsy et al. (2011) evaluated the nature of biosorbent and metal ion interaction by infrared (IR) technique. IR analysis of bacterial biomass revealed the presence of amino, carboxyl, hydroxyl, and carbonyl groups, which are responsible for biosorption of Cd(II) and Zn(II).

Huang et al. (2012) investigated the biosorption characteristics of Cd(II), Cu(II) and Pb(II) by the fruiting body of *Auricularia polytricha* to study the mechanisms of the biosorption process. A Fourier transform infrared spectroscopy analysis indicated that carboxyl, amine/hydroxyl, amino, phosphoryl, and C–N–C were the main functional groups to affect the biosorption process. Synergistic ion exchange and surface complexation were the dominant mechanisms in the biosorption process. The work revealed the potential of fruiting body of *A. polytricha* to remove toxic heavy metals from contaminated water.

2.10.2 LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROSCOPY (LC-MS)

Direct coupling of reverse phase liquid chromatography to a mass spectrometer provides the advantages of characterizing stress proteins by its retention time along with its mass spectral signature. This is normally done by splitting the flow coming from the high pressure liquid chromatography (HPLC) using a splitter that conveys only a fraction of elutent into the mass spectrometer. Eletrospray Ionisation (ESI), and sometimes the Atmospheric Pressure Chemical Ionization (APCI), has been mostly used to ionize the components prior to mass analysis (Deziel et al. 1991, 2000; Haba et al. 2003; Benincasa et al. 2004; Monterio et al, 2007). In negative ESI, the molecular weight of the psedomolecular ion $[M-H]^-$ can be directly obtained. This provides some information on the nature of stress components produced by the bioaccumulators such as mushrooms, eluting from the column at that retention time. In order to improve ionization, ammonium acetate is added to both solvents of water/acetonitrile gradient (Daziel et al. 2000). Ionized molecules are selected by mass analyzer according to their mass to charge ratio (m/z) and are subsequently detected.

Hansen et al. (2007) investigated the stress factors produced during metal exposure in *Boletus edulis.* The presence of phytochelatins (PCs), a family of cystine rich oligopeptides was confirmed in Cd-containing cell extracts by HPLC-MS. The

interactions of Phytochelators, PC and Glutathione, GSH in heavy metal interactions etc were explained. They are the pioneers in reporting the phytochelatins in mushrooms.

Robin et al. (2011) used a simple, highly selective, sensitive and reproducible liquid chromatography-electrospray ionization mass spectrometry method in their studies for the direct and simultaneous determination of reduced (GSH) and oxidized (GSSG) glutathione in micro dialysis samples from human dermis. Chromatographic separation was carried out on an Modulo cart qs kromasil 5C18 (250 mm×2 mm ×5 µm) analytical column at a flow rate of 0.25 ml/min. An isocratic mode was used and consisted of acidified water and acetonitrile (50/50, v/v). To improve the sensitivity, silver nitrate was added as post-column reagent. A trap mass spectrum was used equipped with an ESI interface. The limits of detection and quantification were respectively 0.12 and 0.4 ng/ml for GSH and 0.2 and 0.5 ng/ml for GSSG. Intra-day and inter-day accuracy and precision were determined and the variability was less than 6.2% (R.S.D.).

Squellerio et al. (2012) reported the central role of Glutathione in the defense against oxidative damage and in signalling pathways. Upon oxidation the reduced glutathione (GSH) is transformed to glutathione disulfide (GSSG). The concentration of GSH and GSSG in samples and their ratios is a useful indicator of the oxidative stress status and/or disease risk. In their work they adopted a liquid-chromatographic method coupled with tandem mass spectrometry (LC–MS/MS). The results obtained were compared with a high-performance liquid chromatographic method with electrochemical detection (HPLC-ECD). The method performed well in terms of validation parameters, i.e. linear range (0.01–50 µM for both GSH and GSSG), precision (intra and inter-day coefficients of variation were less than 10% for both GSH and GSSG), accuracy (bias% varied between −2.1 and 7.9% for both analytes), and quantification limits (LLOQs were 0.5 µM and 0.0625 µM for GSH and GSSG respectively). They also described the major benefits of LC–MS/MS like the

improved selectivity, precision and accuracy, the higher sensitivity and the unaltered capacity of detection with time in contrast to Electron Capture Dissociation (ECD).

2.10.3 SCANNING ELECTRON MICROSCOPY (SEM) AND ENERGY-DISPERSIVE X-RAY MICROANALYSIS (EDX).

SEM is used to study the surface morphology of the system and also helps to detect the presence of protrusions, or folding produced upon metal stress. The basic principle is that a beam of electrons is generated by a suitable source, typically a tungsten filament or a field emission gun. The electron beam is accelerated through a high voltage (20 kV) and the high energy electrons were passed through a system of apertures and electromagnetic lenses to produce a thin beam of electrons., then the beam scans the surface of the specimen by means of scan coils (like the spot in a cathode-ray tube "old-style" television). Electrons are emitted from the specimen by the action of the scanning beam and collected by a suitably-positioned detector. The image obtained can be photographed and analysed by comparing with that of control.

EDX helps to generate histogram which gives the elemental profile of the sample. The principle of EDX is that the electron beam generates X-rays within the specimen. Many of these X-rays have energies characteristic of the elements that emitted them. So, if the energy of the X-rays can be measured, elements present in the specimen may be known.

Lu et al. (2006) used SEM micrographs to explain the effect of heavy-metal-free and metal-loaded *Enterobacter sp.* J1 It was observed that the cell-surface morphology considerably changed after metal biosorption. The SEM micrographs also showed that the surface of metal-loaded cells looked vague and distorted and seemed to be damaged by the heavy-metal ions. The alteration in morphology may also result from secretion of extracellular polymeric substance during metal biosorption, as reported by Chen et al. (2000) who utilized *Desulfovibrio desulfuricans* to adsorb zinc and copper. Moreover, the EDS analysis was done to

confirm the presence of metal adsorbates on the cell mass, giving a direct detection of metals on cells.

Cao et al. (2010) performed SEM and EDX analysis to determine the surface structure of *Tricoderma lobayenypesse* after metal uptake along with its concentration.

Remenarova et al. (2011) studied about the metal interaction in single and binary metals systems affecting the metal uptake potential. The mechanism of biosorption of Cd (II) and Zn (II) by sewage sludge was determined by FTIR, SEM-EDX analysis and chemical blocking of functional group. The SEM analysis was used to determine the surface morphology and EDX was used for metal ion determination.

2.11 KINETICS OF METAL REMOVAL

The study of kinetics of metal removal provides an insight into the possible mechanism of metal uptake along with the reaction pathways. There are limited studies reported on kinetics of metal removal from soil by mushrooms. Several reports are available on biosorption kinetics. The biosorption generally follows a three-stage process: boundary layer diffusion, intra particle diffusion, and biosorption on binding sites (Basha et al. 2008; Zolgharnein and Shahmoradi 2010). As per their literature study report, the specific surface area of the fruiting body was 5 m^2 g^{-1}, indicating that the micropores available for biosorption inside the biomass were limited and the intra particle diffusion stage was negligible. In general, the pseudo-second-order kinetic describes well the long process period, while the pseudo-first-order model fits the experimental data well for an initial period of the first reaction step. The assumption of the pseudo-second-order model is that metal ions are adsorbed onto two surface sites and that chemisorption occurs involving valency forces through the sharing or the exchange of electrons between the fruiting body and the divalent metal ions (Ho 2006; Zolgharnein and Shahmoradi, 2010). Similar heavy metal removal kinetics was also reported by Sar and Tuzen (2009) and

Morsy et al. (2011). The metal removal from the soil by bioaccumulation process may involve all the above mentioned three steps along with the transport of metals from the surface of the fungi to the accumulation sites. If the biosorption at the surface of the mycelia is the rate limiting step, then there is a possibility of first or second order kinetic equations used for biosorption also hold good for metal removal by bioaccumulation in mushrooms (Mishra et al. 2010). The kinetic studies may help in determining the rate of metal removal and the rate controlling step in the process of metal removal by bioaccumulation.

2.12 CHELATE ASSISTED BIOACCUMULATION

Chelating agents are known to increase the bioavailability by forming neutral complexes (Michele et al. 2007). These neutral complexes can be easily accumulated by passive adsorption. Based on the bioremediation studies reported in the past decades it is understood that the chelating agents play a significant role in increasing the bioaccumulation efficiency without diminishing their yield. From a thorough literature studies it is clear that various kinds of chelaters, chemical or biological origin were used widely for phyto remediation. Their influence in soil heavy metal removal were widely discussed in the studies of Khan et al. (2000) Chen et al. (2001), Michel et al. (2007), Machuca et al. (2007), Sinhal et al. (2010), Zhao et al. (2010) Sun et al. (2011), Ullah et al. (2011). The most commonly studied chelaters were Ethylene diamine triaceticacid (EDTA) and N-(2- hydroxyethyle) ethyl diamine triacetic acid (HEDTA), iminodisuccinic acid (IDSA), Ethylenediamine-N,N'-disuccinic acid (EDDS)various synthetic aminopolycarboxylic acids, such as ethylene diamine tetraacetic acid, and natural ones such as, ethylene diamine disuccinate and nitrilotriacetic acid, Diethylene triamine pentaacetic acid (DTPA), citric acid, oxalic acid, vanillic acid, and gallic acid. These chelaters can easily form complexes which are less toxic and increase the bioavailability of the metals ions.

2.13 MULTI-METAL INTERACTION IN METAL UPTAKE FROM SOIL

Toxic heavy metals, especially copper (Cu), cadmium (Cd), lead (Pb), and zinc (Zn), are increasingly being released into the environment from industrial wastewaters and other human activities. Heavy metal contaminations bring potential health hazards to animals and human beings (Volesky et. al.1995). A wide variety of fungi, bacteria, and algae are now under study for possible applications as bioremediating agents for heavy metal remediation (Ahluwalia et al. 2007). These biological agents are not able to effectively remove metals from real industrial effluents attributable to presences of organic or inorganic ligands and presence of metal mixtures. Simultaneous interactions among multiple heavy metals with microorganisms may result in synergic, antagonistic, or additive effects. These mechanisms of interactions may be particularly complex and unique, depending on the combinations of heavy metals and microbial strains. The combined effects (e.g. growth stimulation or toxicity) of multiple metals in the same microbial consortium are usually different from the additive effects from the individual metals involved (Petros et al 2008). Hence bioaccumulation studies in multi metal environment are necessary. The effect of presence of multi-metals in the soil on the bioaccumulation efficiency of various macro fungi were also reported by Thomet et al. (1999), Gabrel et al. (2008), Cao et al. (2010), Remenarova et al. (2011) and Lin et al. (2012).

Gopal et al. (2002), Stirk and Staden (2000), Addour et al., 1999 and Opeolu et al. 2010 discussed about the heavy metal biosorption using fungi like *Phanerochaete chrysosporium, Rhizophora mangle, Saccharomyces cerevisiae* The bioaccumulation efficiency studies of these fungal species were performed for copper (II), lead (II), and cadmium (II) to evaluate the effectiveness and to optimize the operational parameters using response surface methodology (RSM). The operational parameters chosen were initial metal ion concentration and pH of the medium. RSM was used to explain the relation between metal removal efficiency and the operational parameter values to be optimized. These researchers have used design of experiments

(DOE) strategy to reduce the number of experiments to study the effect of operational parameters. The study also showed that the removal efficiencies followed the order Pb(II) > Cu(II) > Cd(II). The metal removal efficiency was found to decrease as the initial metal ion concentration increased. Design of experiment strategy can be used to design the experiments involving multiple factors to reduce the number of experiments. Response plots can be obtained through RSM using the results of experiments designed as per DOE. These response plots help in analyzing the effect of factors and to study the interaction effect between the factors. Box Behnkam and Central Composite Designs are most often used experimental designs.

From the through literature studies it is understood that the exact mechanism of reduction in bioaccumulation potential at multi-metal level when compared to a single metal system is not known. But it is clear that the presence of metals together in medium suppressed the uptake of individual metal ions. The interpretation of the multi- metal system has proved to be complex and may be a function of one or all of the following parameters: ionic radii, electronegativity, system, pH, and the availability of the active sites for uptake (Mohan et al. 1996; Sari et al. 2009; Chatterjee et al. 2010).

2.14 ESTIMATION OF HEAVY METAL CONTENT IN MUSHROOMS

The heavy metal associates themselves with organic compounds in the soil, causing the metal ions immobile or partially so within the soil structure. To completely release the metal ions into liquid phase for analysis, an extraction process has to be applied to the soil sample. Some methods of determination require the sample to undergo chemical digestion to release the metals so that analysis can be achieved *viz.*. Atomic absorbtion spectroscopy (AAS) (Anderson et al. 1982; Kojo and Lodenius. 1988 Vetter 1994; Jorhem et al. 1995; Cibulka et al.1996; Svoboda et al. 2000 Kalac et al. 1991; Demirbas 2002; Isildak et al. 2003), Inductive coupled plasma analyzer (ICP) (Abdechi et al. 2010; Guven and Akinci 2011). Non destructive methods like XRF (X-Ray fluorescence) (Zhang et al. 2008;

Chen et al. 2009) are the primary accredited methods with proven high precision but all of the quality analysis systems can be negated by a non representative sample. As per the literature reports the mushrooms are capable of accumulating wide range of metals like cadmium, chromium, copper, tin, gold, lead, zinc, magnesium, mercury, molybdenum. Most of the researchers used US-EPA procedure for metal ion extraction. As per the procedure the soil/ biomass was digested using concentrated hydrochloric acid (HCl) at 90ºC (wet digestion method) and the product is analyzed for metals using any of the following: Flame atomic absorption spectrophotometry (FLAA), Graphite furnace atomic absorption spectrophotometry (GFAA), Inductively coupled plasma atomic emission spectrometry (ICP-AES) or Inductively coupled plasma mass spectrometry (ICP-MS). Guven and Akinci (2011) modified EPA's Acid digestion procedure 3050B for a short digestion time, less acid consumption and high extraction ability using HCL and nitric acid (HNO_3) mixture. Researchers like Tuzen et al. (2003), Mustafa et al. (2004), Carvalho et al. (2005), Juan et al. (2008), Chen et al. (2009) and Chengham et al. (2010) developed a modified acid digestion procedure for an efficient metal removal. They also reported that the metal ions in mushroom can be determined by digesting them with an acid mixture consisting of hydrochloric acid and perchloric acid in the ratio 1:3 per gram of biomass.

The review of literature revealed that several mushroom species have the ability to accumulate the metals from their immediate surroundings. The metal bioaccumulation potential of the mushrooms may be exploited for mycoremediation of soil by removal of metals using the mushroom species. It is necessary to isolate and to select the mushrooms species which has good metal tolerance and bioaccumulation potential. Mushroom species grown in metal polluted soil may have high bioaccumulation potential. Thus mushroom species may be isolated form metal polluted soil and the potent mushroom species for mycoremediation may be selected based on their metal tolerance and bioaccumulation potential studies. In order to further understand and improvise the process of mycoremediation the mechanism of metal uptake has to be investigated. It is also important to study the effect of other

metals on bioaccumulation so as to test the efficiency of the mushrooms for metal removal in multi-metal contaminated environment.

Based on the extensive review of literature, the objectives of the present study were formulated and are presented in the following section.

2.15 OBJECTIVES OF THE STUDY

The main objective of this research is to remediate heavy metal contaminated soil using macro fungi (mushrooms) belonging to Basidiomycetes family.

The specific objectives are:

- To isolate heavy metal tolerant mushrooms from municipal waste dump yards.
- To screen the isolated mushrooms based on heavy metal tolerance and mycelial growth
- To study the factors affecting the uptake of heavy metals of high priority by mycelia of the selected mushrooms.
- To optimize soil pH and incubation time for efficient soil remediation through metal bioaccumulation.
- To identify the selected mushroom isolates
- To study metal bioaccumulation efficiency of the mycelial biomass and fruiting body of the selected mushroom species and to determine the bioaccumulation factor
- To identify the site of bioaccumulation in the fruiting body of the selected mushroom species
- To study the relevant bioaccumulation mechanism and deduce an appropriate mechanism for heavy metal uptake by the selected mushroom isolate.

- To study the kinetics of metal removal from soil by the selected mushroom isolate.
- To study the effect of addition of chelating agents into soil on heavy metal bioaccumulation
- To study the effect of multi metal interaction on heavy metal removal by the selected mushroom isolate through bioaccumulation.

CHAPTER – 3

MATERIALS AND METHODS

This chapter describes the experimental details of various methodologies adopted in the present study. The detailed procedure of isolation of heavy metal tolerant mushrooms, its *in-vitro* establishment involving methods of spawning and casing, bioaccumulation studies of mycelia and fruiting bodies, removal rate kinetic studies, methods in understanding the mechanism and methods to study the effect of chelators, multi-metal interaction effects are discussed in detail.

3.1 MATERIALS

The materials used in the present study are presented in Table 3.1.

Table3.1 Description of chemicals used in the study

Chemicals	Company and Grade
Sabourauds dextrose agar media, Nutrient Agar, yeast extract.	Hi media Laboratories Pvt Ltd, Mumbai, India.
Sucrose, Tris-HCl, Phenol, Acetone, Ethanol, Perchloric acid, Sulphuric acid.	Nice Chemicals, Cochin, India
Hydrochloric acid, Ammonium cholaride, Copper sulphate, Zinc sulphate, Lead sulphate, Pottasium dichromate, Cadmium Sulphate, Calcium chloride, Ethylene diamine tetra acetic acid, Sodium hydroxide, Potassium dihydrogen phosphate, Ferrous sulphate, Di sodium hydrogen phosphate, Sodium nitrate, Ammonium nitrate, Citric acid, Gallic acid, Ferrous chloride, Calcium carbonate, Sodium chloride, Acrilamide, Bis acrilamide, Sodium dodecyle sulphate, Ammonium acetate, Acetic acid, Mercapto ethanol, Gluteraldehyde,	Merck India Ltd, Bangalore, India.
Paddy seeds, Saw dust	Locally available
Protein marker	Genei Ltd, Bangalore, India.

3.2 METHODS

Detailed methodologies adopted in the present study to fulfill the specified objectives are as follows:

3.2.1 SELECTION OF SALTS OF HEAVY METALS FOR *IN-VITRO* STUDIES

Toxicity of any chemical in the environment can be regarded as a function of the chemical's solubility in water. Insoluble compounds as well as the metallic forms often exhibit negligible toxicity. Heavy metal gets in to the system in their soluble forms and imitates the action of an essential element in the body, interfering with the metabolic process to cause illness. Hence in the present study heavy metal salts were selected based on their solubility and hence their access to environment in their potent form. For the present study heavy metal salts like $CuSO_4$, $CdSO_4$, $K_2Cr_2O_7$, $PbNO_3$ and $ZnNO_3$ were selected as these are highly soluble in water and thus act as potent pollutants in soil.

3.2.2. PREPARATION OF STOCK SOLUTIONS

The aqueous stock solutions of 10,000 mg/L of Cu(II), Cd(II), Cr(VI), Pb(II) and Zn(II) were prepared by dissolving suitable quantity of analytical grade $CuSO_4$, $CdSO_4$, $K_2Cr_2O_7$, $PbNO_3$ and $ZnNO_3$ in deionized water, respectively. The stock solutions were used to contaminate the soil to attain the desired concentrations of these heavy metals (50 and 100 mg/kg). Similar heavy metal salts were also used in heavy metal bioaccumulation studies conducted by Oei (1996) and Orazio et al. (2010).

3.2.3 COLLECTION OF MUSHROOMS SPECIES

Macro fungi belonging to *Basidiomycota* phylum that can yield fruiting body were collected from various municipal waste dump yards of Dakshina Kannada District, Karnataka, India. The district lies between **12 57' and 13 50' North Latitude and 74 and 75 50' East Longitude.** The mushrooms belonging to *Basidiomycota* phylum are generally known as *Basidiomycetes.* The mushrooms (fruiting body) were excised using sterile scalpel, washed with deionized water and subjected to surface sterilization using 70% alcohol. The samples were stored in clean zip bags at 4°C until sub culturing.

3.2.4 INITIAL SCREENING OF COLLECTED MUSHROOMS

The collected mushrooms were identified to their genus level by detailed observation of morphological characteristics (color of the fruiting body, gills, spore print and other significant features). The descriptions of the mushroom species collected are shown in Table 3.2. Sub-culturing of these mushrooms was done by inoculating the explants on to sabouraud's dextrose agar medium (SDA) for *in-vitro* growth. The composition of the media used for sub culturing is given in Appendix-I.The totipotent regions of the mushrooms were excised using sterile surgical blades and were inoculated on SDA amended with the selected heavy metals. The objective was to perform preliminary screening of the isolated mushrooms based on their ability to grow in a medium contaminated with heavy metals like Cu(II), Cd(II), Cr(VI), Pb(II) and Zn(II). The mushrooms were tested for their growth in SDA medium amended with the salts of heavy metals like Cu(II), Cd(II), Cr(VI), Pb(II) and Zn(II) at a concentration of 100 mg/L individually for studying individual effect of these metals (Bai 2002). The plates were then incubated at room temperature for 5 days at 23±2°C. The isolates which could grow successfully in the medium were selected for further studies.

Table 3.2 Description of mushroom samples collected

Sl. no	Name of the sample	Sample Description	Location
1	M1	Stem: Flexious Cap: Smooth Spore: black Color: Off white with black spot in the center	Road sides near Kudremukh Iron Ore Company Ltd, India and Municipal waste deposit areas of Mangalore, Dakshina Kannada District, Karnataka, India.
2	M2	Stem: Equal Cap: Rugulose Spore: white Color: white and dusty	
3	M3	Stem: Clavate Cap: Glabrous Spore: Brown Color: Dark Brown	
4	M4	Stem: Equal Cap: Sericeous Spore: Brown Color: Slight brownish	
5	M5	Stem: Terete Cap: Glabrous Spore: Brown Color: Pinkish white	
6	M6	Stem: Equal Cap: Smooth Spore: Off white Color: Creamish White	
7	M7	Stem: Short thick or absent Cap: 2” Broad Spore: Yellow Color: Yellowish white	
8	M8	Stem: Short Cap: Flower like Spore: Dark brown and dusty Color: Dark brown	
9	M9	Stem: Clavate Cap: Rivulose Spore: White Color: White	
10	M10	Stem: Slender and yellow Cap: Flat Spore: Off white or Yellow Color: White	

3.2.5 SCREENING OF ISOLATED MUSHROOMS FOR THEIR METAL TOLERENCE

The mushrooms which could successfully grow in metal laden conditions (100 mg/L) were selected for further studies. The totipotent regions of the mushrooms were aseptically isolated and were inoculated on to a sabouraud's dextrose agar medium (SDA) amended with Cu(II), Cd(II), Cr(VI), Pb(II) and Zn(II) at concentrations from 100 to 1000 mg/L in increments of 100 mg/L. This process helps to study the heavy metal tolerance of mushrooms *in-vitro* by growing them on nutrient medium i.e SDA broth. The plates were then incubated for 5 days at 23 ± 2°C. The growth patterns of the fungal species were observed and the minimum inhibitory concentration (MIC) or metal tolerance of heavy metals was determined by visual observation (Aboulroos et al. 2006; Chen et al. 2000). The fungal species which have shown good tolerance against most of the heavy metals were chosen for further screening studies (Martinez et al. 2000).

3.2.6 ARTIFICIAL CONTAMINATION OF SOIL

A soil slurry was prepared by mixing 10 g of garden soil of grain size >2 mm with adequate amount of sterile distilled water containing desired concentration of metal salts to form a slurry (85-90% moisture). It was further cured for a period of 10 to 15 hours at 40^{o}C in hot air oven. The cured soil slurry containing the salts of heavy metals was used for mycelia bioaccumulation studies. The heavy metal salt solution was mixed with the soil to attain required metal concentration. The mixture was then dried at room temperature. The metal amended soil was used for metal bioaccumulation in fruiting body stages (Chen et al. 2000; Dermirbas 2002).

3.2.7 ANALYSIS OF HEAVY METAL CONCENTRATION

In the present studies, the analysis of concentrations of metals in biomass and in soil was necessary. Many analytical instruments and methods have been developed over past 30 years to determine the concentrations of metals in our ecosystem, atmosphere, water, soils and sediments (Sandroni et al. 2003). Highly sensitive spectroscopic techniques such as flame or electro thermal atomic absorption spectroscopy (FAAS, ETAAS) and inductive coupled plasma mass spectroscopy (ICPMS) and inductively coupled plasma optical emission spectroscopy (ICP-OES) are the most widely used methods to determine metals' concentration in liquid samples (Sastre et al. 2002; Guven and Akinci, 2011). Since the concentrations in solid samples cannot be analyzed directly by these instruments, the metal content in soil and biomass samples had to be transferred to liquid form by digestion.

Atomic absorption spectroscopy was proposed for metal analysis in the present study. Pretreatment of *Galerina vittiformis* biomass was very important before atomic absorption spectroscopy (AAS: GBC-6000) as it can analyze only liquid samples. Similarly digestion steps were required to leach out metals from its complex form to simple ionic forms. There are mainly three methods available for digestion, namely wet digestion, dry digestion and microwave digestion. Both microwave and wet digestion were found to be efficient as per the studies of Kucak and Vlanusa, (1998), Guven and Akinci (2011). Due to its simplicity and ease, wet digestion was followed in the present study. Acid mixtures are commonly used for digestion to release the metal ions from the solid biomass. Acid mixture selection for digestion also plays a critical role in extraction procedures; most commonly used acid mixtures are as follows HNO_3, HNO_3-HF and HNO_3-HCL. Among the acids used for wet digestion, HCl (boiling point 110^oC) is useful for salts of carbonates, phosphates, some oxides and some sulfides. HNO_3 (Boiling point 123^oC) oxidizes many samples which are not dissolved by HCl. HF is usually avoided in the digestion procedures because of its toxic nature.

The soil and biomass samples after bioaccumulation process were dried in an oven at 60^{o}C. The acid digestion was carried out to leach out the metal ions from the solid samples. The digestion of mycelia samples were carried out as per the procedures reported by Haswell(1991) and digestion of mushroom fruiting body samples were carried out according to the procedures reported by Tuzen et al (1998) and Demirbas, (2001). The procedures for pretreatment and acid digestion of the solid samples (mycelia, fruiting body and soil) for analysis of metal concentrations are outlined in section 3.2.7.1, section 3.2.7.2 and section 3.2.7.3. Acid digestion of the samples was done to leach out the metal ions from the soil efficiently for atomic absorption spectroscopy (Haswell, 1991, Chen et al. 2009). The metal concentrations in the digest were analyzed using AAS, Model: GBC-6000.

3.2.7.1 ANALYSIS OF METAL CONTENT IN MYCELIA

The mycelial biomass was pretreated and acid digestion was carried out as per the procedures reported by Haswell (1991). For an effective heavy metal analysis, the dried biomasses were acid digested .1g of the dried mycelial biomass samples were mixed with 2 ml of 65% HNO_3 and 6 ml of perchloric acid and then digested in a microwave digester (CEM- MARS, USA) at 600 W for 20 min. The digested mixtures were cooled and were made up to 50 ml using deionized water. The cooled mixture was then filtered using Whattman No.1 filter paper. These samples were analyzed for metal contents using, Atomic absorbtion spectrometer (Model AAS: GBC-6000). The heavy metal content in the biomass was then calculated. Similarly the metal concentration in the soil was analyzed using oven dried soil samples.

3.2.7.2 ANALYSIS OF METAL CONTENT IN MUSHROOM FRUITING BODIES

The fruiting body biomass was pretreated and acid digestion was carried out as per the procedures reported by Tuzen et al. (1998) and Demirbas (2001). Every gram

of dried mushroom fruiting body biomass were mixed with 6 ml of 98% concentrated oxi-acid mixture (HNO_3:H_2SO_4:H_2O_2 in the ratio 4:1:1 v/v) and heated for 3h at 75°C. The digested mixtures were cooled and 20 ml of deionized water was added to it. Further the mixture was heated for 4h at 150°C with occasional stirring. Heating was continued till the orange vapors escape completely. The digest was then cooled and the final volume was made up to 25 ml with demineralized water after cooling. The cooled mixture was then filtered using Whattman No.1 filter paper. These samples were analyzed for metal contents using, Atomic absorbtion spectrometer (Model AAS: GBC-6000).

3.2.7.3 ANALYSIS OF METAL CONTENT IN SOIL SAMPLES

The metal content in soil samples were analyzed according to the method reported by Srivastava et al. (2006). To measure the metal concentration in soil slurry used for the study, the oven dried soil after every bioaccumulation study was digested with 2ml of 65% HNO3 and 6 ml of HCl per gram of soil at 600 W in microwave digester (Make: MARS:CEM, USA). The digest was then filtered and analyzed by AAS.

3.2.7.4 ANALYSIS OF METAL CONCNETRATION USING ATOMIC ABSORPTION SPECTROMETER

The AAS was recalibrated for analysis of metal concentration in liquid samples using three known concentrations of standard metal ion solutions. The concentration ranges of these standard solutions were selected based on the sensitivity of metal ion to the instrument. The standard solution concentrations used for calibration for different metal ion analysis are presented in Table 3.3. The AAS: GBC-6000 is programmed to take a mean of all the samples readings five times to ensure the accuracy of metal analysis. The instrument also ensures to analyze the sample after obtaining a best fit calibration curve. The same acts a pre check of the

instrument. All the samples are prepared using Millipore pure water for preventing clogging of spraying nozzle. 5µl of the sample was drawn every time for the analysis which was then sprayed on to the furnace. The furnace will maintain at a temperature of 1000ºC with the help of acetylene gas. At this high temperature the metal ions get atomized casing a change in the intensity of light. Automatic wavelength setting 185 to 900 nm. Continuously adjustable slit width 0.2 to 2.0 nm with automatic setting. Asymmetric modulation reduces noise by up to 40%. Ultra Pulse fast background correction, at up to 2.5Abs. The difference in the light intensity is compared to the blank and concentrations can be deduced using Beer lamberts law. The light source used for each metal analysis is different. This also increases the sensitivity of the instrument.

Table 3.3 Concentration range of standard solutions for calibration of AAS

Metal ions	Calibration range(ppm)
Cu (II)	0.1- 60 ppm
Cd (II)	0.5-25 ppm
Cr(VI)	0.1-3 ppm
Pb (II)	0.3-5 ppm
Zn (II)	0.2-10 ppm

3.2.8 IN-*VITRO* ESTABLISHMENT OF MUSHROOM ISOLATES IN SOIL

All mushroom isolates that showed good growth and tolerance on SDA media were selected for this second level of screening. In this study the ability of mushroom species to grow on soil environment *in-vitro* was analyzed. The mushroom isolates were grown on metal laden soil environment independently with different metals under study for a period of 20 days at 30 ± 2°C. Incubation period was extended to 20 days as no sufficient amount of biomass was observed within 5 days of incubation. The isolates were grown in 100 ml conical flask containing the soil slurry with heavy metals, Cu(II), Cd(II), Cr(VI), Pb(II) and Zn(II) individually at a concentration of

50 mg/kg to 250 mg/kg. A thin top layer of metal solution was formed after thorough mixing. The pattern of growth was observed carefully and the observations in terms of growth of isolates were recorded. The recorded observations were further analyzed for selecting metal tolerant isolates in soil for further studies.

3.2.9 STUDY OF FACTORS AFFECTING MYCELIAL BIOACCUMULATION

The bioremediation process is a complex system and is controlled by many factors. These factors include the existence of a mushroom population capable of accumulating heavy metals, the availability of heavy metals from soil to mushroom, the contact time and the environment factors (type of soil, soil pH, temperature, atmospheric humidity) (Morsy et al. 2012; Chen et al 2000). Among them, pH and incubation time play a very important role. Hence, the studies on effect of soil pH (ranging from 5 to 8) and incubation time for heavy metal accumulation by mushroom mycelia were carried out in a flask system. The fungi were grown in flasks containing soil slurry with the metal under study for a period of 20 days, harvested at every 5 days intervals and analyzed for metal concentration in both soil and biomass by atomic absorption spectrometer, after pretreatment and digestion.

3.2.9.1 SOIL pH

To estimate the effective soil pH for maximum bioaccumulation in mushrooms, the mycelial mat of 1cm were grown in metal contaminated soil slurry maintained at different initial pH ranging from 5 to 8. The disc of mycelial mat can be removed aseptically using cork borer. For every pH under study two sets of flasks were maintained. Generally fungal mycelium showed maximum growth of fungi at slightly acidic pH *i.e* pH 6.8. Hence the pH range of the soil was studied between pH 5 to pH 8 to standardize the effective soil pH for maximum mushroom growth. The system was incubated at room temperature ($30\pm2^{o}C$) for 20 days. The mycelia

mat obtained were further digested with acid mixture for analyzing metal content using AAS. The detailed procedure for metal analysis is mentioned in sections 3.2.7.1.

3.2.9.2 INCUBATION TIME

To study the effective incubation time required for maximum bioaccumulation, 5 g of spawn bits were grown on metal contaminated soil slurry maintained at pH 6.8 in 100ml conical flasks. From the literature it was understood that the fungal mycelia grow efficiently in a medium pH of 6.8. At an interval of every 10 days two sample flasks were harvested and the biomass (fungal mat) was analyzed for metal concentration using AAS. The process was continued for 40 days.

3.2.10 MYCELIAL BIOACCUMULATION STUDIES

Fungal mycelia discs were inoculated on to flasks containing 10g of metal laden soil slurry. A minimum of 3 flasks were assigned for each metal ion and the studies were conducted with two concentrations (50 and 100 mg/kg). The systems were maintained at 30 ± 2°C for the best suitable incubation period to attain maximum mycelia biomass and bioaccumulation. The mycelia mat formed on the surface were further harvested by skimming. The skimmed mycelia mats were further washed with sterile water to remove the traces of soil media using a funnel and a pre weighed Whatman's Grade No: 1 filter paper. The mycelium was dried in a hot air oven at 60^{o}C until a consistent mass of the biomass was obtained. The exact mass of these dried biomass were determined. The metal content in the mycelial biomass and soil were analyzed by following the acid digestion procedure described in section 3.2.7.1 and 3.2.7.3 respectively.

3.2.11 IN-VITRO CULTIVATION OF MUSHROOMS

The selected mushrooms after surface sterilization were dissected for totipotent regions using fine sterile surgical blades without any tissue damage. The totipotent regions were inoculated onto SDA and incubated at room temperature for experimentation purpose. Growth of the fungal species was observed for the formation of uniform mycelial mat. Martínez-Carrera et al. (2000), Flegg et al. (1985), Chang and Miles (1989) and van Griensven (1988) had also adopted the criterion of formation of uniform mycelia mat as an indication of growth for *in-vitro* cultivation of mushrooms.

3.2.11.1 SPAWNING

Fungal mycelial mat free of SDA medium were inoculated on to a sterilized substratum. Substratum can be either rice grains or wheat grains or dung depending on the ease of availability (Oei, 2005; Chang, 2003). In the present study, rice grain was used as a substratum. Calcium carbonate was added to the selected substratum to control the moisture content.

Pre Treatment of substratum: 1 kg rice grain was soaked overnight. The seeds were then washed 3 times with tap water and boiled for 30 minutes. Water was drained as and when the grains split and blot dried to room temperature. 10% $CaCO_3$ was mixed thoroughly with the grains to avoid clumps, $CaCO_3$ was used to control the moisture content of the grains so that effective growth of mycelia is promoted. 100g of the grain mixture was then dispensed to 500ml conical flask. 2 loops full of fungal culture were inoculated on to the mixture, the flasks were cotton plugged and were incubated at suitable conditions (90-94% humidity, 23ºC) for 25-30 days until a proper mycelia mat was formed.

3.2.11.2 CASING OR LAYERING

Matured spawns (each grain coated with the fungal mycelia) were crushed using a sterile glass rod to avoid clumps and were overlaid with pretreated substratum (Saw dust) alternatively to obtain 1inch total bed thickness. The preparation and pretreatment of casing substratum was done as described below.

Pretreatment of substratum: To a sterile mixture of soil and saw dust (3:1), 1% ammonium nitrate solution was added, as nitrate concentrations trigger fruiting body formation. 10ml of ammonium nitrate was used per 50 g of soil saw dust mixture. This helps to maintain 80-90% relative humidity.

The bed was then incubated in dark conditions at 23±2ºC for a period of 30 days. Moisture was maintained at 80% to promote maximum growth of fungal mycelia. The overview of casing arrangement is shown in Figure 3.1.

Fig 3.1 Lateral section of casing set up

3.2.12 FRUITING BODIES BIOACCUMULATION STUDIES

5 g of spawns were cased on soil mixture artificially contaminated with the salts of heavy metals. Casing process was carried out in Plastic trays of 25cm×20cm×5cm dimensions, sterilized with 70% alcohol. 1 inch thick soil layer along with saw dust as the bulking agent, in the ratio of 4 :1 (w/w) was mixed with suitable volumes of solutions of $CuSO_4$, $CdSO_4$, $K_2Cr_2O_7$, $PbNO_3$ and $ZnNO_3$ *i.e.*, salts

as source of heavy metals: Cu(II), Cd(II), Cr(VI), Pb(II) and Zn(II), such that concentrations of heavy metals were 50 and 100 mg/kg as desired, along with saw dust as the bulking agent, in the ratio of 3:1 (w/w) for lab scale bioaccumulation studies. Spawns were grown in the dark at 20°C - 24°C and 80 - 90% relative humidity for a period of 25 days with periodical monitoring. At the end of 25th day, the fruiting bodies were harvested using sterile forceps and allowed to dry at room temperature. The dried samples were digested using oxi-acid mixture as per on the detailed procedure presented in section 3.2.7.2.

3.2.12.1 DETERMINATION OF BIOACCUMULATION FACTOR (BAF)

To determine the efficiency of bioaccumulation, the metal concentration in the mushroom was compared to the metal concentration in its environment (Niu et al. 2007; Zhao et al. 2003). The bio accumulation factor/ efficiency factor is defined as given below (Vanloon et al. 1973; Scragg 2005).

$$BAF = \frac{\text{Concentration of metal in dried biomass (mg/kg)}}{\text{Concentration of metal in the soil (mg/kg)}}$$

3.2.12.2 DETERMINATION OF SITE OF BIOACCUMULATION

After the studies on bioaccumulation using mushrooms, it was understood that heavy metals are accumulated in its fruiting bodies than in its mycelia. The fruiting body consists of its stalk and pileus. To study the exact site of accumulation in fruiting bodies the heavy metal content was analyzed differentially. The method for determination of the site of bioaccumulation was as follows: the Spawns were grown on soil mixture artificially contaminated with the salts of heavy metals like $PbNO_3$, $CdSO_4$, $CuSO_4$, $K_2Cr_2O_7$, $ZnNO_3$ at various concentrations like 10, 50 and 100 mg/kg. Plastic trays of 25cm×20cm×5cm dimensions were sterilized with 70% alcohol and were used for efficient bioaccumulation studies. Spawn bits of selected mushrooms species were grown by sandwiching with artificially contaminated soil mixtures as shown in Figure 3.1 This system was maintained in dark at 20- 24°C and 80-90% RH

for a period of 25days. After the 25th day as and when the fruiting body emerges it was harvested using sterile forceps. From the harvested fruiting bodies the cap (pileus) and stipe were separated and dried separately at room temperature. The dried biomass was digested with acid mixtures and heavy metal content in them was analyzed using AAS.

3.2.13 IDENTIFICATION OF HEAVY METAL TOLERENT MUSHROOM SPECIES

The mushrooms samples M5, M6 and M9 which were selected based on the screening process (tolerance study) were sent to Agharker Research Institute, Pune, Maharashtra, India for Internal transcribed spacer sequence analysis (ITS). The detailed procedure for sequencing is mentioned in the appendix-II. The sequence obtained was compared with National Center for Biotechnology Information Gen Bank entries by using BLAST algorithm.

3.2.14 STUDIES ON METAL REMOVAL KINETICS FROM SOIL BY MUSHROOMS

To study the kinetics of heavy metal removal from the soil using the mushrooms, the mushrooms were grown in metal contaminated soil (50 mg/kg) in a tray-soil system under 20-24ºC for 40 days. The biomass was recovered every day after initial 5 days of incubation (lag phase of growth). The biomass and the soil samples were then dried in oven (Orbiteck, India), these dried biomass and soil were acid digested using microwave digesters and the heavy metal content were analyzed using AAS. The detailed procedure for metal content analysis is given in section 3.2.7.2 and 3.2.7.3. The data on metal concentration in soil as a function of time were recorded. Tangents were drawn at various points on concentration *vs* time plots and the corresponding rate of metal removal were obtained from the slope of the tangents. The removal rate – concentration data were analyzed and tested with various

known removal rate kinetic equations such as intra particle diffusion model, first order and second order kinetic models to deduce the rate limiting mechanism during the removal of metals from soil and also to evaluate the valid kinetic model along with the parameters of the model.

3.2.15 CHELATE ASISTED BIOACCUMULATION

Influence of chelaters on metal uptake efficiency was studied using both chemical and biological chelating agents. For the study, biobased chelating agents *viz.* citric acid, gallic acid and chemical chelater; ethylene di-amine tetra acetic acid (EDTA) were used. The soil slurry in conical flask was treated with chelating agent at concentrations of 1, 5 and 10 mmol kg^{-1} and untreated system was used as a control. The soil systems were then cased with *Galerina vittiformis* spores and were incubated in dark conditions at 20-24ºC and 80-90% relative humidity for a period of 25 days with periodical monitoring. At the end of 25^{th} day, the fruiting bodies were harvested using sterile forceps and allowed to dry at room temperature. The dried biomass was subjected to pretreatment, acid digestion and metal analysis using AAS. The method followed for the metal analysis is same as it is mentioned in section 3.2.7.2.

3.2.16 MECHANISM OF BIOACCUMULATION

3.2.16.1 SCANNING ELECTRON MICROSCOPY (SEM) AND ENERGY-DISPERSIVE X-RAY SPECTROSCOPY (EDX)

In order to prove that the fungal species accumulate the metals from the environment, mushrooms were grown in metal contaminated soil slurry. The fungal (mycelia) mat obtained were harvested and dried in oven at 60ºC. These dried biomass were treated as per the protocol described in Section 3.2.7.2 (Susan et al. 2007; Araya et al. 2007; Srivastava, et al. 2006; Gonzalez et al. 2002; Basile et al. 2007). The dried biomasses were pretreated before subjecting for SEM-EDX. The pretreatment methods were as follows: Dried fungal mycelia were

immersed in 10% glutaraldehyde and were incubated for about 10-12 hours at 4°C. Further the biomass was treated with alcohol gradations (10%, 30%, 50%, 80% and 100%) for 2min to remove the water content. The pretreated specimens were then sputtered with gold particles using a sputter coater under vacuum and then observed under a scanning electron microscope (JSM-6380; JEOL, Tokyo) at an accelerating voltage of 12 or 15 kV to capture the images.

3.2.16.2 FOURIER TRANSFORM INFRARED SPECTROSCOPY ANALYSIS (FTIR ANALYSIS)

Three discs containing 5days old mycelia of the selected mushroom species were grown in metal contaminated soil and were incubated for 20 days. The mycelia were isolated and washed with distilled water and dried in oven at 60°C. The dried biomass was powdered and analyzed by Thermo Nicolet 6700 FTIR spectrometer to find out the functional groups that are responsible for the metal binding in cytosol. From the through literature studies it is understood that FTIR spectra of finger print region *i.e* 1000 to 1500 (amide-II and II regions) and 800-1000 cm^{-1}(amide-III regions) which indicates the presence of higher amounts of acids, protenitous and non proteinous compounds by *G.vittiformis* upon exposure to Cd(II), Cu(II), Pb(II), Cr(VI) and Zn(II).

To characterize the stress components produced in this biomass, FTIR was performed on fruiting body extracts. The stress components were extracted using Tris buffer system, 3 g of dried fruiting body was ground using liquid nitrogen in a mortar and pestle, the homogenized extract was mixed with 3X Tris buffer (30 mMTris, 250 mM NaCl, pH 7.6) in ice bath; Centrifuged at 12,000 g for 15 min at 4ºC, the supernatant was collected and stored at -20ºC. The extract was then subjected to FTIR.

3.2.16.3 HIGH PRESSURE LIQUID CHROMATOGRAPHY AND MASS SPECTRAL ANALYSIS STUDIES (HPLC-MS)

Fruiting body obtained after bioaccumulation studies for all the studied metals were dried at room temperature. These dried biomasses were subjected to the extraction procedures reported by Hansen et al. (2008) as given below. All the extracts were subjected for HPLC-MS analysis to detect the presence of non-protein stress factors like glutathione, mettalothionine and phytochelatins.

To extract metals from dried biomass**,** liquid N_2 was added to 3 g of dried mushroom biomass and then ground to a fine powder using mortar and pestal. The biomass powder was then homogenized with 25 ml of 3X volume of Tris buffer (30 mM, 250 mM NaCl, pH 7.6). The mixture was then centrifuged at 12,000 g for 15min at 4ºC. Supernatant was collected and were stored at -80ºC until analysis.

3.2.16.4 PRE REQUISITES FOR LC-MS ANALYSIS

Liquid chromatography was performed using an Accela pump and an Accela auto sampler (Thermo Fisher Scientific, San Jose, CA, USA). Separation of analytes was conducted on a Luna PFP(2) analytical column (100 mm × 2.0 mm, 3 µm). The LC mobile phases were (A) ammonium formate 0.75 mM adjusted to pH 3.5 with formic acid and (B) methanol. Separation was performed under isocratic conditions with 99% mobile phase A at flow rate of 200 µL/min and a column temperature of 35ºC. Total run time per sample was 10 min and all injection volumes were 10 µL. Mass spectrometric analysis was performed using a TSQ Quantum Access (Thermo Fisher Scientific, San Jose, CA, USA) triple quadrupole mass spectrometer coupled with electro spray ionization (ESI) operated in multiple reactions monitoring (MRM) in positive mode. The MRM for GSH (m/z 308.1 → m/z 76.2 + 84.2 + 161.9) and GSSG (m/z 613.2 →m/z 230.5 + 234.6 + 354.8) were performed with collision energy optimized for each transition. The operating conditions for MS analysis were as follows: spray voltage, 2500 V; capillary temperature and voltage, 280ºC and 35 V,

respectively; sheat gas and auxiliary gas flow, 30 and 5 arbitrary units, respectively; tube lens offset, 84 V for GSH and 115 V for GSSG. The mass spectrometer was employed in MS/MS mode using argon as collision gas. Data acquisition and analysis were performed with Xcalibur® software, version 2.0 (Thermo Fisher). Conditions described above were used for the analysis of both phytochelatins and glutathione.

For the analysis of metalothionine in the samples, the samples were infused into the mass spectrometer at a flow rate of 7 μL/min. The conditions selected for good quality analysis were as follows, the heated capillary temperature was kept at 160°C, spray voltage was set to 5.21 kV, and the capillary voltage was at 50 V. The sheath gas flow rate was 39 mL/min.

3.2.16.5 DETERMINATION OF THE EFFECT OF HEAVY METAL STRESS FACTORS

To study the effect of heavy metal stress on total protein content of the *G. vittiformis* total protein was extracted from the dried biomass. The detailed extraction procedure is given below. The extraction method suggested by Leow et al. (2008); Zheng et al. (2010); Hearst et al.(2010) were followed.

Extraction Method: 10g of fruiting body was ground using liquid N_2 in a pre chilled motar and pestle to obtain fine powder. 200 mg of ground power was transferred into pre chilled eppendoff. 500 µl of Tris- bufferd phenol [TBP] was prepared for the extraction. Extraction media was mixed with TBP with biomass for 30min at 26ºC ± 2ºC. The mixture was then centrifuged at 15,000 rpm for 20 min at 4ºC. The top phenol phase was collected. 500µl of TBP was added to top phenol phase and centrifugation step was repeated. The top phenol phase was pooled from each centrifugation step with the previous ones. 5 volumes of ammonium acetate in 100% ethanol was added to top phenol phase, vortexed and incubated at -20ºC overnight. The resultant suspension was then centrifuged at 15,000 rpm for 20 min at 4ºC to pellet down the suspended protein. The pellet obtained was washed twice with 5 volumes of ammonium acetate in 100% ethanol by centrifugation at 15,000 rpm for

20 min at 4ºC. The pellet was further washed with 80% acetone and then with 70% ethanol followed by centrifugation at 15,000 rpm for 20 min at 4ºC. These washing steps help to reduce the moisture content. The dried pellet obtained after the previous step was then dried at room temperature and dried pellets were stored in dry place until analysis. The protein pellet was obtained, which was further dissolved in tris buffer and subjected for LC-MS analysis.

3.2.17 MULTI-METAL INTERACTION DURING BIOACCUMULATION

Galerina vittiformis belonging to *Basidomycota* was isolated from waste dump yards of Dakshin Kannada, India. Due to its higher bioaccumulation potential in single metal system (Cd (852 mg/kg), Pb (900 mg/kg), Cu (800 mg/kg), Zn (700 mg/kg), Cr (30 mg/kg)) this mushroom was selected for studies on its metal uptake potential in multi-metal system.

3.2.17.1 DESIGN OF EXPERIMENT

In order to study the multi-metal interactions in metal removal process by *G. vittiformis* from the soil, parameters like metal concentration and pH were considered (Elekes et al. 2010). The range of metal concentrations to be studied were selected based on the bioaccumulation potential as investigated through preliminary experiments on metal contaminated soil taking one metal at a time. pH was chosen as a factor for multi-metal interaction study on bioaccumulation, as bioaccumulation and multi-metal interactions are influenced by the pH of the environment (Morsy et al. 2012; Chen et al. 2000). Preliminary experiments have also revealed that pH of 5 to 8 results in the fungal growth in the presence of each of the metals studied in single metal system, and hence pH range was chosen as 5 to 8. The influences of factors on bioaccumulation were done individually to select the best range values. In order to reduce the number of experiments, Design of experiments (DOE) strategy was used for multi-metal interaction studies.

Experiments were designed based on central composite design (CCD) with six factors *i.e* concentration of five metals and pH in the soil at five levels each. Table 3.4 presents the factors and levels in coded and un coded form. Ninety experiments were generated using CCD with fourteen replicates of the central points. The experimental conditions for 90 sets of experiments obtained by CCD are presented in Table 3.5. Tray experiments were carried out as per the method described in section (3.2.11). The experiments at the central points were run to determine the curvature and to compensate the lack of fit values which indicates the significance of the model. Percentage removal of each of the metals under study from the soil at the end of 30 days was taken as responses. The system of six factors and five responses were analyzed by response surface methodology (RSM) using MINITAB-14 software. To determine the interaction of the metals on the removal efficiency, three dimensional response surface and contour plots were generated using MINITAB-14 software.

CCD is considered ideal to study the behavior of multi-metal bioaccumulation (Montgomery, 2001). Multiple regression analysis (MRA) was carried out on the system with six factors and five responses as experimental input –output data and multiple regression models were developed to relate each of the responses to six factors.

Table 3.4 Factors involved in CCD

Factors	Symbol	Units	Level				
			-2	**-1**	**0**	**+1**	**+2**
Concentration of Copper	Cu	mg/kg of soil	10	70	130	190	250
Concentration of Cadmium	Cd	mg/kg of soil	10	70	130	190	250
Concentration of Chromium	Cr	mg/kg of soil	10	70	130	190	250
Concentration of Lead	Pb	mg/kg of soil	10	70	130	190	250
Concentration of Zinc	Zn	mg/kg of soil	10	70	130	190	250
pH	pH	pH of soil	5	5.75	6 .5	7.25	8

Table 3.5 Experimental set showing coded and un coded values

Run Order	PtType	Blocks	Coded values						Uncoded values					
			Cu	Cd	Cr	Pb	Zn	pH	Cu	Cd	Cr	Pb	Zn	pH
1	-1	1	-2	0	0	0	0	0	70	190	70	190	190	6.50
2	-1	1	2	0	0	0	0	0	130	130	130	130	130	5.75
3	1	1	1	-1	1	1	-1	1	190	70	190	70	70	7.25
4	-1	1	0	0	0	0	0	-2	190	70	190	190	70	6.5
5	0	1	0	0	0	0	0	0	130	130	130	130	130	7.25
6	1	1	-1	1	-1	-1	-1	1	190	190	190	190	70	6.5
7	1	1	-1	1	1	-1	-1	-1	130	130	130	130	130	5.75
8	1	1	1	1	1	1	-1	-1	190	190	70	190	70	7.25
9	-1	1	0	0	0	0	0	2	190	190	70	70	190	5.75
10	0	1	0	0	0	0	0	0	70	70	190	70	70	5.75
11	1	1	-1	-1	1	-1	1	-1	70	70	70	190	190	6.5
12	-1	1	0	0	0	0	2	0	130	130	130	130	10	5.75
13	1	1	1	1	-1	-1	1	1	190	70	70	70	190	7.25
14	1	1	1	1	-1	1	-1	1	190	70	70	190	190	6.5
15	1	1	-1	1	1	1	1	-1	70	190	70	70	190	6.5
16	0	1	0	0	0	0	0	0	190	70	190	190	190	7.25
17	0	1	0	0	0	0	0	0	70	70	70	70	70	7.25
18	1	1	-1	-1	-1	1	1	-1	130	130	130	130	130	6.5
19	0	1	0	0	0	0	0	0	70	190	190	190	190	7.25
20	1	1	1	1	1	1	1	1	190	70	190	70	190	5.75
21	-1	1	0	0	2	0	0	0	190	70	190	70	70	5.75
22	1	1	-1	-1	1	1	1	1	190	190	190	70	190	7.25
23	1	1	1	-1	-1	-1	1	-1	250	130	130	130	130	7.25
24	-1	1	0	2	0	0	0	0	70	70	190	190	70	5.75
25	1	1	-1	-1	1	-1	-1	1	70	70	70	70	70	5.75
26	0	1	0	0	0	0	0	0	70	70	70	190	190	7.25
27	1	1	1	-1	1	-1	1	1	70	190	190	190	70	5.75
28	0	1	0	0	0	0	0	0	70	190	70	70	190	5.75
29	1	1	1	1	1	-1	-1	1	190	70	70	70	70	6.5
30	1	1	-1	-1	-1	1	-1	1	190	190	70	70	70	7.25
31	1	1	1	1	-1	1	1	-1	70	190	190	70	190	6.5
32	1	1	1	-1	1	-1	-1	-1	190	190	190	70	190	8.621
33	1	1	-1	1	-1	1	-1	-1	190	70	70	70	70	7.25
34	1	1	-1	1	1	-1	1	1	70	190	190	70	70	7.25
35	1	1	-1	1	-1	1	1	1	130	130	250	130	130	7.25
36	-1	1	0	0	-2	0	0	0	10	130	130	130	130	5.75
37	1	1	-1	-1	-1	-1	1	1	130	130	130	130	130	5.75
38	1	1	-1	-1	-1	-1	-1	-1	70	190	70	70	70	5.75
39	-1	1	0	-2	0	0	0	0	70	70	190	70	190	7.25
40	-1	1	0	0	0	0	-2	0	190	70	70	190	70	5.75
41	1	1	1	1	1	-1	1	-1	70	190	70	190	70	5.75
42	1	1	1	-1	-1	-1	-1	1	70	190	70	190	190	6.50
43	1	1	1	1	-1	-1	-1	-1	70	190	190	70	70	7.25
44	1	1	1	-1	-1	1	1	1	190	190	70	190	190	6.5
45	0	1	0	0	0	0	0	0	190	70	190	190	70	7.25
46	0	1	0	0	0	0	0	0	130	130	130	130	130	6.5
47	-1	1	0	0	0	-2	0	0	70	70	190	190	190	7.25
48	1	1	1	-1	-1	1	-1	-1	130	10	130	130	130	5.75
49	-1	1	0	0	0	2	0	0	130	130	10	130	130	7.25
50	1	1	-1	1	-1	-1	1	-1	70	190	190	70	190	5.75

51	1	1	-1	1	1	1	-1	1	190	190	190	190	70	5.75
52	1	1	-1	-1	1	1	-1	-1	130	130	130	250	130	6.50
53	1	1	1	-1	1	1	1	-1	70	70	190	70	190	6.50
54	1	1	1	1	1	1	1	-1	190	190	190	70	70	6.50
55	-1	1	0	0	2	0	0	0	70	70	70	190	70	5.75
56	1	1	-1	-1	1	0	0	0	70	190	70	190	70	7.25
57	1	1	1	-1	-1	1	1	-1	190	70	70	190	70	7.25
58	-1	1	0	2	0	0	0	0	130	130	130	130	130	6.50
59	1	1	-1	-1	1	1	1	1	190	190	70	70	190	5.75
60	0	1	0	0	0	0	0	0	190	70	70	190	190	6.5
61	1	1	1	-1	1	1	1	1	190	190	190	190	190	5.75
62	0	1	0	0	0	-1	1	-1	190	70	190	190	190	6.50
63	1	1	1	1	1	0	0	0	130	250	130	130	130	6.50
64	1	1	-1	-1	-1	-1	-1	1	70	70	190	190	190	6.50
65	1	1	1	1	-1	0	0	0	70	190	70	70	70	7.25
66	1	1	1	-1	1	-1	1	1	70	190	190	190	190	5.75
67	1	1	-1	1	-1	0	0	0	70	70	190	190	70	7.25
68	1	1	-1	1	1	-1	-1	1	190	70	190	70	190	7.25
69	1	1	-1	1	-1	1	-1	1	130	130	130	130	130	5.75
70	-1	1	0	0	-2	1	1	-1	130	130	130	130	130	7.25
71	1	1	-1	-1	-1	-1	-1	-1	190	190	70	190	70	5.75
72	1	1	-1	-1	-1	1	-1	-1	190	190	190	190	190	7.25
73	-1	1	0	-2	0	-1	1	1	70	70	190	70	70	7.25
74	-1	1	0	0	0	1	1	1	70	190	190	190	70	5.75
75	1	1	1	1	1	0	0	0	130	130	130	130	130	7.25
76	1	1	1	-1	-1	-1	1	1	130	130	130	130	130	7.25
77	1	1	1	1	-1	-1	-1	-1	130	130	130	130	130	5.75
78	1	1	1	-1	-1	0	0	0	130	130	130	10	130	5.75
79	0	1	0	0	0	0	-2	0	130	130	130	130	250	5.75
80	0	1	0	0	0	-1	1	-1	130	130	130	130	130	5.75
81	-1	1	0	0	0	-1	-1	1	130	130	130	130	130	5.75
82	1	1	1	-1	-1	-1	-1	-1	70	70	70	70	190	6.5
83	-1	1	0	0	0	1	1	1	190	70	70	70	190	7.25
84	1	1	-1	1	-1	0	0	0	190	190	190	70	70	4.37
85	1	1	-1	1	1	0	0	0	190	190	70	190	190	7.25
86	1	1	-1	-1	1	-2	0	0	130	130	130	130	130	5.75
87	1	1	1	-1	1	1	-1	-1	70	70	70	190	70	6.50
88	1	-1	1	1	0	-2	0	0	70	70	70	70	190	6.50
89	1	-1	-1	1	0	0	0	-1	130	130	130	130	130	6.50
90	1	1	-1	1	1	1	1	1	190	190	70	70	70	7.25

3.2.17.2 EXPERIMENTAL METHOD FOR MULTI-METAL INTERACTCION STUDIES

50 g of mushroom spawn (seed of the mushroom) were grown in small trays containing 100 g of laterite soil (<2 mm grain size) artificially contaminated with desired concentrations of heavy metals under study. 1000 mg/L of the stock solutions of the five metals Pb(II), Cd(II), Cr(VI), Zn(II) and Cu(II) were prepared by

dissolving salts like $CuSO_4$, $CdSO_4$, $K_2Cr_2O_7$, $PbNO_3$ and $ZnNO_3$ respectively in distilled water and were used to contaminate the soil for the desired concentrations, *in-vitro* (Isildak et al. 2007; Gast et al. 1988 and Elekes et al. 2010). 15 ml of basal salt media consisting of $CaCl_2$, $MgSO_4$, KH_2PO_4, NH_4NO_3 and glucose (1%) were added to 10 g of the metal contaminated soil (metals in concentrations of 10 to 250 mg/kg soil slurry). The pH of the soil was varied from 5 to 8 by adding HCl / NaOH solution. The tray with the above mentioned contents were incubated at 24 ± 2°C for 30 days for an efficient bioaccumulation study (Chen et al. 2009). The fruiting bodies and the soil samples collected after 30 days of incubation were subjected to digestion with acid mixtures according to procedures described in section 3.2.7.2 and section 3.2.7.3 and the solutions were analyzed for metals by atomic absorbtion spectrometer (Model AAS: GBC-6000) (Oei 1996 and Dermirdas, 2002).

CHAPTER – 4

RESULTS AND DISCUSSION

This chapter presents the illustrations of the experimental results obtained by the methodologies presented in Chapter 3. The results of the preliminary studies like isolation of mushroom species, tolerance study along with its *in-vitro* establishment and molecular identification of potent mushrooms by ITS analysis are presented in brief. The effect of pH and incubation time on bioaccumulation, metal removal kinetics studies; effect of presence of multi metals and chelaters in metal bioaccumulation are discussed with their relevant findings and literature. The proposed mechanism of metal uptake by the selected mushroom species is also presented. The results obtained have been analyzed and discussed with the help of relevant available literature reports. The data are presented as mean values of triplicate experimental results.

4.1 ISOLATION OF HEAVY METAL TOLERANT MUSHROOMS

Since 1988, researchers have reported that mushrooms are efficient in accumulating heavy metals from metal polluted soil samples (Gast et al. 1988; Volesky 1995; Sesli et al. 2008; Elekes et al. 2010). In the present study ten fungal species have been collected from two different municipal waste dump yards of Dakshina Kannada District, Karnataka, India. Table 4.1 presents the morphological characteristics of the collected fungal species, their genus and habitat.

The photographic image of the collected mushroom samples is presented in Fig. 4.1. All the mushroom isolates were labeled as M1, M2, M3, M4, M5, M6, M7, M8, M9 and M10 respectively, The morphological characteristics of which are presented in Table 4.1. Out of the 10 isolates, 9 fungal isolates (M1, M2, M3, M4, M5, M6, M7, M8 and M9) were found to grow successfully on Sabourauds Dextrose Agar medium (SDA) amended with 100 mg/L of the individual test metals initially. However, literature reveals that metal accumulation potential of mushrooms is a function of their metal tolerance at elevated concentrations (Susan et al. 2008; Chen et al. 2009; Dermirdas et al. 2000; Thomet et al. 1999; Elekes et al. 2010). Hence, all the successfully isolated mushrooms were screened for their heavy metal tolerance.

Table.4.1 Morphological characteristics and habitat of isolated mushrooms

Fungal Name	Morphological Characteristics	Mushroom Genus	Habitat
M1	White colored with slight brownish spot on the center of cap	*Agaricus Sp.*	In woodland, hedgerows and gardens
M2	Pure white colored fleshy stem	*Clitocybe Sp.*	In woodland, hedgerows and gardens
M3	Chocolate brown colored stem and cap	*Unidentified*	In woodland
M4	Flesh colored stem and cap	*Pholiota Sp.*	In woodland, hedgerows and gardens
M5	Large coral shape having golden yellow colored gills and white colored outer covering	*Pleurotus Sp.*	Soil rich in decaying logs
M6	Small brownish small slender stem	*Galerina Sp.*	In mixed woods
M7	Reduced stem with yellow spores	*Pleurotus Sp.*	Soil rich in decaying logs
M8	Dark brown small and slender stem	*Coprinus Sp.*	In woodland, hedgerows and gardens
M9	Blackish large jelly cup appearance.	*Pachyella Sp.*	In woodland, hedgerows and gardens
M10	Star like appendages with puff ball like sporangia bearing black spores.	*Geastrum Sp.*	In woodland, hedgerows and gardens

The photographic image of the collected mushroom samples is presented in Fig. 4.1. All the mushroom isolates were labeled as M1, M2, M3, M4, M5, M6, M7, M8, M9 and M10 respectively, The morphological characteristics of which are presented in Table 4.1. Out of the 10 isolates, 9 fungal isolates (M1, M2, M3, M4, M5, M6, M7, M8 and M9) were found to grow successfully on Sabourauds Dextrose Agar medium (SDA) amended with 100 mg/L of the individual test metals initially. However, literature reveals that metal accumulation potential of mushrooms is a function of their metal tolerance at elevated concentrations (Susan et al. 2008; Chen et al. 2009;

Dermirdas et al. 2000; Thomet et al. 1999; Elekes et al. 2010). Hence, all the successfully isolated mushrooms were screened for their heavy metal tolerance.

Fig 4.1 Overview of collected mushroom samples

4.2 SCREEING OF EFFICIENT METAL ACCUMULATING MUSHROOMS

Fungal species belonging to *Basidiomycetes* shows a haplodiplontic life cycle (both mycelial and fruiting body stage). Thus for an effective bioremediation process, the bioaccumulation by the organism at their different life stages; both mycelia and fruiting bodies need to be monitored. Screening of mushrooms for metal bioaccumulation is based on their metal tolerance and bioaccumulation potential (Yilmaz et al. 2003; Zhu et al. 2010; Abechi et al. 2010). Hence, it is important to test the metal tolerance and metal bioaccumulation potential of the metal tolerant mushrooms through bioaccumulation studies.

4.2.1 SCREENING BASED ON HEAVY METAL TOLERANCE OF MUSHROOM ISOLATES IN SDA MEDIUM

All the successful isolates have been tested for their maximum tolerance level to different heavy metals viz., (Cu(II), Cd(II), Cr(VI), Pb(II) and Zn (II)) at concentrations from 100 to 1000 mg/L in increments of 100 mg/L in SDA medium. The maximum concentration of these heavy metals in soil beyond which the organism fails to grow or exhibit reduced growth was identified as the maximum tolerant concentration for the given mushroom species. Table 4.2 shows the tolerance profile of the isolates at various metal concentrations from which it can be observed that, out of nine isolates, M3, M5, M6, M7 and M9 have been found to grow in the plates having metal concentrations above100 mg/L, while, M1 failed to grow in plates containing Cd(II) and Cr(VI) for concentrations above 100 mg/L. Similarly, M2 also failed to grow in the presence of Cr(VI) and Cu(II) even at 100 mg/L concentration.

According to Table 4.2, M2 is found to have highest tolerance limit for Pb at 1000 mg/L. However, M6 and M7 have also exhibited higher tolerance levels up to 800 mg/L for Pb (II). The tolerance profile of mushroom isolates is presented in Fig. 4.2, which reveals that the mushroom isolates M5, M6, M7 and M9 show tolerance for all the studied heavy metals. Among these mushrooms, M6 showed significant tolerance for all the studied heavy metals compared to others, viz., Cu(II): 300 mg/L, Cd(II): 500 mg/L, Cr(VI): 100 mg/L, Pb(II): 800 mg/L, Zn(II): 400 mg/L. Though M5 showed tolerance for all the studied metals, the maximum tolerance limits were very low when compared to M6, M7 and M9. M7 exhibited comparatively lesser tolerance for all the metals except Pb(II) and Zn(II). Thus, mushrooms that showed higher tolerance for all the five metals viz. M5, M6 and M9 were selected for further screening process based on the tolerance in soil environment and their bioaccumulation potential. The mushroom isolate M7 was not selected for further studies as it showed least tolerance for Cu(II): 100 mg/L, Cd(II): 100 mg/L, Cr(VI): less than 100 mg/L.

Moreover , the observation that, isolate M7 produced spores when grown on SDA medium, indicated that they belong to a group of fungi known as *Ascomycetes*, phylum Ascomycota (Fig.4.3). The mycelial growth of these isolates on SDA medium for selected mushroom isolates is given in Fig.4.3. From mycological studies reported by earlier researchers, it is observed that the fungi belonging to *Ascomycetes* are incapable of producing fruiting bodies; hence M7 was not selected for further studies (Deacon 1997; Mehrotra and Aneja 1990).

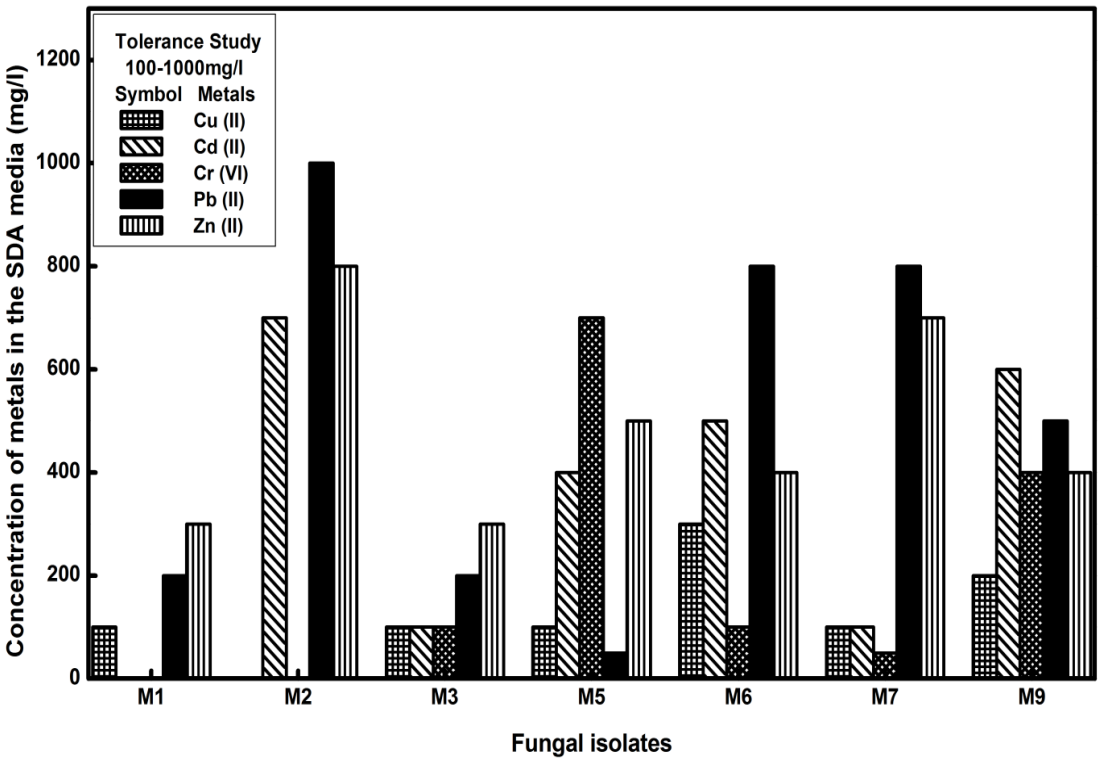


Fig. 4.2 Tolerance profile of fungal isolates at different heavy metal concentrations (100-1000 mg/L)

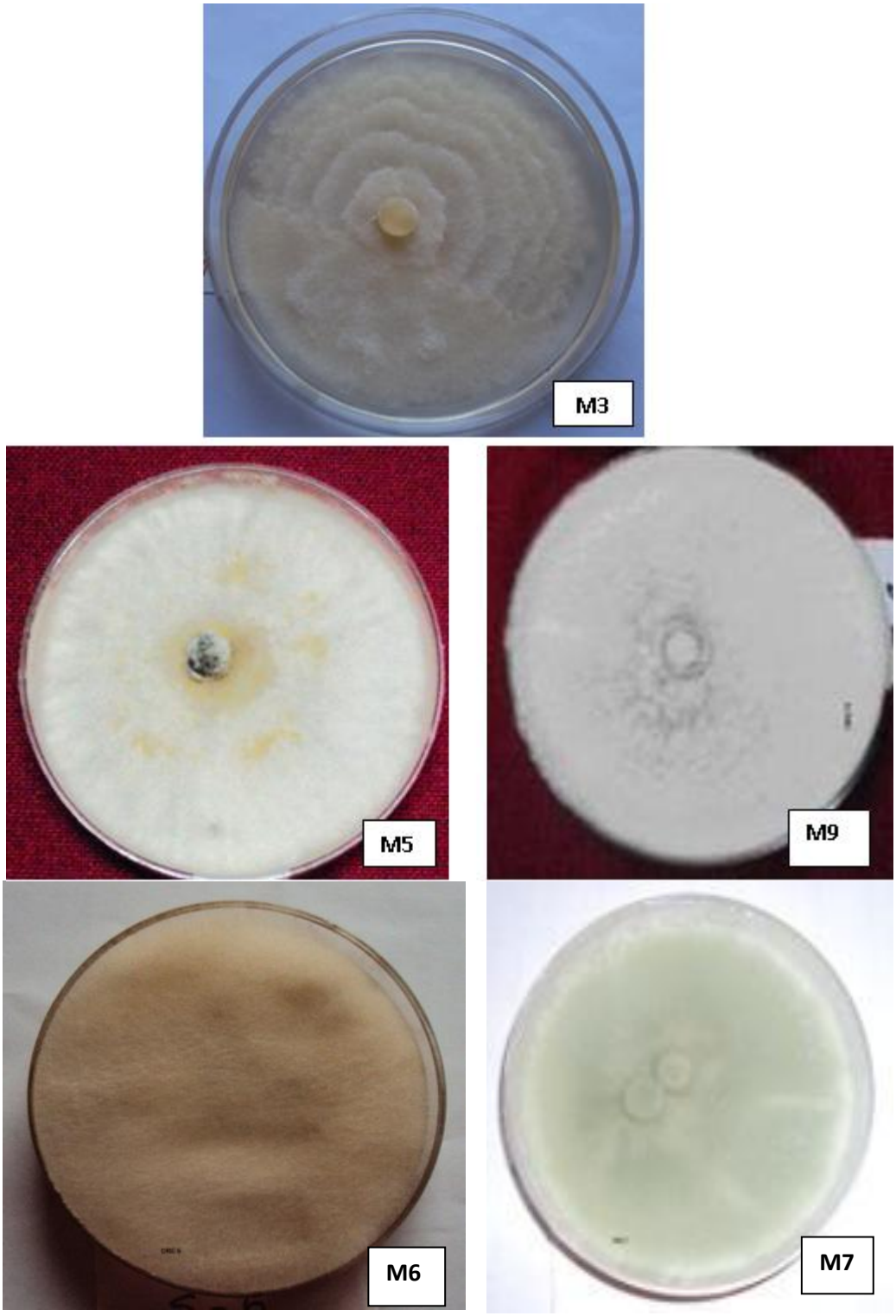


Fig.4.3 Mycelia growth of fungal isolate on SDA medium

Table 4.2 Tolerance profile of isolated mushrooms

Organism	Metals under study	Metal Concentrations in mg/L									
		100	200	300	400	500	600	700	800	900	1000
M1	Cu(II)	+	-	-	-	-	-	-	-	-	-
	Cd(II)	-	-	-	-	-	-	-	-	-	-
	Cr(VI)	-	-	-	-	-	-	-	-	-	-
	Pb(II)	+	+	-	-	-	-	-	-	-	-
	Zn (II)	+	+	+	-	-	-	-	-	-	-
M2	Cu(II)	+	-	-	-	-	-	-	-	-	-
	Cd(II)	+	+	+	+	+	+	+	-	-	-
	Cr(VI)	-	-	-	-	-	-	-	-	-	-
	Pb(II)	+	+	+	+	+	+	+	+	+	+
	Zn (II)	+	+	+	+	+	+	+	+	-	-
M3	Cu(II)	+	-	-	-	-	-	-	-	-	-
	Cd(II)	+	-	-	-	-	-	-	-	-	-
	Cr(VI)	+	-	-	-	-	-	-	-	-	-
	Pb(II)	+	+	-	-	-	-	-	-	-	-
	Zn (II)	+	+	+	-	-	-	-	-	-	-
M4	Cu(II)	-	-	-	-	-	-	-	-	-	-
	Cd(II)	-	-	-	-	-	-	-	-	-	-
	Cr(VI)	-	-	-	-	-	-	-	-	-	-
	Pb(II)	-	-	-	-	-	-	-	-	-	-
	Zn (II)	-	-	-	-	-	-	-	-	-	-
M5	Cu(II)	+	-	-	-	-	-	-	-	-	-
	Cd(II)	+	+	+	+	-	-	-	-	-	-
	Cr(VI)	+	+	+	+	+	+	+	-	-	-
	Pb(II)	+	-	-	-	-	-	-	-	-	-
	Zn (II)	+	+	+	+	+	-	-	-	-	-
M6	Cu(II)	+	+	+	-	-	-	-	-	-	-
	Cd(II)	+	+	+	+	+	-	-	-	-	-
	Cr(VI)	+	-	-	-	-	-	-	-	-	-
	Pb(II)	+	+	+	+	+	+	+	+	-	-
	Zn (II)	+	+	+	+	-	-	-	-	-	-
M7	Cu(II)	+	-	-	-	-	-	-	-	-	-
	Cd(II)	+	-	-	-	-	-	-	-	-	-
	Cr(VI)	+	+	+	+	+	-	-	-	-	-
	Pb(II)	+	+	+	+	+	+	+	+	-	-
	Zn (II)	+	+	+	+	+	+	+	-	-	-
M8	Cu(II)	-	-	-	-	-	-	-	-	-	-
	Cd(II)	-	-	-	-	-	-	-	-	-	-
	Cr(VI)	-	-	-	-	-	-	-	-	-	-
	Pb(II)	-	-	-	-	-	-	-	-	-	-
	Zn (II)	-	-	-	-	-	-	-	-	-	-
M9	Cu(II)	+	+	-	-	-	-	-	-	-	-
	Cd(II)	+	+	+	+	+	+	-	-	-	-
	Cr(VI)	+	+	+	+	-	-	-	-	-	-
	Pb(II)	+	+	+	+	+	-	-	-	-	-
	Zn (II)	+	+	+	+	-	-	-	-	-	-

❖ ***Symbol '+':- Growth of mycelium; Symbol '-':- No growth***

4.2.2 SECOND LEVEL SCREENING BASED ON METAL TOLERANCE POTENTIAL OF MUSHROOM ISOLATES IN SOIL

Selected metal tolerant mushroom isolates (M5, M6 and M9) were subjected to second level of screening process in which their tolerance efficiency in soil environment in terms of growth has been studied in detail. In comparison to the metal tolerance property, the hyper accumulator phenotype of the mushroom isolates enhances the bioremediation efficiency to a greater extent (Svoboda et al. 2000; Anderson et al. 1982; Vetter 1994; Jorhem et al. 1995; Cibulka et al.1996; Chen et al. 2009). The selected mushroom species was subjected for growth in soil containing metal concentrations ranging from 50 to 250 mg/kg of soil. It has been observed that the growth rates of the organisms had reduced with increase in metal concentrations. The observations on *in-vitro* metal tolerance study are presented in Table 4.3. From the results it is observed that the mycelia can effectively grow in soils contaminated with heavy metals at 50 and 100mg/kg concentrations.

It can also be observed from the Table 4.3, that the mushroom isolates M5, M6 and M9 at their mycelia stage have potential to grow in soil in the presence of all the studied heavy metals at concentration of 50 mg/kg. Further Table 4.3 indicates the metal tolerance level of various mushroom species under study *i.e* M5, M6 and M9. The mushroom species M5 showed a significant growth in the presence of all the heavy metals except Pb(II) at 100 mg/kg. For concentrations above 150 mg/kg of all the metals, mushrooms M5 and M9 have found to have negligible growth whereas, M6 showed maximum growth for all the heavy metals under study except Cr(VI) at 100 mg/kg. A significant growth by M6 has been observed for Pb(II) up to 200 mg/kg concentration indicating a higher tolerance for Pb(II). In case of M9, good growth was observed for all the studied heavy metals up to 50 mg/kg concentrations. Among all heavy metals understudy, M9 showed a maximum tolerance to Cd(II) up to 150 mg/kg. Hence it can be concluded that M5,M6 and M9 show a definite tolerance level of 50mg/kg in terms of all the heavy metals under study

M5 and M6 exhibited higher tolerance for four of the studied heavy metals at 100mg/kg of soil except Pb(II) by M5 and Cr(VI) by M6. While the mushroom isolate, M5 could tolerate Cd(II) and Zn(II) concentrations up to 150 mg/kg, the isolate M6 could tolerate Pb(II) up to 200 mg/kg and M9 upto 150 mg/kg of Cd(II).

Table 4.3 Growth patterns of mushroom isolates M5, M6 and M9 in the soil

Organism	Metal concentration in soil slurry (mg/kg)	Heavy metals under study	Observation
M5	50	Cu(II),Cd(II),Cr(VI),Pb(II) & Zn(II)	Significant growth
	100	Cu(II),Cd(II),Cr(VI) & Zn(II)	Good growth
		Pb(II)	No growth
	150	Cd (II) & Zn (II)	Less growth
		Cu(II),Cr(VI) & Pb(II)	No growth
	200	Cu(II),Cd(II),Cr(VI),Pb(II) & Zn(II)	No growth
	250	Cu(II), Cd(II), Cr(VI), Pb(II) & Zn(II)	No growth
M6	50	Cu(II), Cd(II), Pb(II) & Zn(II)	Significant growth
		Cr(VI)	Less growth
	100	Cu(II), Cd(II), Zn(II) & Pb(II)	Good growth
		Cr(VI)	No growth
	150	Pb(II)	Good growth
		Cu(II),Cd(II),Cr(VI) & Zn(II)	Less growth
	200	Pb(II)	Less growth
		Cu(II), Cd (II),Cr(VI) & Zn(II)	No growth
	250	Cu(II), Cd (II),Cr(VI),Pb(II) &Zn(II)	No growth
M9	50	Cu(II), Cd (II),Cr(VI),Pb(II) & Zn(II)	Good growth
	100	Cd (II),Cr(VI) & Pb(II)	Less Growth
		Cu (II) & Zn (II)	No growth
	150	Cd (II)	Less Growth
		Cu(II), Cr(VI), Pb (II) & Zn(II)	No growth
	200	Cu(II),Cd (II),Cr(VI),Pb (II) & Zn(II)	No growth
	250	Cu(II),Cd (II),Cr(VI),Pb (II) & Zn(II)	No growth

All the studied mushroom isolates exhibited a growth inhibition for all the above mentioned heavy metals at concentrations above 150 mg/kg. From this tolerance study result, it was also observed that the mushroom species; M5, M6 and M9 showed good growth for all the studied heavy metals at 50 mg/kg concentration and moderate growth with most of the metals under study at 100 mg/kg concentration.

Hence further bioaccumulation studies were conducted with both 50 and 100 mg/kg concentrations.

4.3 STUDIES ON THE EFFECT OF HEAVY METALS ON BIOMASS PRODUCTION OF THE SELECTED MUSHROOMS

In addition to the tolerance and bioaccumulation potential a significant biomass yield is important for the effective removal of metals form soil. Boaccumulation potential is presented as metal accumulation per unit mass of the biomass. Hence larger the biomass faster will be the rate of metal removal from soil. Thus, the growth profile of mushroom isolates M5, M6 and M9 were studied in conical flasks containing soil slurry in the metal free environment. The results as amount of biomass per kg soil are presented in Fig 4.4. From Fig. 4.4, it is evident that after 40 days of incubation the biomass profile of all the studied mushroom isolates remained the same indicating the attainment of stationary phase of mushroom growth due to nutrient limitation. Hence for further studies on growth pattern, 40 days of incubation period was considered suitable. The co-relation between the mycelial growth and metal concentration in the environment were further studied by growing these isolates on metal laden environment.

Fig 4.5 to Fig. 4.7 present the growth profiles of M5, M6 and M9 respectively in both control and in the presence of heavy metals with initial concentration of 50 mg/kg of soil. From Fig.4.5 it is observed that for M5 produces higher amounts of mycelial biomass in metal free soil compared to the metal contaminated soil for the given experimentation period. The amount of mycelial biomass produced in the metal containing environment for M5 followed the order; Cr(VI) > Zn(II) > Cd(II) > Cu(II) > Pb(II). Upon comparing the biomass production profile with the tolerance study results given in Table 4.3, it can be concluded that the biomass production profile of the mushroom isolate M5,were similar to their tolerance profile, in the order of Cr(VI) > Zn(II) > Cd(II) > Cu(II) = Pb(II). The result of this study indicates their metal ion preference for growth in soil environment. The above study also showed a reduced

growth for M5 in the presence of heavy metals, indicating the effect of metal toxicity on their growth and early attainment of stationary phase compared to that of control. This reduced growth pattern may be because of the metal toxicity effect (Liu et al. 2005; Yan and Viraraghavan 2003; McGrath et al. 2001; Salt et al. 1995).

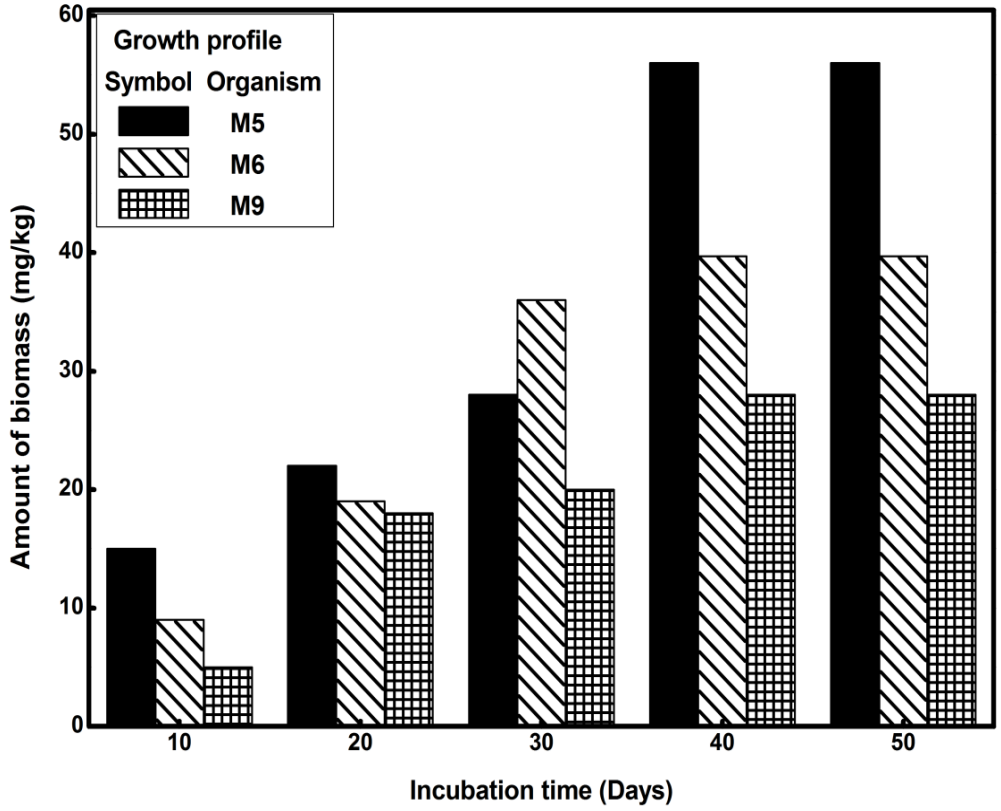


Fig.4.4 Biomass profile of mushroom isolates; In metal free environment

In the case of mushroom isolates M6 and M9, the mycelial biomass produced in the metal contaminated environment is higher than that of those grown in control environment which indicates a stimulatory effect of metals on the growth of mycelial biomass of M6 and M9 in metal contaminated soil environment. The order of metal ions preferred for the growth of biomass is found to be Pb(II) > Cd(II) > Zn(II) > Cu(II) > Cr(VI) for M6 and Cd(II) > Pb(II) > Cr(VI) > Zn(II) > Cu(II) for M9. From Table 4.3, the order of metal tolerance profile for M6 is found to be Pb(II) > Cd(II) > Zn(II) > Cu(II) > Cr(VI) and for M9 it is Cd(II) > Pb(II) > Cr(VI) = Zn(II) > Cu(II).

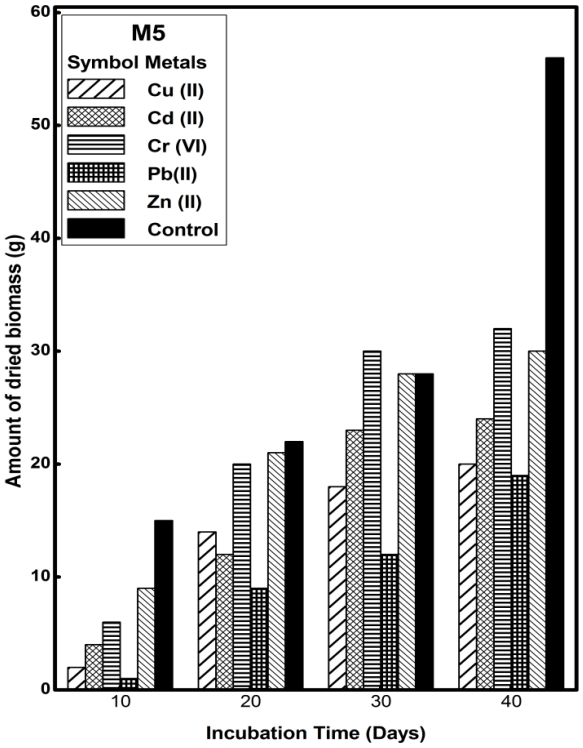


Fig. 4.5 Amount of biomass produced by M5 in metal laden environment

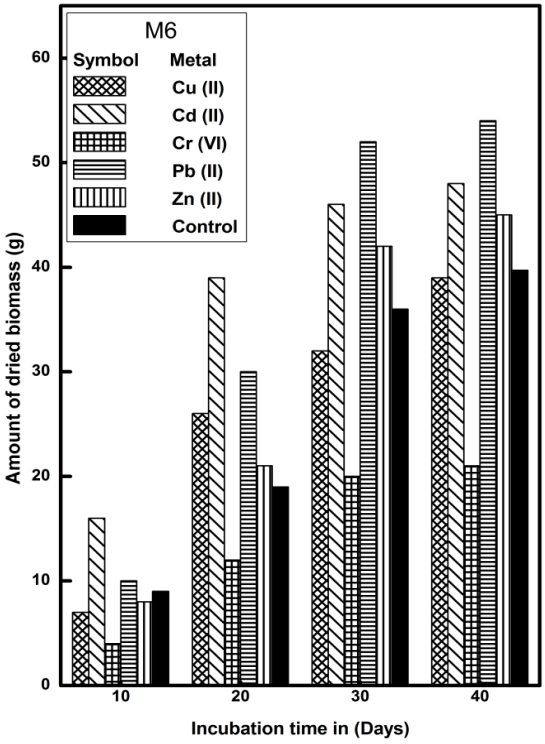


Fig. 4.6 Amount of biomass produced by M6 in metal laden environment

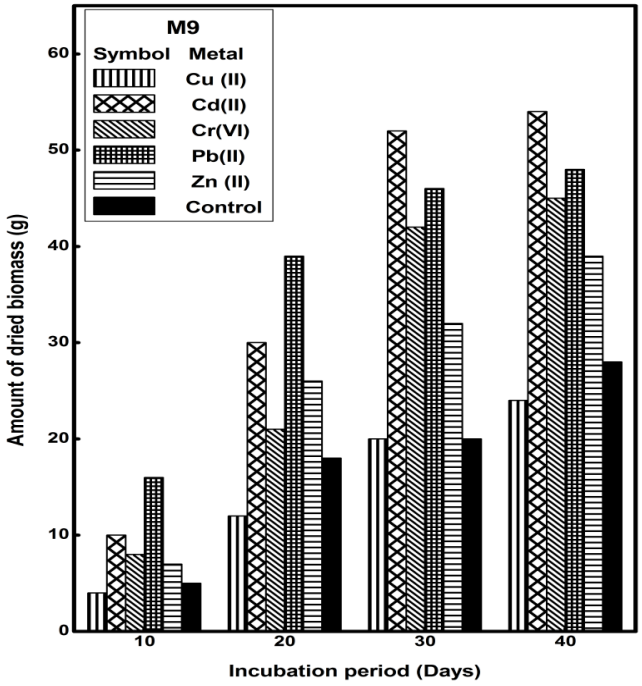


Fig. 4.7 Amount of biomass produced by M9 in metal laden environment

Similar mycelial growth pattern in the presence of heavy metals were also reported in fungal mycelia of *Aspergillus* Sp and *Rhizopus* Sp, *Basidiomycetes* Sp. *Alternaria* Sp., *Geotrichum* Sp., *Fusarium* Sp., *Rhizopus* Sp., *Monilia* Sp. and *Trichoderma* Sp. by various scientists *viz*., Baldrian (2003), Zafar et al. (2007). Certain concentrations of heavy metals (below the tolerance level) can be essential micronutrients as they are found critically involved in the functional activities of large numbers of proteins that are responsible for sustaining growth and development of organelles in the organism. However, at concentrations above their tolerance level, these metal ions can become detrimental to living organisms (Ahmad 2005; Sannasi et al. 2009; Hossain et al. 2012).

4.4 MYCELIAL BIOACCUMULATION STUDIES

4.4.1 FACTORS AFFECTING MYCELIAL BIOACCUMULATION

Literature studies revealed that, certain mushrooms like *Paxillus Sp.,* and *Boletus Sp.,* are naturally hyper bioaccumulators when compared to other mushroom species (Kalac and Svoboda, 2006; Zhu et al. 2011; Ge et al. 2011). The hyper accumulating potential of these mushrooms may be attributed to their superior metal influx systems and the capability of accumulating high levels of metals in their tissues in a harmless state, likely via chelating using metallothioneins and phytochelatins and other such mechanisms (Thomet et al. 1999; Paraskiewicz et al. 2011). Furthermore, there are several other environmental factors that affect the bioavailability of the metals in soil. These include soil pH, soil water content, presence of organic matter, level of soil fertility that supports overall mushroom growth as reported by Srivastava et al. (2006). Since, biomass growth is an important factor that influences the metal uptake capacity of the mushrooms, it is important to study the factors that influence the growth profile of the mushroom species and their metal bioaccumulation potential. Incubation time and the soil pH may significantly influence the growth and bioaccumulation potential of the mushroom species and hence further experiments were conducted to study the effect of these factors.

The present study directs attention towards the similiarity in the biomass production profile of mushrooms with their tolerance studies as given in Table. 4.3 and discussed earlier in Section 4.4.1, from which the trend of the growth profile and tolerance profile of the mushrooms is observed. Hence, it can be concluded that the metal ions preference for the growth of mushrooms are species specific and follow the trend of their tolerance limits (Thomet et al. 1999; Tuzen 2003; Turkekuel et al. 2004). The effects of incubation time and soil pH are other two important parameters that reveal the adaptation of the mushrooms and preference on the ionic states of the metals during the uptake. Hence, it is important to study the effect of these parameters on each of the metal-species interactions.

4.4.1.1 EFFECT OF INCUBATION TIME ON BIOMASS PRODUCTION AND BIOACCUMULATION

Bioaccumulation studies by Kalac and Svoboda, (2006) and Zhu et al. (2011) have revealed that, the metal accumulation efficiency and choice of metal uptake are species specific. Since, each species has different growth rates over incubation time and yield different quantity of biomass in presence of the above mentioned heavy metals, the influence of the metals over the mushroom biomass yield and their ability to bioaccumulate for different incubation time need to be studied. The requirements for adequate biomass production and maximum bioaccumulation for few metals have been studied by Muraleedharan et al., 1995 and Vimala and Das, 2009.

The amounts of biomass produced by M5, M6 and M9 in the presence of heavy metal concentrations at 50 mg/kg for 40 days were compared with that of its biomass produced from metal free environment. The bioaccumulation profiles of M5, M6 and M9 for a period of 40 days at an initial concentration of 50 mg/kg are tabulated in Table 4.4. From Table 4.4 it is observed that for all the mushroom isolates, hardly any metal accumulation occurred during the initial 10 days of incubation, indicating their adaptation to the metal environment, *i.e* lag phase of

growth cycle. Table 4.4 also indicates that, there is no significant increase in bioaccumulation after the 30th day of incubation by the mushrooms.

Fig 4.8, Fig.4.9 and Fig. 4.10 show the bioaccumulation profiles of M5, M6 and M9 for 40 days. Upon comparing the bioaccumulation profile of these three mushrooms with respect to their biomass profile, it can be concluded that these organisms showed very less change in their biomass yield after the 30th day of incubation (Fig 4.5, Fig 4.6 and Fig 4.7). Based on these observations, all future bioaccumulation studies were conducted only for initial 30 days of incubation time during the life cycle of the organism.

Similar studies on mycelial bioaccumulation were discussed by various researchers viz. Chen et al. (2009), Demirbas (2002), Elekes et al. (2010) regarding the biomass yield of the mushrooms under heavy metal stressed conditions. The above literature revealed that, there was hardly any progress in the biomass yield by the mushrooms after certain period of their incubation time. Thus the presence of these heavy metals imposes stress on the organism and reduces their biomass yield.

Table 4.4 Metal uptake profile at various Incubation time

Mushroom isolates												
	M5				**M6**				**M9**			
Metal under study	**Incubation time (Days)**											
	10	**20**	**30**	**40**	**10**	**20**	**30**	**40**	**10**	**20**	**30**	**40**
	Metal concentration in biomass (mg/kg) (Soil pH 6.5 & Initial metal concentration 50 mg/kg)											
Cu (II)	42	120	150	152	200	280	340	338	25	96	139	120
Cd (II)	15	98.0	122	128	298	390	492	489	40	123	175	176
Cr (VI)	BDL	30.0	58.0	49.0	19.0	32.0	39.0	40.0	BDL	40.0	52.0	50.0
Pb (II)	78.5	138	165	162	390	580	620	622	29.0	130	196	193
Zn (II)	BDL	93.0	110	112	200	240	245	243	60.0	98	134	133

- **BDL: Below Detection Limit**

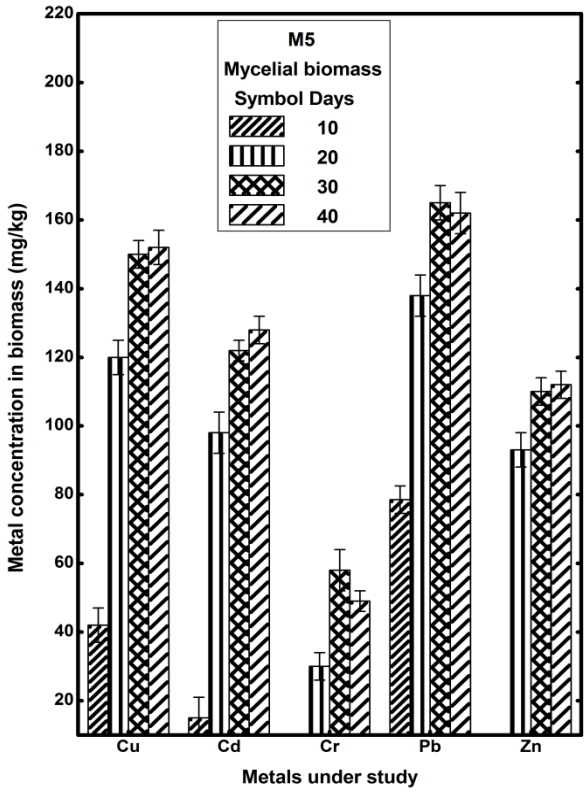


Fig. 4.8 Bioaccumulation profile of mushroom M5

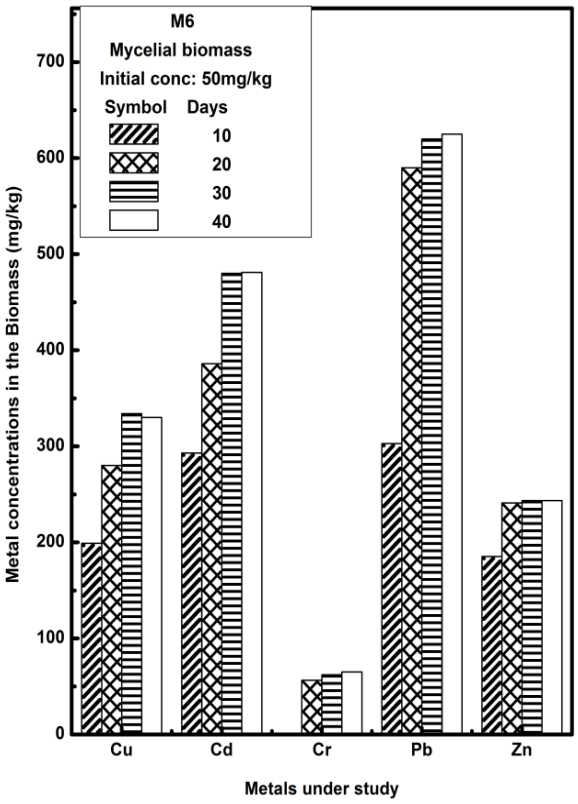


Fig. 4.9 Bioaccumulation profile of mushroom M6

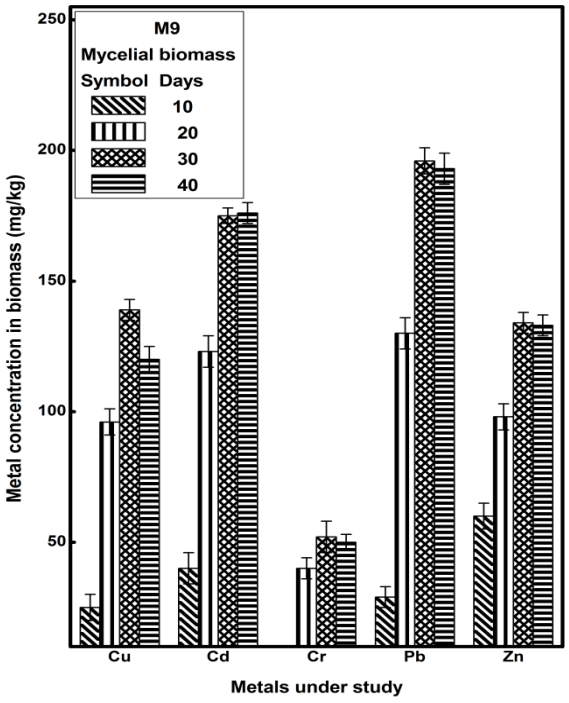


Fig. 4.10 Bioaccumulation profile of mushroom M9

4.4.1.2 EFFECT OF SOIL pH ON METAL BIOACCUMULATION

Soil pH can be regarded as one of the critical parameter in controlling the growth and heavy metal uptake by fungi (Chen et al. 2000; Wuyep et al. 2007). The effect of soil pH on bioaccumulation efficiency have been studied for M5, M6 and isolates for 30 days at initial soil pH values ranging from pH 5 to pH 8. The effect of soil pH on bioaccumulation efficiency of mushroom isolates, M5, M6 and M9 are shown in Fig 4.11, Fig 4.12 and Fig 4.13 respectively. From the bioaccumulation studies it is evident that the optimum soil pH for M6 and M9 are pH 6.5 and pH 5.5 respectively. It is also observed that, the metal uptake efficiency of these isolates above their optimum pH has been found to decrease in their bioaccumulation efficiency. The results obtained are found to be in accordance with reports on heavy metal accumulation by Dermirdas (2002) and Gast et al. (1988) for the mushrooms like *Pleurotus Sp., Agaricus Sp., Aspergillus Sp., Rhizopus Sp. etc*. From the literature study it is also incurred that, the metal uptake efficiency of fungi reduces with increase in pH, as the metals form hydroxide colloids under alkaline conditions. These hydroxide colloids have large molecular size and hence results in reduced cell permeation. Moreover, the availability of the metals to the mushrooms might have reduced due to precipitation under alkaline pH (Niu et al. 2007). Thus changes due to osmotic pressure and hydrolyzing effects might retard the metal uptake process from the soil under alkaline pH conditions (Zhu et al. 2011; Dermirbas, 2001). From the results of this study, the optimum pH has been found to be species specific but not metal specific. It may be due to the fact that pH not only affects the metal mobility in soil and availability to the fungi for bioaccumulation, but also the growth of the fungi. Surface charges may be altered by the pH of the environment and thus initial biosorption step is governed by pH (Mancini and Bruno 2011; Michael et al. 2007).

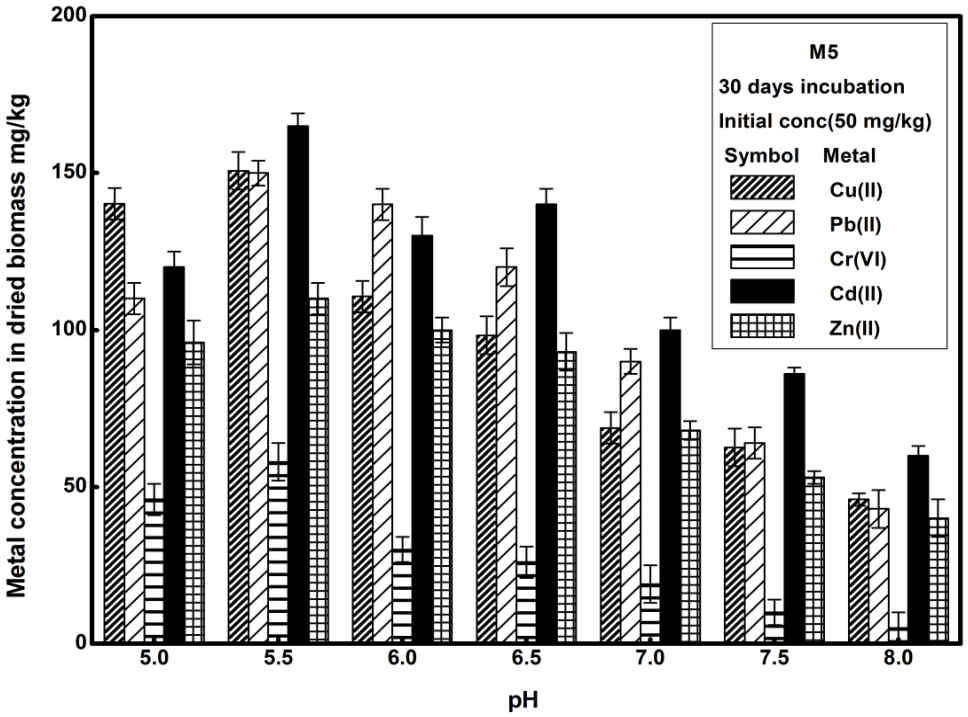


Fig. 4.11 Bioaccumulation profile of M5 at various pH's

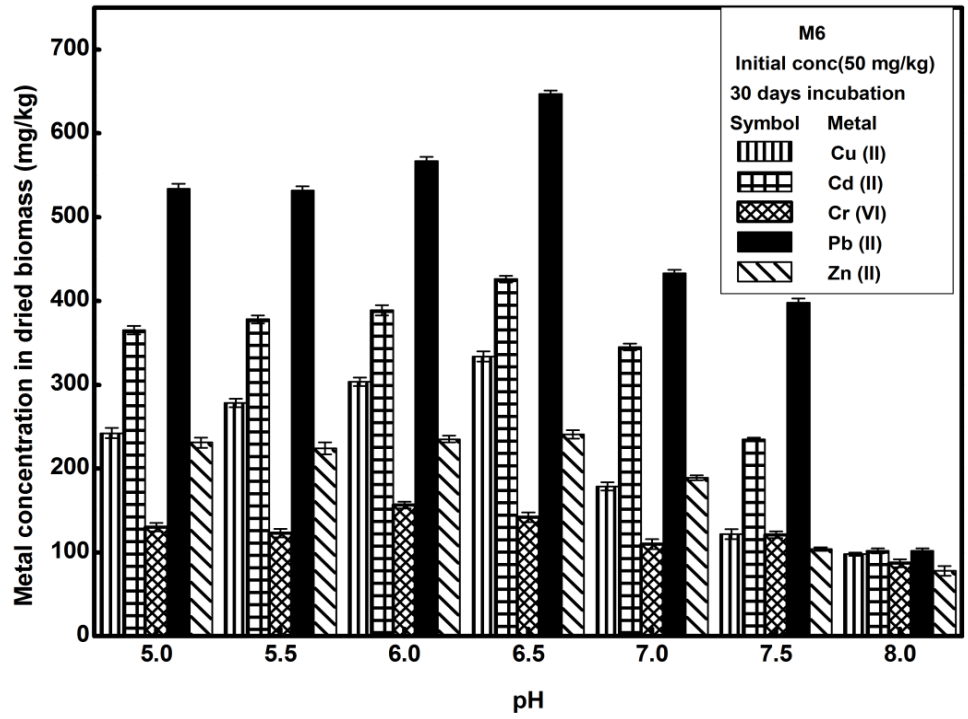


Fig. 4.12 Bioaccumulation profile of M6 at various pH's

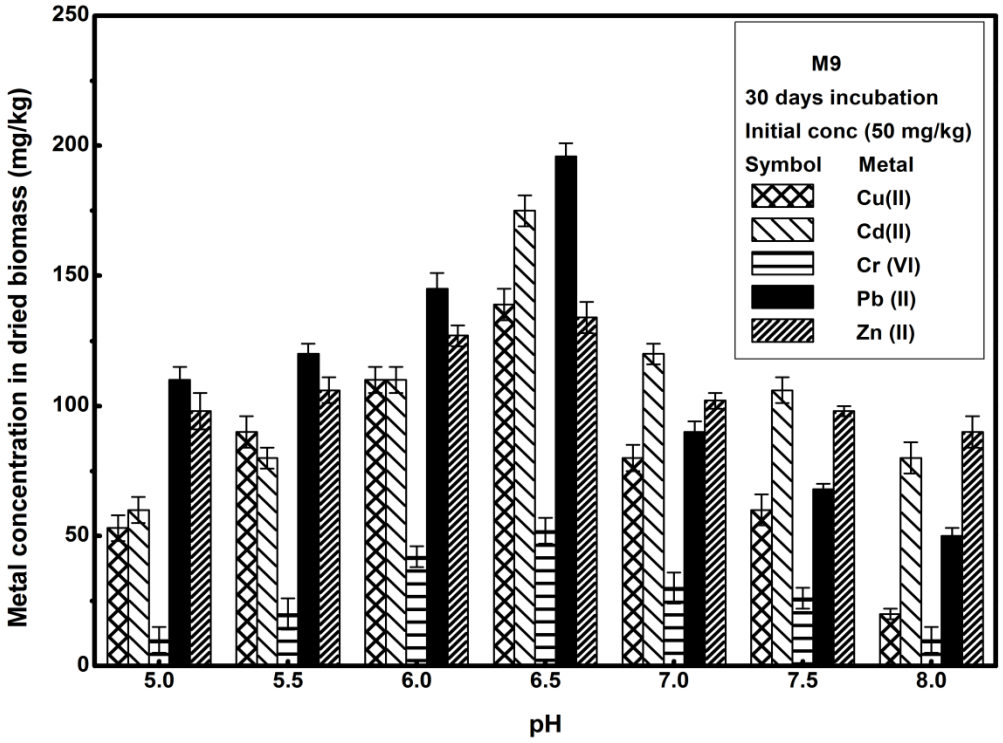


Fig 4.13 Bioaccumulation profile of M9 at various pH's

From the above results it was observed that, M5 isolate is more efficient in bioaccumulating all the studied heavy metals at a soil pH of 5.5, wheras M6 and M9 showed maximum accumulation at pH 6.5. Hence, for bioaccumulation studies an optimum soil pH of 5.5 for M5 and 6.5 for M6 and M9 are maintained.

4.4.2 SCREENING OF MUSHROOMS SPECIES BASED ON BIOACCUMULATION POTENTIAL

In order to further understand the bioaccumulation potential of the selected mushroom species, M5, M6 and M9, a detail study on bioaccumulation has been conducted for bioaccumulation of metals from soil slurry holding concentrations of 50 and 100 mg/kg of the selected metals under study. The bioaccumulation profile of three isolates (M5, M6 and M9) at an initial metal concentration of 50 mg/kg and 100 mg/kg of soil are shown in Fig. 4.14 and Fig 4.15 respectively for Cu(II), Cd(II), Cr(VI), Pb(II) and Zn(II). The studies were conducted at corresponding optimum soil

pH for all the isolates (Section 4.4.2). From the results it was found that the order of bioaccumulation potential of M5, M6 and M9 during its mycelial stage under the given conditions is Pb(II) > Cd(II) > Cu(II) > Zn(II) > Cr(VI). The mushroom M6 was found more efficient in bioaccumulating all the metals under study as compared to M5 and M9. The bioaccumulation potential for the mushroom species followed the order M6 > M9 > M5. However, M5 showed a slightly better bioaccumulation of Cr (VI) than M9 at 50 mg/kg concentration in the soil slurry.

The bioaccumulation profile was found to follow the order: Pb(II) > Cu(II) > Cd(II) > Zn(II) > Cr(VI) with M5; Pb(II) > Cd(II) > Cu(II) > Zn(II) > Cr(VI) with M6 and Pb(II) > Cd(II) > Zn(II) > Cu(II) > Cr(VI) with M9. However, it was observed that the bioaccumulation potential order for different metals does not follow similar order as the biomass growth profile or tolerance profile. It indicates that the bioaccumulation potential may not be entirely growth related. Bioaccumulation may also been influenced by certain other parameters like metal toxicity and effects related to metal toxicity. From Table 4.4 it is also evident that the metal uptake efficiency of mushroom isolate M6 is significant compared to the other mushroom isolates. M6 was found to be more efficient in accumulating heavy metals when compared to other non-edible mushroom species reported by Isildak et al. (2003) and Huang et al. (2012), hence it is evident that a novel non edible mushroom species has been identified for accumulating higher concentrations of heavy metals from the soil in the current study. The heavy metals, Cu(II), Cd(II), Cr(VI), Pb(II) and Zn(II) accumulating efficiency of the mycelial stage of M6 within a short period of 30 days was found to be very much significant when compared to other fungal species reported in the literature (Table 4.5) (Isildak et al. 2003; Huang et al. 2012). The biomass profile for M6 in the metal laden environment also follows a similar pattern *i.e* Pb(II) > Cd(II) > Cu(II) > Zn(II) > Cr(VI) (Fig 4.8). Thus the organism, M6 being a non edible macro fungi has been found to be an efficient bioaccumulating agent with shorter incubation time, among the other reported species in literature or the other mushroom species reported in the current study .

Table: 4.5 Heavy metal content in sporocarp of various tolerant mushrooms

Sl. No	Mushroom Species	Metal content in sporocarp, mg kg^{-1} of dry wt.	References
1	*Agaricus bisporous*[1] *Boletus edulis*[1] *Lepiota rhacodes*[2] *Paxillus rubicondulus*[1]	Pb (4), Cd (3.48), Cu (5.) Cu (66.4), Cd (6.58), Pb (3.03) Pb (66), Cd (3.7) Pb (0.69), Cd (0.78), Cu (51.0) Zn (16.8)	Srivastava et al. 2006
2	*Agaricus bisporous*[1]	Cu (107),Pb (1),Zn (57.)	Turkekuel et al. 2003
3	*Havlvella leucomelaena*[2] *Pleurotus sp.*[1]	Pb (4.8), Cd (.0) Pb (3.4), Cd (1.18), Cu (13.6), Zn (9.8)	Mitra et al. 1994
4	*Tricholoma terreum*[1] *Havlvella leucomelaena*[2]	Cu (5), Zn (179), Cd (0.56), Pb (4.4) Pb (3.1), Cd (1.1)	Dermirbas 2001
5	*Paxillus involutus*[2] *Rhizopogonaceae luteolus*[1] *Omphalotous olearius*[2] *Hygrophorous hedyricii*[2] *Ciocybe dealbata*[2] *Lepiota alba*[2]	Cu (57.0), Pb (1.6.0), Fe (991), Cd (0.84), Pb (3) Cu (13), Zn (30), Mn (13), Fe (620), Cd (0.26), Pb (2.8). Cu (21), Zn (27), Mn (36), Fe (95), Cd (1.3), Pb (5.2). Cu (37), Zn (97),Mn (11), Fe (395), Cd (1.2), Pb (2.7) Cu (41), Zn (115), Mn (30), Fe (386), Cd (0.86), Pb (3.2) Cu (29), Zn (86), Mn (22), Fe (779), Cd (0.8), Pb (5.8)	Yilmaz et al. 2003
6	*Tricholoma terreum*[2] *Agaricus bisporous*[1]	Pb (4), Cd (1.6), Cu (35.8), Zn (48.0) Pb(0.8), Cd(0.78)	Zhu et al. 2011
7	*Pseudevernia furfuraceae*[2] *Scorpiurum circintum*[2]	Al (12.51), As(0.23), Cd (0.19), Cu (2.5), Cr(0.11),Pb (5.1), Zn(17.9), Mn(12.9) Al(17.51), As (0.32), Cd(0.35), Cu (3.2), Cr (1.1), Pb(6.3), Zn (46.1), Mn (46.7)	Basile et al. 2007
8	*Aspergillus foeitidus*[2]	Al (32.5), Co (5.95), Cr (6.23), Mg (44.9), Zn (2.4), Ni (189.5)	Ge et al. 2011
9	*Poria Sp.*[2] *Nectria cinnabarina*[1] *Gonoderma lucidium*[1] *Paragyrodous sphaerosporous*[1] *Polyporous frondosis*[1]	Zn (90.3), Cu (30.8), Pb (1.0), Mn(31.3), Cd (0.1) Zn (30.1), Cu (29.3), Pb(1.9), Cd(0.2), Mn (19.3) Zn(60.1), Cu (43.8), Pb (0.7), Mn (30.4), Cd (0.31) Zn (115), Cu (34.4), Pb (0.4), Mn (37.3), Cd (0.2) Zn (120.1), Cu (34.4), Pb (0.4), Mn (37.3), Cd (0.2)	Ita et al. 2006

10	*Phellinus badius*[2] *Phellinus sanguineus*[2]	Cd (110), Cu (60), Hg (61), Ni (56) Cd (80), Cu (42), Hg (35), Ni (66)	Baldrian 2003
11	*Tricoloma terreum*[2] *Boletus badius*[1] *Russula delica*[1]	Pb (3.64), Cu (34.86), Cd (0.67), Zn (54.13), Cr (2.54) Cu (44.54), Pb (4.48), Cd (0.91), Zn (34.17), Fe (264.62), Cr (2.86) Cu(19.55), Pb (2.02), Cd (1.22), Zn (38.5), Cr (6.95)	Isildak et al. 2007
13	*Pleurotous platypus*[1] *Agaricus bisporous*[1]	Cd (34.9), Pb (27.10) Cd (33.7), Pb (29.67)	Vimala et al. 2009
14	*Lactarius delicious*[1] *Rhizopogon roseolous*[1] *Russula delica*[1]	Cd (0.26), Cr (0.12), Cu (6.15), Pb (0.73), Zn (76.7) Cd (0.18), Cr (0.10), Cu (21.2), Pb (2.03), Zn (36.7) Cd (0.42), Cr (0.27), Cu (52.2), Pb (0.77), Zn (58.2)	Cayır et al. 2010
15	*Sarcosphaeera crassa*[1] *Cantharellus cibarius*[1] *Suillus luteus*[1] *Morchella rigida*[1] *Agarocybe aegerita*[1]	Ag (0.044), As (8.03), Cd (0.016), Cr (0.98), Pb(0.02) Ag (0.022), As (0.03), Cd (0.036), Cr (0.69), Pb (0.04) Ag (0.015), As (0.15), Cd (0.034), Cr (0.15), Pb (0.06) Ag (0.087), As (0.24), Cd (0.007), Cr (0.44), Pb (0.02) Ag (0.074), As (0.44), Cd (0.010), Cr (0.25), Pb (0.018)	Konuk et al. 2007
16	*Agaricus arvensis*[2] *Agaricus silvicola*[1] *Macrolepiota procera*[1] *Lycoperdon perlatum*[1]	Cd (117) Cd (67.9) Pb (53.8) Pb(50.0)	Petkovsek and Pokorny 2013
17	*Pleurotus sajor-caju*[1]	Zn (30.0)	Jibran and Milsee Mol 2011
18	*Pleurotus ostreatus*[1]	Cd (103)	Tay et al. 2011
19	*Pleurotus tuber-regium*[1]	Cd (0.16), Cr (5.6), Cu (21.2), Pb (2.03), Zn (46.7)	Oyetayo et al. 2012
20	*Agaricus bisporous*[1]	Pb (76.07),Cu (69.6),Cr (40.0), Ar (30.0)	Chauhan and Suhalka 2014
21	***Galerina Sp.*[2]**	**Cr (VI) : 28 mg/kg, Pb (II) : 889 mg/kg , Cu(800),Zn (II) : 698 mg/kg**	This study

❖ **[1]: Edible; [2]: Non edible Mushrooms**

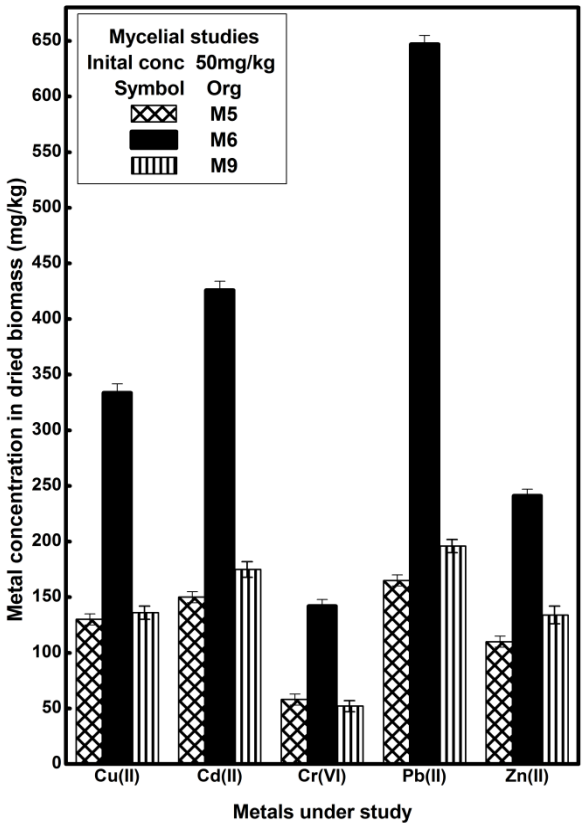


Fig.4.14 Bioaccumulation profile in mycelial stage at respective optimum soil pH

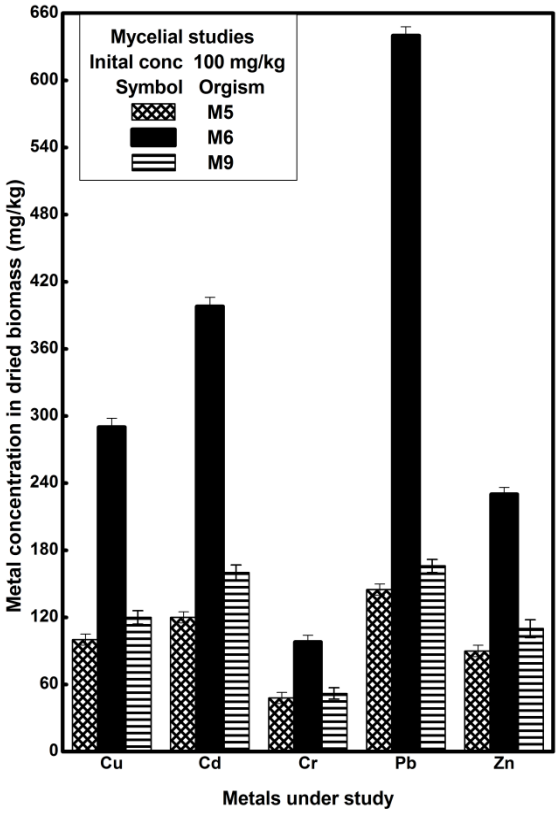


Fig. 4.15 Bioaccumulation profile in mycelial stage at respective optimum soil pH

The metal accumulation potential is found to be higher at an initial metal concentration of 50 mg/kg as compared to 100 mg/kg. The variation in the metal accumulation efficiency among the isolates may be because of the genetic variations among mushroom species. The mechanisms of metal uptake, accumulation, exclusion, translocation, osmoregulation and compartmentation vary with each mushroom species and their specific role in mycoremediation. Thus variations exist for hyperaccumulation of different metals among various mushroom species and within populations (Pollard et al. 2002; Lone et al. 2008).

4.5 STUDIES ON BIOACCUMULATION IN FRUITING BODIES OF MUSHROOM ISOLATES

Metal bioaccumulations in fruiting body stage of successful mushroom are studied. The isolates which were selected based on their growth, tolerance profile and bioaccumulation potential in mycelial stages was tested for their ability to form fruiting bodies *in-vitro*. The mushroom isolates which formed fruiting bodies were selected for further bioaccumulation studies.

4.5.1 *IN-VITRO* ESTABLISHMENT OF MUSHROOMS BY PRODUCING FRUITING BODIES

Thorough literature studies reveals that mushrooms can build up large concentrations of some heavy metals when compared to green plants, (Stijve and Roschnic, 1974; Kuusi et al., 1981). An important factor is the absorption of these elements in the body after ingestion. Mushrooms having haplodiplontic life cycle, the mycelia accumulate heavy metals from soil and are accumulated in the fruiting body which are formed during its reproductive phase of growth cycle (Isildak et al. 2007; Ita et al. 2003; Yilmaz et al. 2003). Hence fruiting body plays an important role in determining the bioaccumulation potential of mushrooms.

The significance of spawning and casing steps in *in-vitro* establishment of mushrooms were explained by Miles et al. (2008) and Stamets (1984) in their studies on mushroom cultivation (Section 3.5). The mushroom isolates M5, M6 and M9 showed the ability to produce spawn after 30 days of incubation. The spawning is characterized by the formation of white net work of mycelia around each rice grain during incubation period as shown in Fig. 4.16(a) and 4.16(b). Thus all the three isolates exhibiting the above morphological characteristic quality were selected for the casing studies (second step of *in-vitro* establishment). The study results indicate that only mushroom isolate M6 shows the potential to produce fruiting bodies *in-vitro*

while other isolates show only mycelial growth on soil mixture. Thus, mushroom M6 was selected for further bioaccumulation studies.

The initial metal concentrations for bioaccumulation studies were taken as 50 and 100 mg/kg in accordance with the results of initial tolerance study discussed in Section 4.2.2. From Table 4.6 it was observed that, while the yield of fruiting bodies is better for all the metals' concentrations at 50 mg/kg. M6 for 100 mg/kg of the metal concentrations, failed to produce large number of fruiting bodies in soil containing metals, Cr(VI), Cu(II) and Zn(II). Mushroom, M6 grown on soil mixture contaminated with 50 mg/kg of heavy metals produced many bunches of primodias (Fig 4.17). The fruiting bodies produced by M6 at an initial concentration of 100 mg/kg and 50 mg/kg of all the metals are presented in Fig.4.18 (a) and (b) respectively. It was observed that M6 grown at 50 mg/kg initial concentration produced large fleshy fruiting bodies compared to 100 mg/kg concentrations. The number of fruiting body production was found to be reduced with increase in soil heavy metal concentrations. Similar observations were also reported by Elekas et al. (2010) in their studies on bioaccumulation in fruiting bodies of *Agaricus* Sp., *Lycoperdon* Sp., *Cantharellus* Sp.

Upon casing, M6 started to produce fruiting bodies by 25 days and the production continued for next 5 days. The fruiting bodies produced were harvested at the end of the 30^{th} day as they may get decayed easily later. The harvested fruiting bodies were dried at room temperature and then analyzed for heavy metal content. The bioaccumulating potential of fruiting bodies, as concentration of accumulated metal in the dry biomass at both 100 mg/kg and 50 mg/kg initial concentrations are shown in Fig. 4.18(a) and Fig. 4.18(b). It is evident from Fig.4.18 (a) and (b) that M6 exhibited higher bioaccumulation potential and more fruiting bodies when grown at an initial concentration of 50 mg/kg, Hence 50 mg/kg can be selected for further studies.

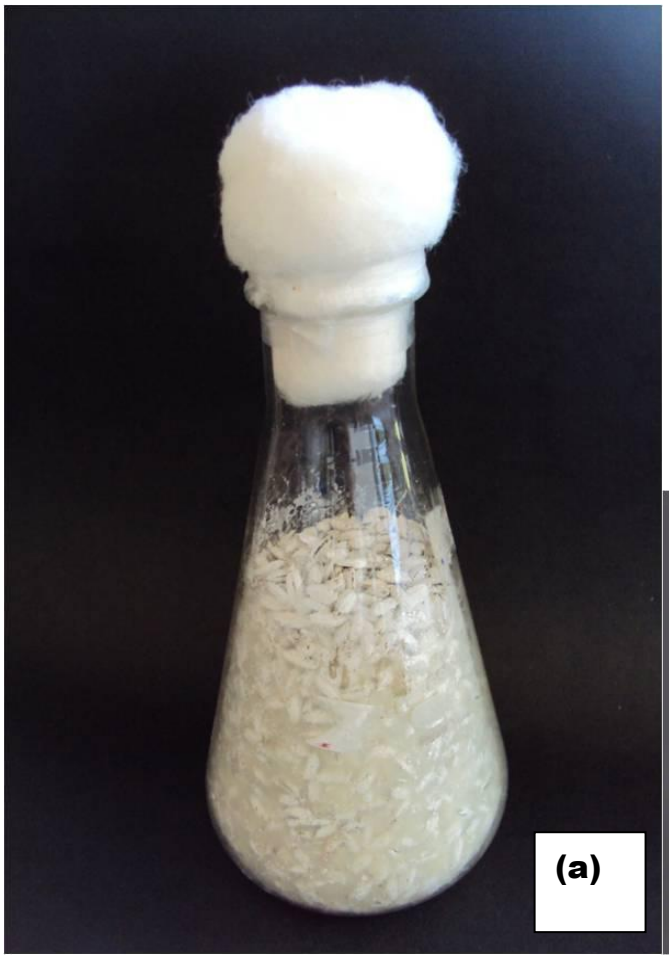


Fig. 4.16 (a) Conical flask containing spawn of mushroom isolate M6

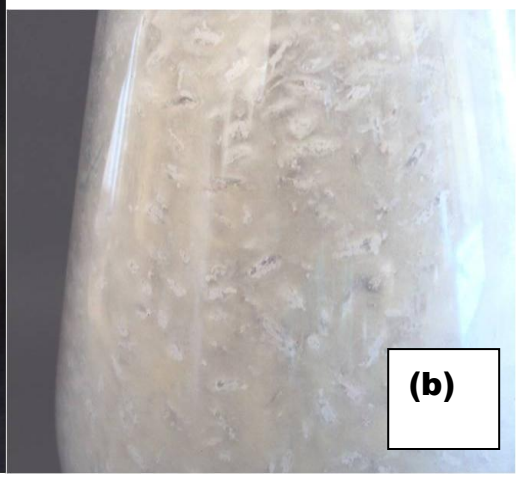


Fig. 4.16 (b) Grains spawn showing white cottony growth

Fig 4.17 Mushroom primodias formed after casing

The levels of heavy metals accumulated in the fruiting bodies for 50 mg/kg are as follows Cu(II): 789 mg/kg, Cd(II): 839 mg/kg, Cr(VI): 28 mg/kg, Pb(II): 889 mg/kg and Zn(II): 698 mg/kg. The bioaccumulation potential of fruiting bodies of M6 were found to be in the following order Pb(II) > Cd(II) > Cu(II) > Zn(II) > Cr(VI). The order of bioaccumulation of metals in fruiting body followed in the same order as the mycelia.

Fig 4.18 (a) Fruiting bodies formed (100 mg/kg)

Fig 4.18(b) Fruiting bodies formed (50 mg/kg)

The soils in the trays were also analyzed for heavy metal concentration once the fruiting body formations terminated (after 30 days of incubation) to determine the bioaccumulation factor which are presented in Section 4.5.4. The termination of fruiting body formations may be because the mushroom, M6 takes 30 days to complete its reproductive stage when grown in soil. On comparing the

bioaccumulation potential of M6 fruiting bodies (Fig. 4.19) to its mycelial form (Fig. 4.9) it is evident that the fruiting bodies are more efficient in heavy metal accumulation from polluted soil than the mycelia. This difference in their bioaccumulation potential may be because the fruiting body stage of mushroom life cycle has efficient mechanism to tolerate heavy metal stress and thereby accumulating higher concentrations of heavy metals compared to their mycelia. It may be also because of the higher biomass and fleshy nature of the fruiting bodies than the thin mycelia. Volesky and Holan (1995), Thomet et al. (1999), Turkekuel et al. (2004), Tuzen (2003), Cao et al. (2010) and Vimala and Das (2009), have also reported that the bioaccumulation potential of mushroom fruiting bodies are higher when compared to its mycelia.

Table 4.6 Casing study results for Mushroom, M6

Metals under study	**Initial metal conc. In soil (mg/kg)**	
	100 mg/kg	50 mg/kg
	No. of primodias formed per grid (2.5×2.5×2.5)	
Cu (II)	1	12
Cd(II)	16	46
Cr(VI)	0	8
Pb(II)	23	30
Zn (II)	9	15

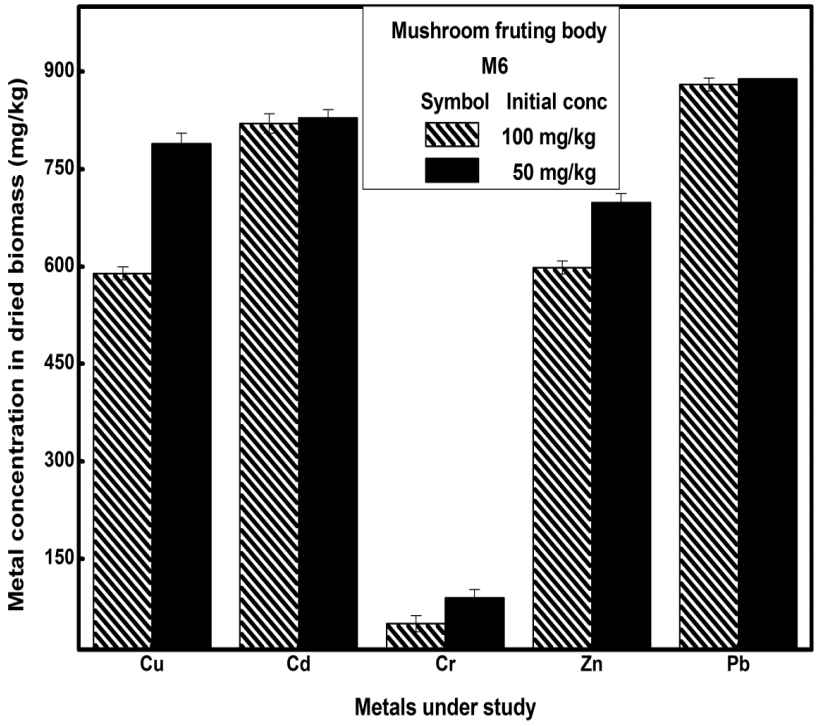


Fig. 4.19 Bioaccumulation profile of M6

4.5.2 PREFERENCE OF METAL ION BY MUSHROOM ISOLATE M6

As per the bioaccumulation study results discussed in section 4.5.1 and Fig.4.19 it is observed that, mushroom M6 prefers the metal ions in the order Pb(II) > Cd(II) > Cu(II) > Zn(II) > Cr(VI). Since, the soil has been contaminated with metal ions using nitrate, phosphate and sulphate salts and their solubility in water is high, the mycelial part of the mushroom has been found to absorb easily and quickly along with other nutrients and water molecules from the soil. However, the bioaccumulation studies have proved that the metal ion movement in the soil and their transport into the mushroom are not only due to absorption of soluble salt forms, but also depends on various other factors like, physical and chemical properties of the metal ions i.e its molecular mass, ionic radii and eletronegativity and on the tolerance mechanism prevailing in the organism at different stages of its growth / life cycle

(Nass et al. 2007; Lu et al. 2008; Azila et al. 2008; Cao et al. 2010). Thus, the relationship between metal ion properties, metal mobility and various physico-chemical properties of the heavy metals can be incurred from Table 4.7. It can be also observed that Cu(II) and Zn(II) having similar molecular mass, ionic radius and eletronegativity, were found to accumulate on the fungal mycelium (roots of fungi) in similar quantities. Among the above said parameters, electronegativity has been found to have major influence on the bioaccumulation process and Pb(II), which has the highest eletronegativity compared to other studied metal ions, found to have higher rates of accumulation by the fungal mycelia. The bioaccumulation patterns shown in Fig.4.19 are in agreement with the literature study reports (Tuzen 2003; Cao et al. 2010; Allan and Brow 1995). A detailed study on the metal bioaccumulation mechanism has to be performed for understanding the underlying bioaccumulation mechanism.

Table 4.7 Properties of metal ions under study (Robina et al. 2011, Ho et al. 1995, Allan and Brown, 1995)

PROPERTIES	Cu(II)	Cd (II)	Cr (VI)	Pb(II)	Zn (II)
Molecular mass (g/mol)	63.57	112.4	51.99	207.21	65.38
Ionic radius(A^0)	0.72	0.95	0.44	1.21	0.74
Eletronegativity of atom (Pauling units)	1.69	1.90	1.66	2.33	1.65

4.5.3 DETERMINATION OF SITE OF BIOACCUMULATION IN M6 FRUITING BODIES

In the present study, determination of heavy metal accumulation sites in mushrooms' pileus and stalks have been analyzed using AAS after acid digestion and the results are presented in Fig.4.20. It is found that, from Fig.4.20, the amount of metal accumulation in the pileus region of mushroom is higher than that of its stalk.

The mushroom, were found to accumulate about 70-80% of heavy metals like Cu(II), Cd(II), Cr(VI), Pb(II) and Zn(II) into the fleshy pileus than in stalks. Pileus area in mushrooms was found to have fleshy tough outer cover biomass compared to the thin hollow stalk. During fruiting body formation in mushrooms, maximum amount of cell division was found to occur in the pileus region, which might have accelerated the transportation of metal ions from soil into the pileus region. Thus pileus may intend to accumulate more metal ions compared the stalk. Similar phenomena have been reported by various researchers in their studies on metal accumulation by mushrooms (Zhu et al. 2010, Chen et al. 2009, Falandysz et al. 2007, Yilmas et al. 2003, Cayer et al. 2009).

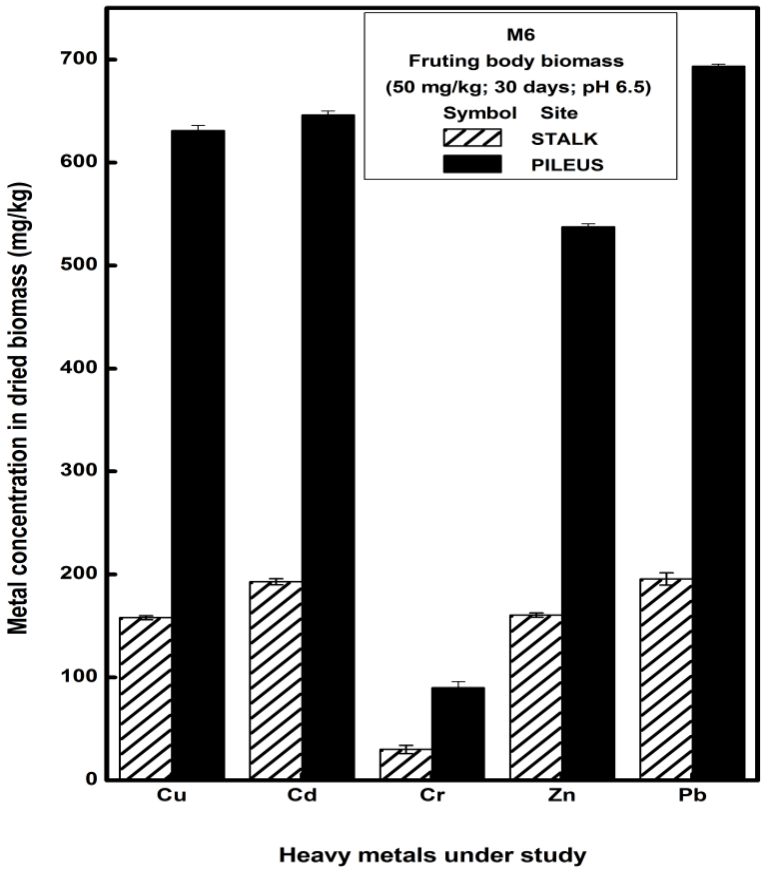


Fig. 4.20 Bioaccumulation potential of fruiting body stalk and pileus tissues in M6

The bioaccumulation potential of M6 was found to be higher than the other mushroom species reported in literature (summarized in Table 2.1). From Table 2.1, it is thus observed that non edible mushroom species accumulate higher amounts of metal ions than the edible species. However, the bioaccumulation profile indicates that metal accumulation capability is species specific and mainly depends on its accumulation mechanism (Wuyep et al. 2007; Niu et al. 2007; Mitra et al. 1994; Yilmas et al. 2003; Turkekuel et al. 2003; Zhu et al. 2011).

4.5.4 DETERMINATION OF BIOACCUMULATION FACTOR (BAF) FOR M6

To determine the efficiency of bioaccumulation by the mushroom species, the metal concentration in the mushroom (both mycelia and fruiting body) was compared to the metal concentration in its environment (Niu et al. 2007; Zhao et al. 2010). The BAF of mushroom M6 both in mycelial form and in fruiting bodies in the soil are presented in Table 4.8. It was observed that BAF values for M6 decreases with increase in initial metal concentration, indicating that bioaccumulation capability may be reduced due to enhanced metal stress. A BAF value of above '1' indicates that the metals have higher affinity to accumulate in the mushroom rather than the soil (Zhao et al. 2010). This aspect proves to be an advantage for the application of mushrooms as bioremediating agent. The BAF values of M6 for Cu(II), Cd(II), Pb(II) and Zn(II) were found to be above '1' at an initial metal concentration of 50 mg/kg indicating its applicability as bioremediation agent. Based on the values of BAF presented in Table 4.8, the mushroom M6 can be regarded as hyper accumulator for metals like Cu(II), Cd(II), Pb(II) and Zn(II). Whereas for Cr(VI) M6 cannot be considered as a good bioremediating agent as its BAF value is less than '1' even though it is tolerant for 50 mg/kg of Cr(VI). The BAF values for mycelial stage of M6 were found to be lesser than those with the fruiting bodies for all the metals under study. Hence, fruiting body stage of the life cycle of M6 can be considered to have higher potential in remediating the soil as compared to that of the mycelial stage.

Thus harvesting the fruiting bodies leads to easy removal of heavy metals from the soil which can be considered as an added advantage of mycoremediation in remediation of metal contaminated soil.

Table 4.8 Bioaccumulation Factor (BAF) for metals in mushroom, M6.

Mushroom isolates	Metal Conc. (mg/kg of soil)	Cu(II)		Cd(II)		Cr(VI)		Pb(II)		Zn(II)	
		M	F.B	M	F.B	M	F.B	M	F.B	M	F.B
M6	50	0.66	1.04	0.64	1.01	0.330	0.220	0.88	1.16	0.52	1.05
	100	0.53	0.69	0.58	0.88	0.008	0.005	0.79	0.91	0.45	0.76

- **M-Mycelia; F.B-Fruiting body**

The BAF values of the fruiting bodies of M6 for Cd(II) and Pb(II) metals at 100 mg/kg concentrations was found to be higher than that for other metals. It may be because; the mushroom failed to yield fruiting body at 100 mg/kg concentrations of Cr(VI) and yielded very less number of fruiting bodies in the presence of Cu(II) and Zn(II). Thus the toxicity of heavy metals, Cu(II), Cr(VI) and Zn(II) on M6 was severe enough to suppress and skip the yield of fruiting bodies. These results were found to be in accordance with reports of Tuzen (2003) on bioaccumulation of heavy metals like Cd(II) and Zn(II) from soil using *Agaricus macrosporous*, *Agaricus silvicola* and *Stropharia rugosoannulata*. Hence, the efficiency of mycoremediation of heavy metal contaminated soil can be considered to be specific for metal concentration and mushroom species. Similar results are observed in the studies of Regvar and Mikus (2008) and Usman et al. (2012).

Fig. 4.21(a) Mushroom M6 at the site of isolation

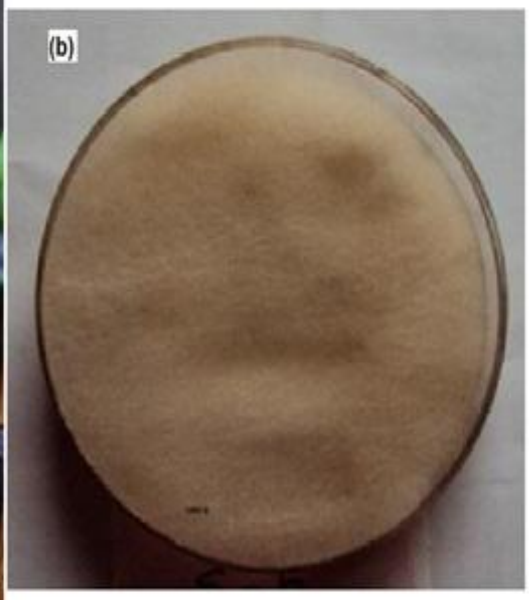


Fig. 4.21 (b) Petri plate showing M6 mycelia

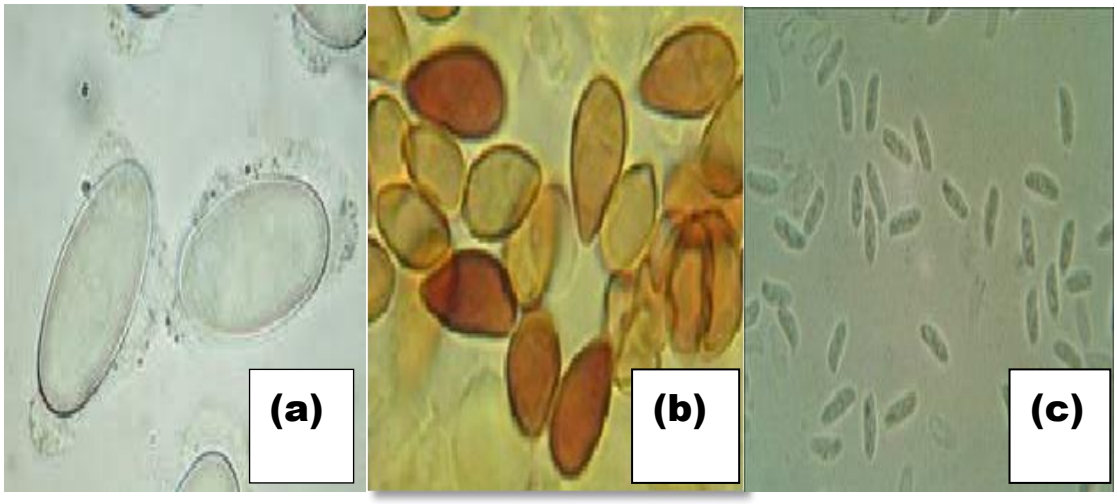


Fig. 4.22 Microscopic view of mushrooms spores (a) M5, (b) M6 and (c) M9.

4.6 IDENTIFICATION OF MUSHROOM ISOLATES

Mushroom isolates M5, M6 and M9 were found to be efficient in bioaccumulating heavy metals compared to their counterparts. Morphological characteristics of these isolates were compared to the mushroom features described in mycology reference books (Moser 1983; Breitenbach et al. 1995). Table 4.9 describes the basic analysis results of mushroom M5, M6 and M9. Analysis of Mycological characteristics presented in Table 4.9 reveals that mushroom species M5, M6 and M9 belong to the genus *Pleurotus* Sp., *Galerina* Sp. and *Polyporous* Sp. respectively.

Table 4.9 Mycological characteristics of isolates M5, M6 and M9

FEATURES	M5	M6	M9
Gills	Present	Present	Present
Cap	Flat	Convex	Concave
Stipe ring	Absent	Absent	Absent
Spore colour	Pink	Brown	yellow
Genus	*Pleurotus* Sp	*Galerina* Sp	*Polyporus Sp*

4.6.1 MOLECULAR CHARACTERISTICS OF M6

A genotype analysis, Internal Transcribed Sequence analysis (ITS analysis) was carried out using 250 bp DNA of M6 at Agharkar Research Institute, Pune, Maharashtra, India to identify the species of the mushroom. The ITS analysis result, (Fig. 4.23) showed about 98% similarity with the standard genome of *Galerina vittiformis* with accession number AJ871544 in the gene sequence from the Gen Bank and hence the species was identified as *vittiformis* under the genus *Galerina.*

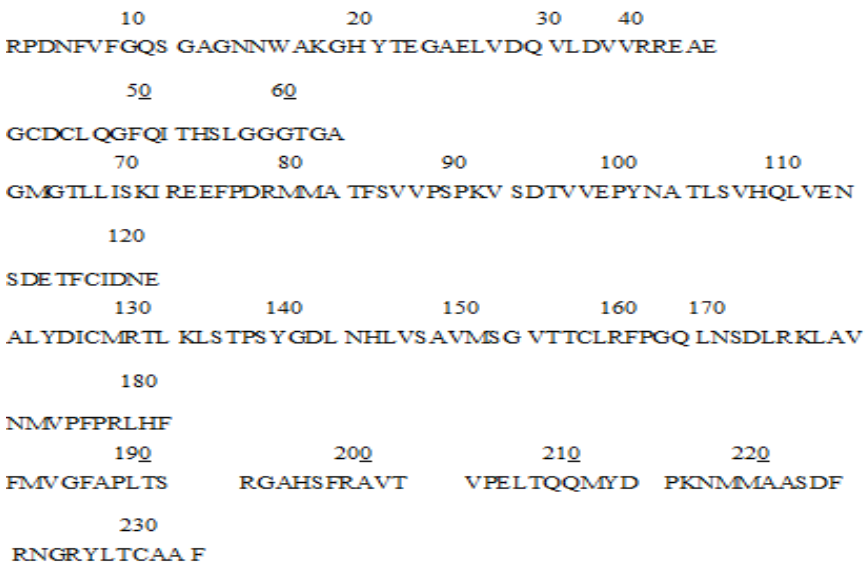


Fig. 4.23 Overview of ITS analysis for *Galerina vittiformis* (M6)

4.7 REMOVAL KINETICS FOR METAL IONS

Metal uptake by fungi involves various processes like metal desorption from soil particles, transport of soluble metals to the stalk of the mushrooms through the mycelial surfaces *via* active transport, chelaters or organic ligands like polysaccharides (Huang et al. 2012; Mata et al. 2009; Davis et al. 2003; Mau et al. 2001). The metal ions from the stalk are further translocated in to the pileus for accumulation. So, in bioaccumulation two distinctive processes can occur. In the first step metal ions are bound to the surface of the cells-the process is metabolically passive and is identical with biosorption. In the second stage, the metal ions are transported to the cellular interior. In order to perform this step, the cells must be metabolically active. The entire process of bioaccumulation of metal from the soil to the fungal cells on removal of metals from soil consists of the following steps (i) transport of metal in the soil near to the mycelial surface (ii) diffusion across the liquid film surrounding the mycelial surface (iii) diffusion of soluble metals in the liquid contained in the pores in the mycelia along the pore walls (iv) sorption and desorption on the external surface of mycelia or within the mycelial pore surface (v)

transportation to the cellular interior. Any of these steps may be the rate controlling steps or the combination of the steps governs the rate of metal uptake. To test for possible mechanism governing the rate of metal uptake by *Galerina vittiformis* from the soil, initially $\sqrt{t}$ test was made to check if the diffusion of soluble metals in the liquid contained in the pores of the mycelia along the pore walls (intra particle diffusion) is the rate controlling step (Ho et al. 2000). For removal kinetic studies, tray experiments were conducted for metal removal from soil over a period of 45 days. The metal uptakes (q_t) by the mushroom biomass (M6) were analyzed at every 5 days interval. The experimental data are presented in Table 4.10.

4.7.1 INTRA PARTICLE DIFFUSION KINETICS

A more appropriate quantitative approach to distinguish between kinetic and diffusion rate control is to perform the square root of contact time analysis. According to Weber and Morris (1964), if the rate limiting step is intra particle diffusion, a plot of solute uptake against square root of contact time (incubation) should yield a straight line passing through the origin (Poots et al. 1976). The most widely applied intra particle diffusion equation for biosorption system is given by Weber and Morris (1963).

$$q_t = k_{id} \sqrt{t} + C \qquad (4.1)$$

where, q_t (mg/kg) is the metal uptake at time t (day), k_{id} (mg $kg^{-1}day^{-0.5}$) is the intra particle diffusion rate constant, and C is a constant (mg/kg). The experimental data obtained from bioaccumulation experimental run for 40 days (presented in Table 4.10) were used to test for the validity of intra particle diffusion model. Plots of $\mathbf{q_t}$ vs $\sqrt{t}$ for intra particle diffusion kinetics models are presented in Fig.4.24 to Fig. 4.28 for Cd(II), Cu(II), Cr(VI), Pb(II) and Zn(II) respectively. Experimental data were found to fit well with the intra particle diffusion model with R^2 value ranging from 0.8556 to 0.9556 as shown in Table 4.11. It shows that intra particle diffusion is one of rate controlling steps. The plot of $\mathbf{q_t}$ vs $\sqrt{t}$ passing through origin indicates that

intra particle diffusion is the sole rate limiting step. From Fig.4.24 to Fig.4.28, it is clear that the intercept of the linear fit of the diffusion equation, does not pass through the origin for all the studied metals indicating that intra particle diffusion is not the only rate limiting step for the metal removal process (Hameed et al. 2008).

Table. 4.10 Experimental qt and t data for all the studied heavy metals

Cu(II)		**Cd(II)**		**Cr(VI)**		**Pb(II)**		**Zn(II)**	
q_t	**t**	**q_t**	**t**	**q_t**	**t**	**q_t**	**t**	**q_t**	**t**
116.11	5	42.8	5	20.0	5	44.7	5	142.92	5
132.17	6	46.1	6	20.3	6	44.1	6	144.60	6
128.43	7	39.5	7	22.8	7	43.6	7	144.33	7
152.32	8	76.4	8	25.0	8	42.8	8	139.4	8
168.89	9	98.4	9	26.0	9	42.4	9	140.03	9
185.38	10	102.0	10	28.1	10	42.2	10	149.17	10
186.00	11	136.0	11	30.0	11	41.9	11	159.40	11
175.15	12	158.4	12	31.5	12	41.8	12	192.50	12
198.60	13	162.4	13	32.3	13	39.3	13	208.01	13
228.43	14	178.4	14	40.0	14	38.8	14	218.54	14
241.40	15	198.0	15	43.0	15	38.7	15	220.00	15
243.50	16	239.4	16	45.0	16	38.5	16	227.80	16
243.47	17	268.5	17	48.0	17	38.3	17	228.65	17
243.39	18	280.4	18	53.0	18	37.9	18	247.70	18
243.40	19	298.4	19	56.0	19	37.4	19	267.75	19
243.49	20	364.4	20	59.0	20	37.0	20	269.20	20
243.49	25	407.0	25	68.0	25	32.7	25	289.56	25
243.50	26	408.0	26	69.8	26	32.6	26	310.00	26
243.46	30	408.0	30	78.6	30	32.3	30	321.00	30
243.4	35	408.0	35	85.0	35	31.8	35	321.20	35
243.48	40	408.0	40	85.6	40	31.8	40	321.00	40

❖ **q_t (mg/kg); t (days)**

Hence, surface reaction kinetics, external film mass transfer or transportation to the cell interior may also simultaneously control the rate of metal uptake by their significant contribution to the overall rate along with intra particle diffusion. Initial curved portion or multi linear pattern of the $\mathbf{q_t}$ vs $\sqrt{\mathbf{t}}$ plots are attributed to boundary layer effects, surface reaction or transportation to the cell interior. The value of intercept C is proportional to the boundary layer thickness for external film mass transfer (Javed et al. 2010; Preetha and Viruthagiri 2005).

In the case of Zn(II),Cd(II), Cr(VI) and Cu(II) the intercept value is negative which indicates that other processes like surface chemical reaction and transportation into cell interior may contribute to the overall rate of metal uptake, apart from intra particle diffusion and external film mass transfer. Table 4.11 also presents the values of intra particle diffusion rate constant ($\mathbf{k_{id}}$).

Table.4.11 Intra particle diffusion analysis result

Metals Ions under study	**R^2**	**k_{id} (mg $kg^{-1}day^{-0.5}$)**	**C (mg kg^{-1})**
Cu(II)	0.9387	72.983	-53.375
Cd(II)	0.9556	168.76	- 423.51
Cr(VI)	0.9795	18.01	-23.684
Zn(II)	0.9450	70.05	- 66.93
Pb(II)	0.8552	120.39	69.76

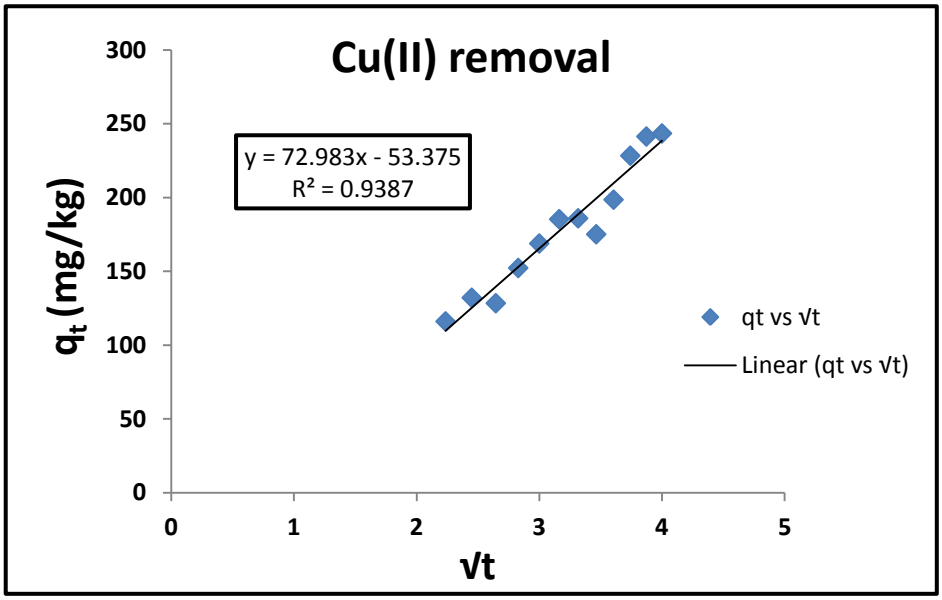


Fig.4.24 Intra particle diffusion kinetic plots for Cu(II) removal

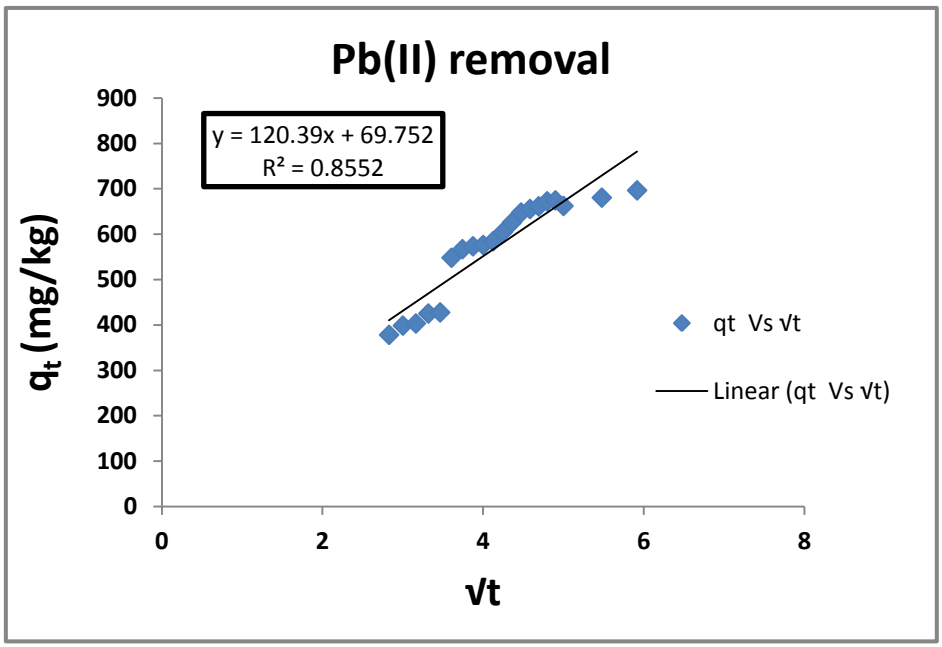


Fig.4.25 Intra particle diffusion kinetic plots for Pb(II) removal

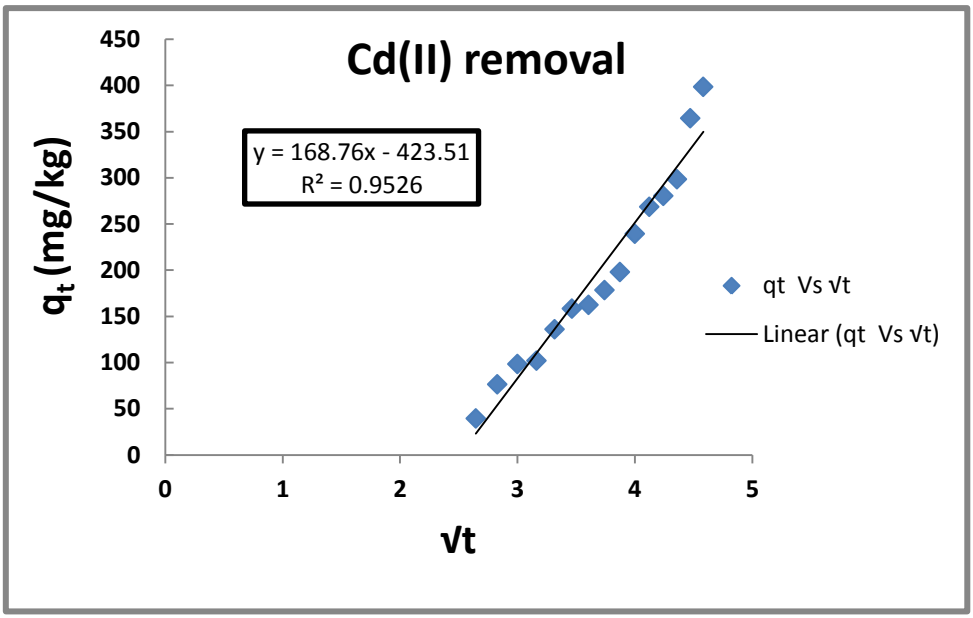


Fig.4.26 Intra particle diffusion kinetic plots for Cd(II) removal

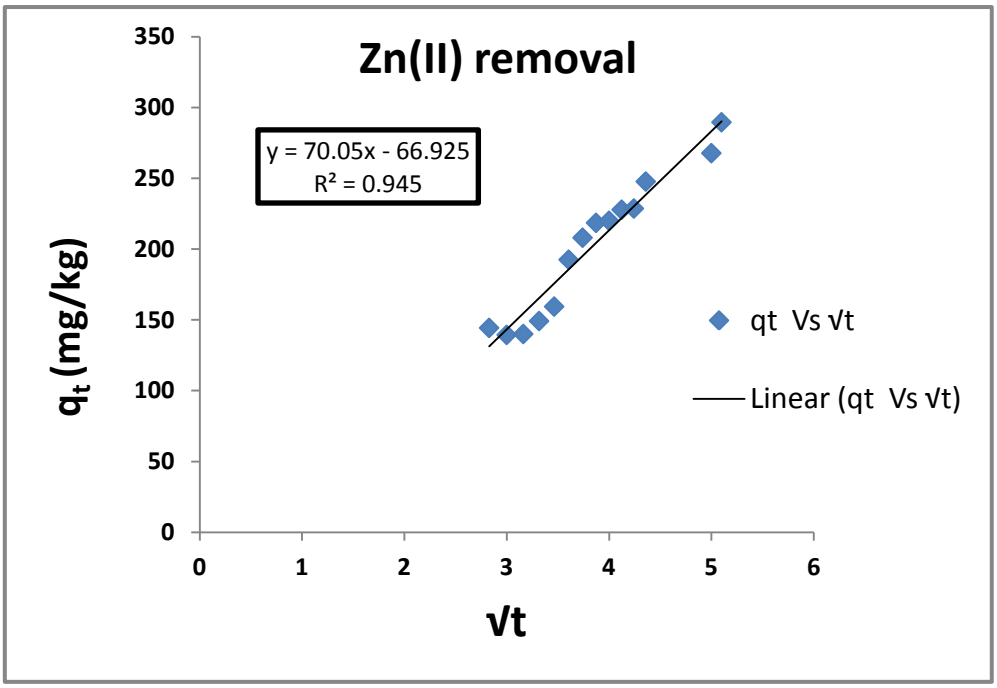


Fig.4.27 Intra particle diffusion kinetic plots for Zn(II) removal

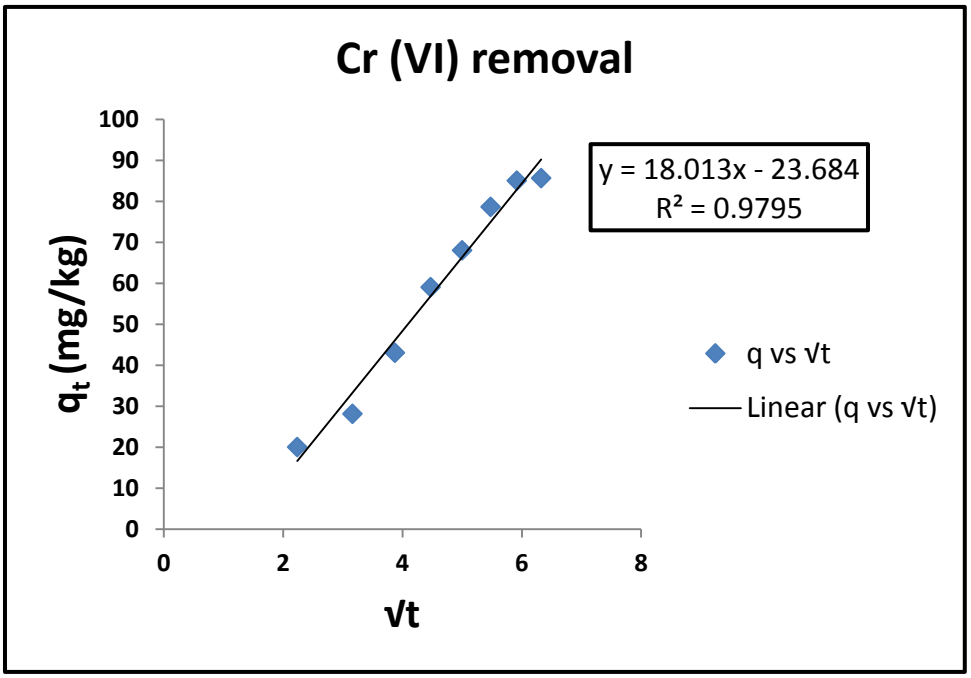


Fig.4.28 Intra particle diffusion kinetic plots for Cr(VI) removal

4.7.2 PSEUDO FIRST ORDER AND SECOND ORDER KINETIC MODELS

As the initial metal uptake is by biosorption, many mathematical models from the literature which describe biosorption kinetics were tested. Lagergren's both pseudo first- order and pseudo-second-order model equations are widely used to explain the kinetics of metal removal (Langergren, 1898).

Boyd et al. (1947) proposed a model to describe biosorption kinetics when external film diffusion surrounding the solid surface is the rate controlling step. The model form proposed by Boyd et al. (1947) is the same as the Lagergren's pseudo first- order rate equation, indicating that differentiating between film diffusion control and pseudo-first order reaction control will be difficult (Ho et al. 2000). This means that, in the case of Lagergren's pseudo first- order rate equation, the metal removal rate depends on the diffusion ability of molecule through boundary liquid film and sorption kinetics as a chemical phenomenon. In the case of metal uptake by mushroom as both the phenomena of (i) transport by diffusion through boundary

liquid film and (ii) the chemical phenomena of binding of metals onto the chelators or organic ligands(secreted by the mycelia) present on the surface may occur, Lagergren's pseudo first- order rate equation was tested for its validity. The experimental q_t and t values are presented in Table 4. 10.

The pseudo-first-order rate equation by the Lagergren's is given as:

$$\frac{dq_e}{d_t} = k_1 \left(q_e - q_t \right) \tag{4.2}$$

Integrating Eq. (4.2) with the boundary conditions of $q_t=0$ at t=0 and $q_t=q_t$ at t=t, yields

$$\log(q_e - q_t) = \log(q_e) - \frac{k_1 t}{2.303} \tag{4.3}$$

Where q_e (mg/kg) and q_t (mg/kg) are the metal uptake on 40^{th} day and at any given time t (day), respectively and k_1 is the rate constant (day^{-1}). Using the experimental data on metal uptake of q_t vs t and the equilibrium uptake q_e (the constant value of uptake reached during experiments), a graph of log ($q_e - q_t$) vs t were plotted for each of the metals under study. The validity of first order kinetic model to represent the experimental data were tested by the linear nature of the plot. The straight line fit of the experimental data to the model was tested based on the value of the coefficient of determination(R^2). The value of rate constants(k_1) were calculated from the slope of the straight line. The fit of experimenatl data to the pseudo-second-order rate expression were also tested for their validity to represent the kinetics of metal uptkae. The second order model describes the kinetic of metal uptake involving valency forces through the sharing or exchange of electrons between the surface and the metal as covalent forces, and ion exchange (Ho and McKay 2002).

The pseudo-second-order rate equation by the Lagergren's is given as:

$$\frac{dq_t}{dt} = k_2 \left(q_e - q_t \right)^2 \tag{4.4}$$

Integrating Eq. (4.4) with the boundary conditions of $q_t=0$ at t=0 and $q_t=q_t$ at t=t, yields

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^{\,2}} + \frac{1}{q_e}\,t \qquad (4.5)$$

Where $\mathbf{k_2}$ is the second order rate constant. Using the experimental data on metal uptake *i.e* $\mathbf{q_t}$ vs **t**, a graph of $\mathbf{t/q_t}$ vs **t** were plotted for each of the metals under study. The value of $\mathbf{k_2}$ can be determined from the slope and intercept(**kg.mg^{-1} day^{-1}**). The straight line fit of the experimental data to the model was tested based on the value of the R^2.

The results of bioaccumulation studies for 40 days were used to plot the graphs for Langergren pseudo first and second order kinetic equations. The data were fitted by a straight line to test for the validity of both the first and second order kinetics. Table. 4.12 shows the values of first order rate consatnt (k_1) obtained from the plots along with their R^2 values for all the metals under study. Fig 4.29 to Fig 4.32 present the plots for linear form of first order kinetics. The plots show that the first order kinetic model fits the Cd(II) and Pb(II) uptake rates well as observed from the R^2 values nearer to one ,whereas the Zn(II), Cr(VI) and Cu(II) removal rates poorly fitted the first order kinetic model. Fig 4.33 to Fig. 4.37 present the plots to test the validity of seocnd order kinetic models and Table 4.13 shows the values of kinetic parameters (k_2) estimated from the plots along with the values for coefficient of determination (R^2). It is clear from the R^2 values for second order kinetic model fit that, the second order model fits the kinetic data for Cr(VI), Cu(II), Pb(II) and Zn(II) better as compared to first order kinetic model. This implies that the kinetics of metall uptake by *G.vittiformis* proceed as per second order kinetic model for Cr(VI), Cu(II), Pb(II) and Zn(II) and first order kinetic model is followed for Cd(II) uptake as shown in Table 4.12 and Table 4.13. The applicable rate equation and the values of kinetic rate constants for removal of the metals under study from soil are shown in Table 4.14. The results suggest the applicability of pseudo second-order kinetics for Cu(II), Pb(II), Cr(VI) and Zn(II) uptake, based on the assumption that in the metal uptake by *G. vittiformis*, surface reaction that involves valence forces may be a rate

limiting step. Initial sharing or exchange of electrons between mycelia and the metals, further lead to sequence of other steps involved in bioaccumulation. Thus second order kinetic model provides the best correlation of data in explaining the kinetics of uptake of Cr(VI), Cu(II), Pb(II) and Zn(II) (Preetha and Viruthagiri 2005; Xiangliang et al. 2005; Javaid et al. 2010). Cd(II) uptake follows first order kinetic model indicating that Cd(II) removal rate depends on the diffusion ability of molecule through boundary liquid film and sorption kinetics as a chemical phenomenon.

Low et al. 1995 explained the adsorption phenomenon of methylene blue dye on water hyacinth root with pseudo first order equation. Similarly the removal of Cr(VI) and Cu(II) using Moss were also explained by first order kinetics by Lee et al. (1996). The metals removal mechanism was reported to be governed by psedo first order reactions by various researchers like Panday et al. (1984), Gupta et al. (1990), Sharma et al. (1990), Namasivayam and Yamuna, (1992), Singh and Rawat (1994), Namasivayam and Ranganathan (1993), Mishra and Singh (1996), Ho et al. (2004).

Table 4.12 Pseudo-first order kinetics analysis result

Metal Ion	**Cd(II)**	**Cu(II)**	**Cr(VI)**	**Zn (II)**	**Pb(II)**
R^2 Value	0.9314	0.6524	0.3420	0.8661	0.9618
k_1 in day^{-1}	0.086	0.2950	0.0045	0.1138	0.1485

Table 4.13 Pseudo-second order kinetics analysis result

Metal Ion	**Cd(II)**	**Cu(II)**	**Cr(VI)**	**Zn (II)**	**Pb(II)**
R^2 Value	0.8402	0.9635	0.9464	0.9244	0.9648
k_2 in $kg.mg^{-1} day^{-1}$	Not fitted (shows negative slope)	8.108×10^{-4}	3.015×10^{-4}	2.64×10^{-4}	1.73×10^{-3}

Table4.14 Kinetic model validity and constants

Metal removal	Valid Kinetic model	Kinetic rate constant
Cd(II)	First order	0.086 day^{-1}
Cu(II)	Second order	8.108×10^{-4} $kg.mg^{-1}$ day^{-1}
Cr(VI)	Second order	3.015×10^{-4} $kg.mg^{-1}$ day^{-1}
Zn(II)	Second order	2.64×10^{-4} $kg.mg^{-1}$ day^{-1}
Pb(II)	Second order	1.73×10^{-3} $kg.mg^{-1}$ day^{-1}

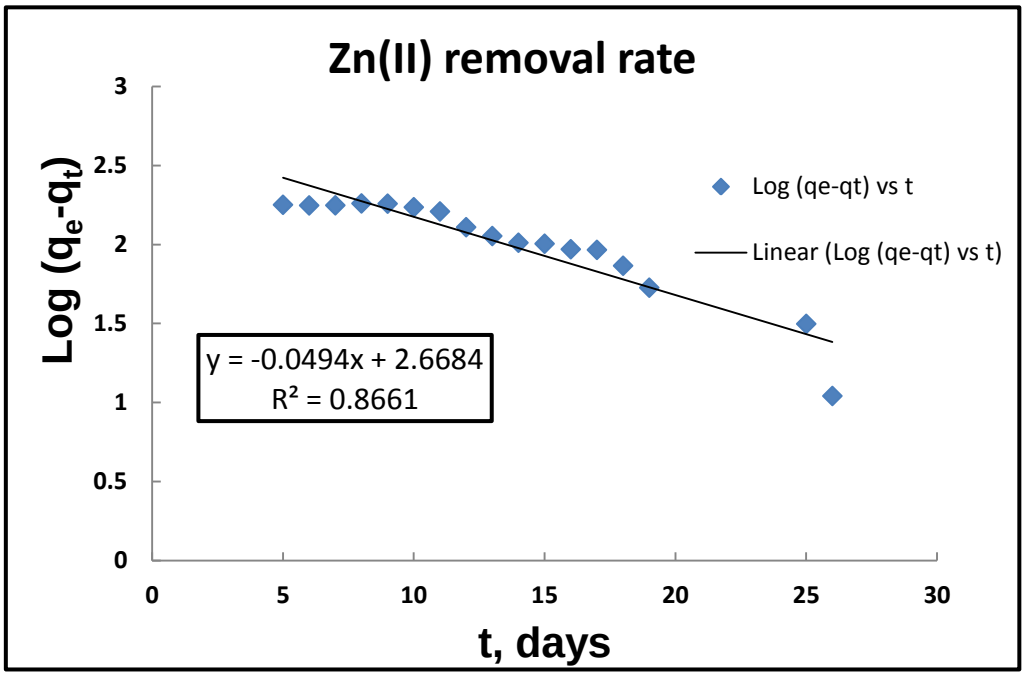


Fig.4.29 Pseudo first order kinetic plot for Zn(II) removal

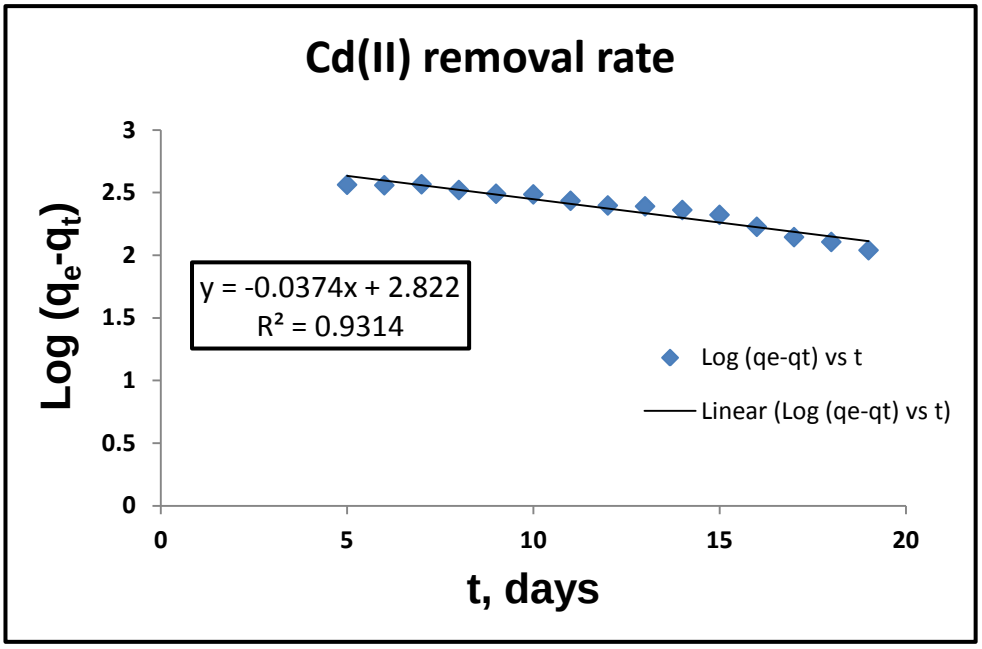


Fig.4.30 Pseudo first order kinetic plot for Cd(II) removal

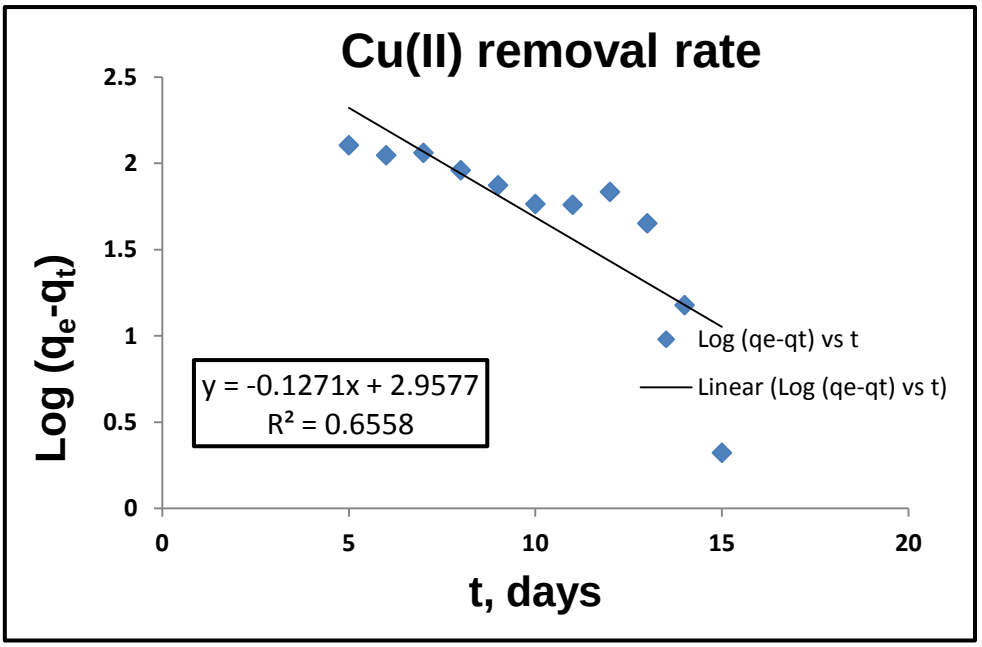


Fig.4.31 Pseudo first order kinetic plot for Cu(II) removal

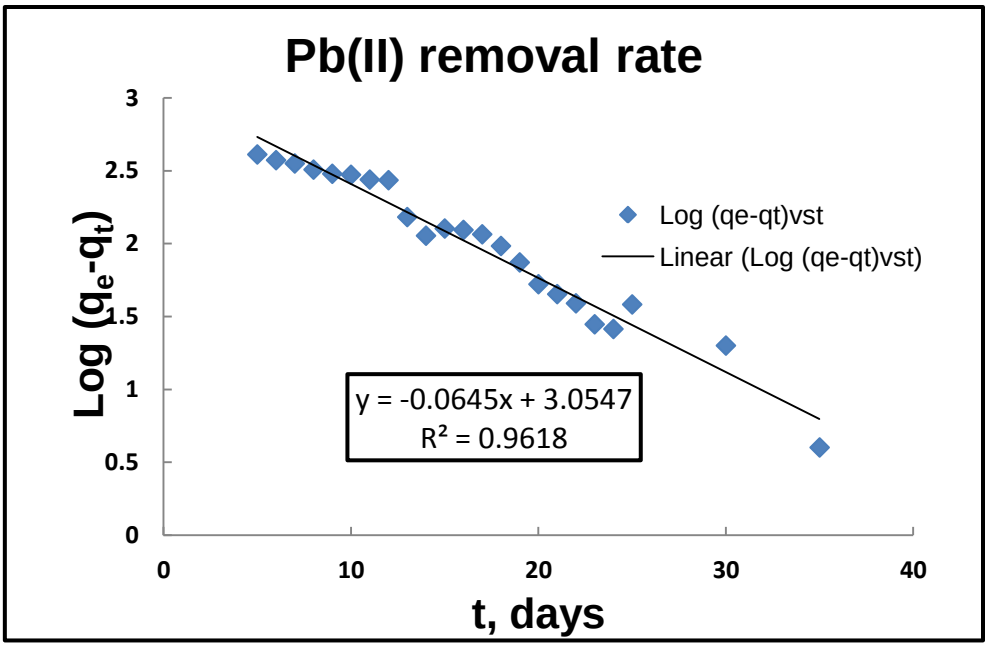


Fig 4.32 Pseudo first order kinetic plot for Pb(II) removal

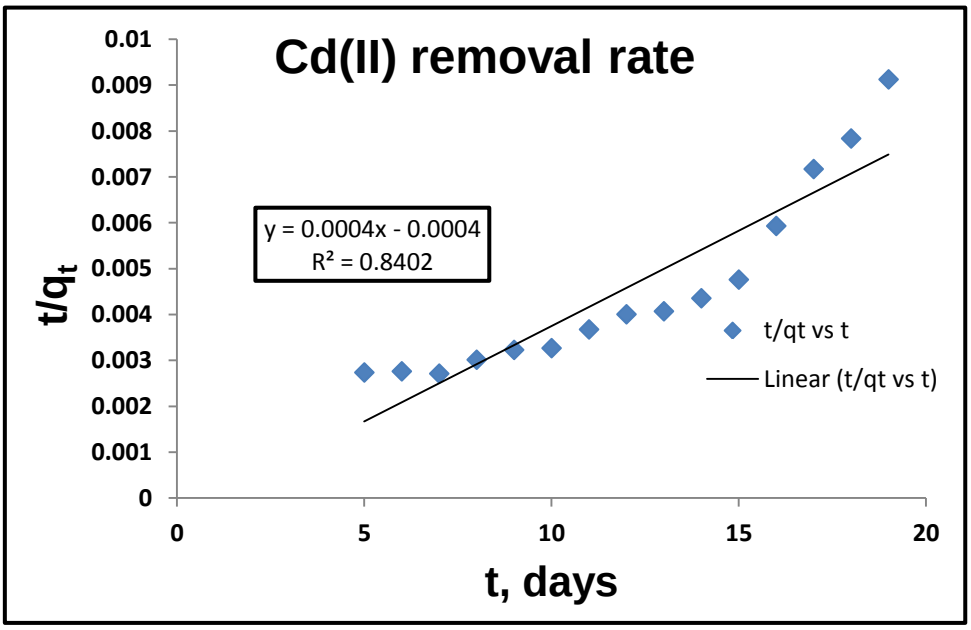


Fig. 4.33 Pseudo Second order kinetic plots for Cd(II) removal.

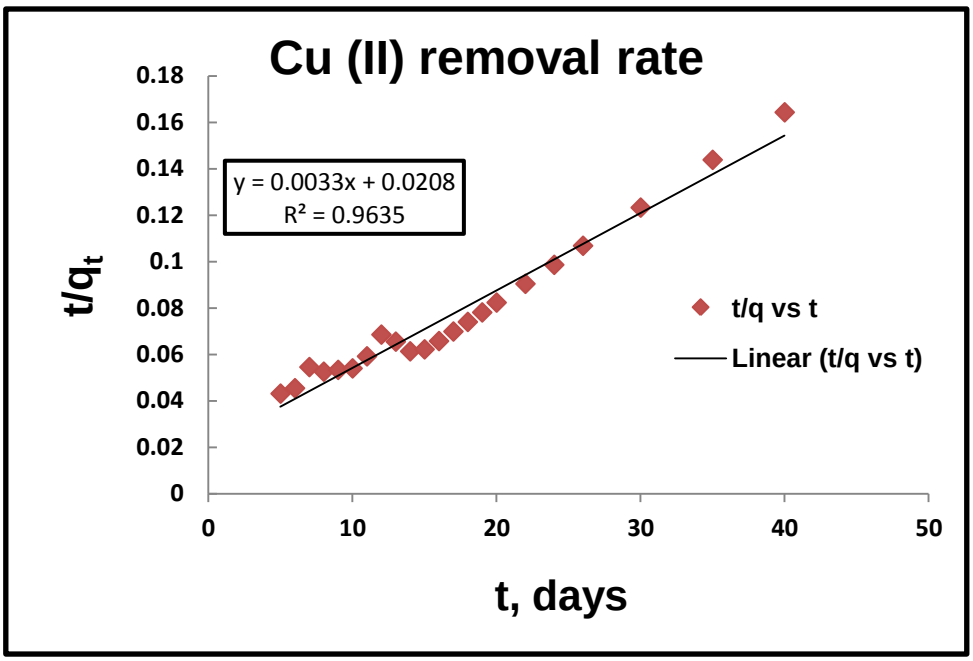


Fig.4. 34 Pseudo Second order kinetic plots for Cu(II) removal.

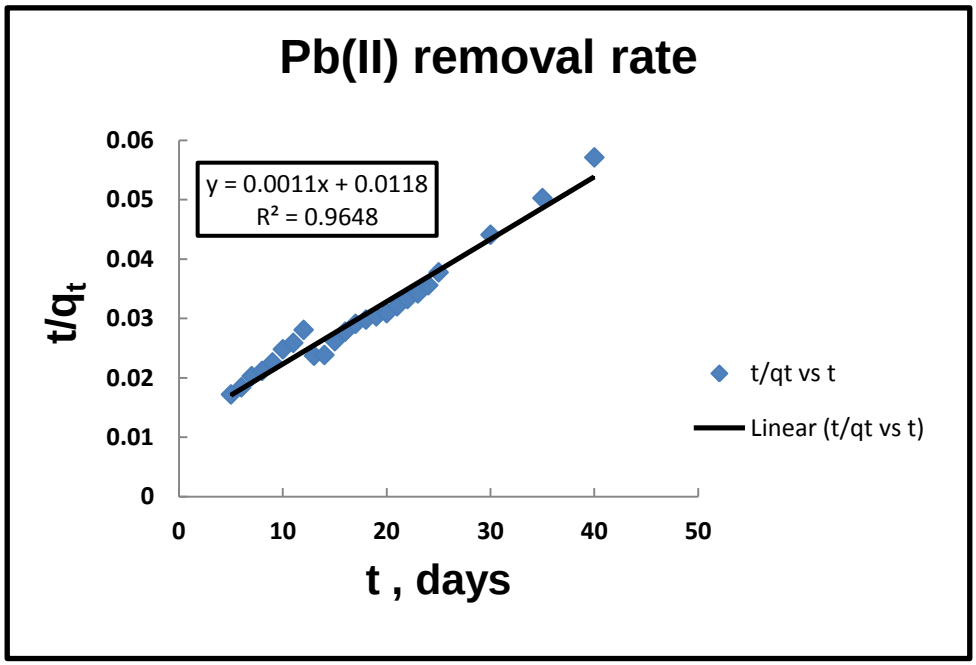


Fig.4.35 Pseudo Second order kinetic plots for Pb(II) removal.

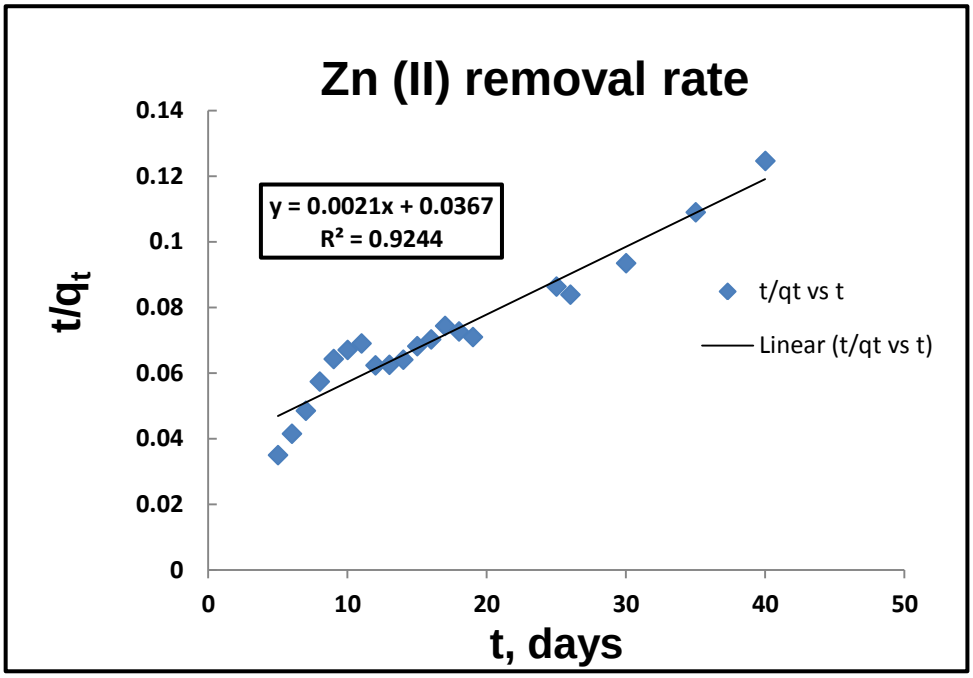


Fig.4.36 Pseudo Second order kinetic plots for Zn(II) removal.

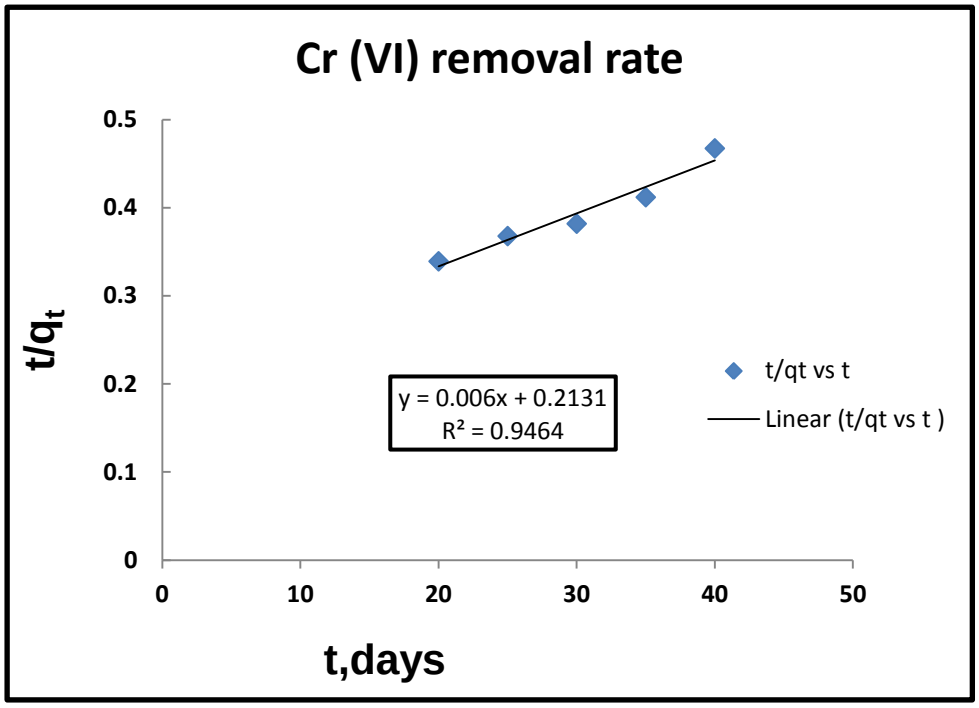


Fig.4.37 Pseudo Second order kinetic plots for Cr(VI) removal.

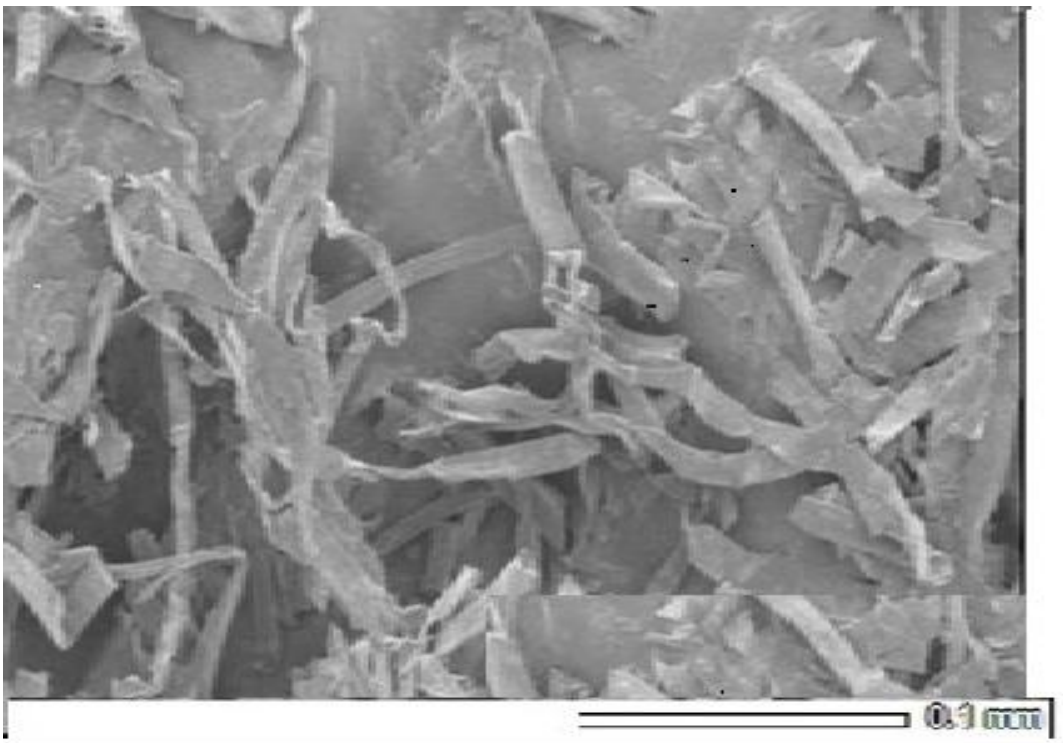
4.8 METAL BIOACCUMULATION MECHANISM

Heavy metals are known to act as a general protoplasmic poison, inducing the denaturation of proteins and nucleic acids (Ge et al. 2011; Joho et al. 1995). They can also break apart biological molecules into even more reactive species (such as: reactive Oxygen Species) which will also disrupt biological processes. Hence only those species which can successfully tolerate these physiological stresses can successfully survive in heavy metal laden environment. Mushrooms respond to metal stress in the environment by producing stress compounds of proteinous and non proteinous origin. The pileus (cap) of the mushrooms has been found to produce stress factors which help in sequestering the accumulated metal ions into their vacuoles. The most common stress components produced by plants and fungi are metalothionine (MT), glutothionine (GSH), phytochelatins (PC), organic acids and plastocyanine (Hall 2002). From the studies of Inouhe et al. (1996), Mehra et al. (1988), Miinger and Lerch (1985), Lerch (1980), it is observed that macro fungi have evolved metal tolerance and accumulation mechanism compared to micro fungi. Understanding the mechanism of metal uptake from the soil to the fruiting bodies would help us to improvise the process by advanced molecular biology tools. Hence study on the uptake mechanism plays a significant role in developing better remediation technique. The mechanism of metal uptake by *Galerina vittiformis* is proposed in the present study through morphological studies by Scanning Electron Microscopy (SEM) with Energy Dispersive X-Ray Analysis (Edx), FTIR analysis and through LC-MS analysis.

4.8.1 MORPHOLOGICAL STUDIES BY SCANNING ELECTRON MICROSCOPY (SEM) WITH ENERGY DISPERSIVE X-RAY ANALYSIS (EDX).

The effect of heavy metal on the morphology of *Galerina vittiformis* mycelia during the bioaccumulation process was studied through SEM image analysis. Fig. 4.38 reveals that the hyphae of *G. vittiformis* are cylindrical, septate and branched before exposure to heavy metal. As shown in Fig. 4.39 characteristic change in the morphology, curling and formation of hyphae coils in response to Cd(II) stress (at concentration of 50 mg/kg) is observed. Similar observations were made when exposed to other heavy metals (Fig 4.40 - Fig. 4.43). Canovas et al. (2004) reported that the surface of, *Aspergillus* Sp., also had rough texture due to protrusions on the hyphae on exposure to 50mM of arsenate solution. Such modifications on the surface of fungi indicate the production of intracellular compounds due to heavy metals stress and which results in increase in pressure within the mycelia leading to the outward growth of the cell wall structures (Parazkeiwicz et al. 2011). Courbot et al. (2005) have also observed that the impact of metal stresses had led to production of thiol compounds, especially GSH and MT due to intracellular detoxification of cadmium in the fungi, *Paxillus involutus.* According to them, the cell wall protrusions indicate increased formation of intracellular vacuoles that serve as storage compartments for thiol containing compounds. These compounds are responsible for the binding of metal ions into the intracellular regions and accumulate them in the vacuoles, thereby reducing their toxicity in the cytoplasm and improving tolerance levels. The Energy Dispersive spectroscopy (EDS) analysis was done to analyze the metal ionic concentrations in the mycelial surface indicating mycofilteration. The results of EDS analysis for the control mycelia (Fig. 4.38) and mycelium treated with Cd(II) are shown in Fig. 4.39. Only traces of Cd(II) were observed in EDS spectral analysis. Whereas EDS of the mycelia exposed to other metals such as Cu(II), Cr(VI) Pb(II) and Zn(II) showed lower concentrations for the metals (Fig.4.40- Fig. 4.42 and Fig. 4.43), indicating the small levels of metal ions on the surface of the mycelia. Presence of lower concentrations of metal ions or small metal peaks in the EDS spectra

indicate that the metal removal by the mycelia of *Galerina vittiformis* may be attributed to vigorous intracellular bioaccumulation mechanism, rather than permanent adsorption on to the surface.

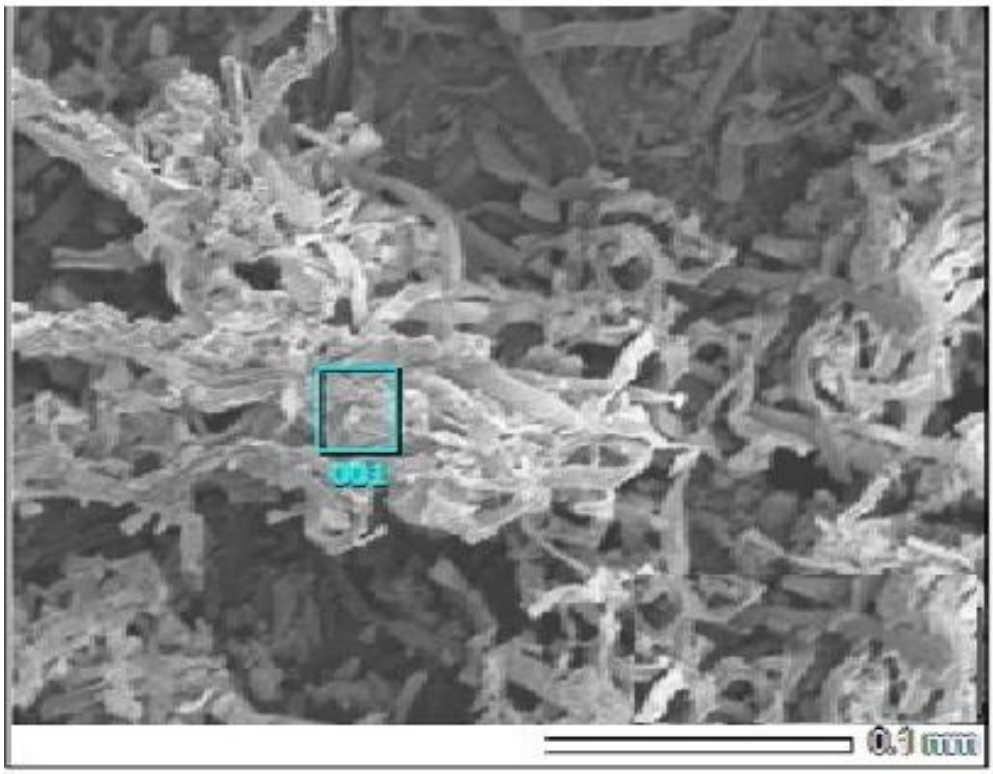


ZAF Method Standardless Quantitative Analysis

Fitting Coefficient : 0.5727

Element	(keV)	mass%	Error%	At%	Compound
C K	0.277	59.75	0.17	66.41	
O K	0.525	40.25	1.08	33.59	
Total		100.00		100.00	

Fig. 4.38 SEM and EDX analysis of *G. vittiformis* mycelia in untreated soil environment (at 700X magnification)

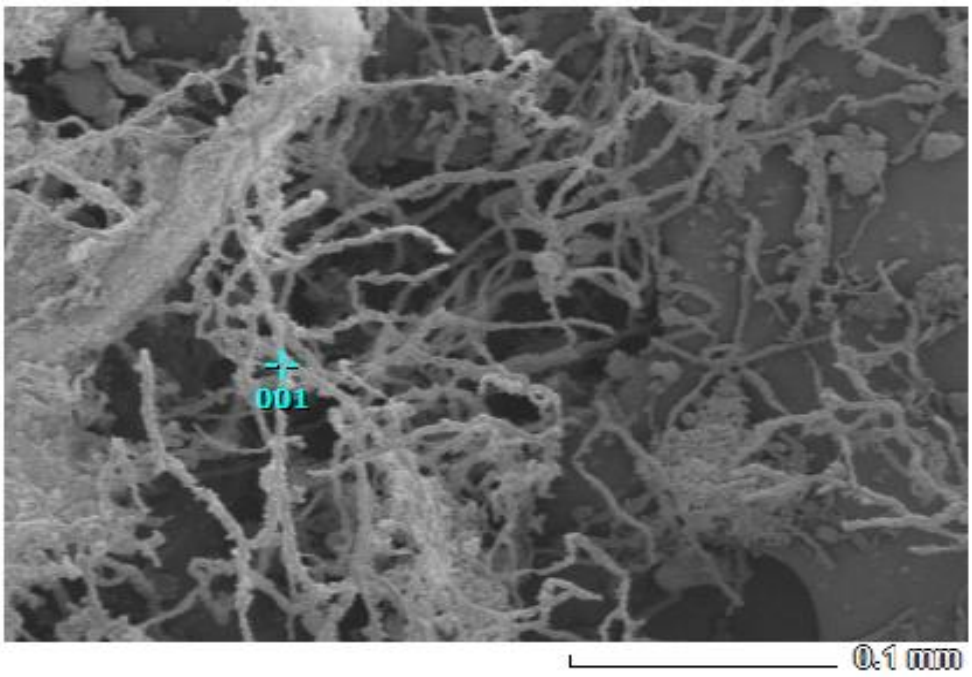


ZAF Method Standardless Quantitative Analysis

Fitting Coefficient : 0.5343

Element	(keV)	mass%	Error%	At%	Compound
C K	0.277	50.25	0.15	57.62	
O K	0.525	49.15	0.72	42.30	
Cd L	3.132	0.60	0.72	0.07	
Total		100.00		100.00	

Fig. 4.39 SEM and EDX analysis of *G. vittiformis* mycelia from Cd (II) treated soil environment (at 700X magnification)

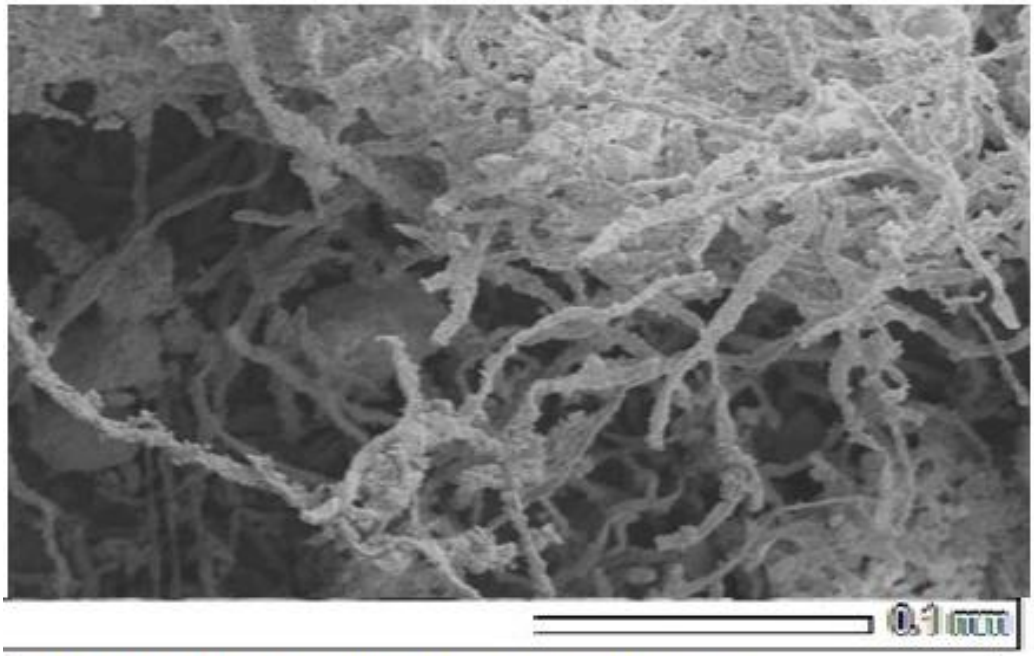


ZAF Method Standardless Quantitative Analysis

Fitting Coefficient : 0.6391

Element	(keV)	mass%	Error%	At%	Compound	mass%
AlK	1.486	37.21	1.37	40.26		43.9102
SiK	1.739	52.09	2.23	54.15		43.3598
CuK	6.398	10.69	5.59	5.59		12.7300
Total		100.00		100.00		

Fig. 4.40 SEM analysis of mushroom *G. vittiformis* treated with Cu(II)

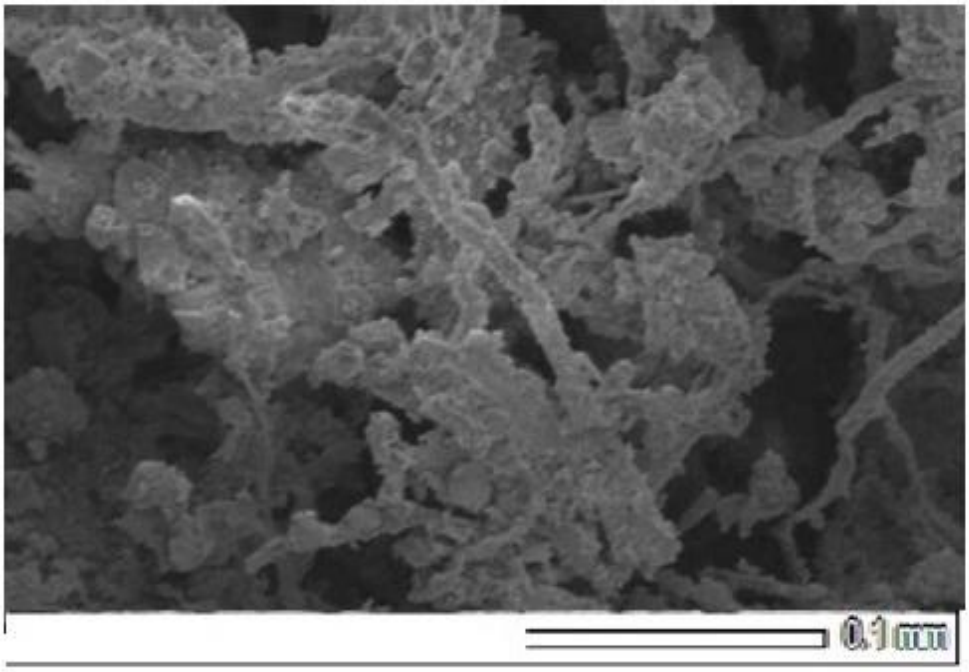


ZAF Method Standardless Quantitative Analysis

Fitting Coefficient : 0.4815

Element	(keV)	mass%	Error%	At%	Compound	mass%
Al K	1.486	36.29	0.82	40.04		41.4687
Si K	1.739	48.50	1.28	51.40		40.3332
K K	3.312	2.04	1.43	1.55		2.3700
Cr K	6.398	13.16	3.18	7.02		15.8281
Total		100.00		100.00		

Fig. 4.41 EDS analysis of mushroom *G. vittiformis* treated with Cr(VI)

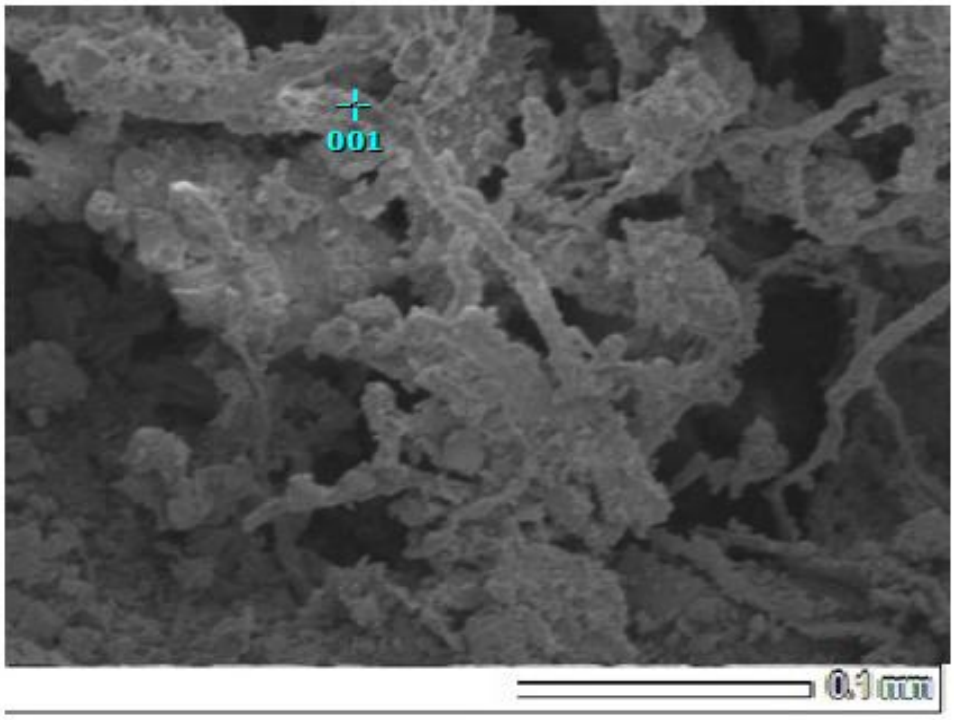


ZAF Method Standardless Quantitative Analysis

Fitting Coefficient : 0.5549

Element	(keV)	mass%	Error%	At%	Compound	mass%	Cation	K
Al K	1.486	35.49	1.04	38.75			41.1946	
Si K	1.739	52.17	1.64	54.73			44.0060	
Pb K	6.398	12.34	4.13	6.51			14.7994	
Total		100.00		100.00				

Fig. 4.42 EDS analysis of mushroom *G. vittiformis* treated with Pb(II)



ZAF Method Standardless Quantitative Analysis

Fitting Coefficient : 0.5993

Element	(keV)	mass%	Error%	At%	Compound	mass%
Al K	1.486	36.08	1.36	39.91		41.0191
Si K	1.739	48.37	2.13	51.40		40.2771
K K	3.312	1.65	2.37	1.26		1.9218
Zn K	6.398	13.90	5.27	7.43		16.7819
Total		100.00		100.00		

Fig. 4.43 EDS analysis of mushroom *G. vittiformis* treated with Zn(II)

4.8.2 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

FTIR spectra of fruiting bodies extracts of *Galerina vittiformis* after bioaccumulation studies were analyzed to determine the presence and disappearance of any functional groups involved in metal accumulation mechanism. The FTIR spectrum absorption bands of *G. vittiformis* grown on metal (Cu(II), Cd(II), Cr(VI), Pb(II) and Zn(II)) (50 mg/kg) contaminated soil were assessed in comparison with the spectrum obtained from control (Fig 4.44). Changes in the finger print region *i.e* 1000-1500 cm^{-1} (amide-I and II regions) and 1000-800 cm^{-1} (amide-III regions) indicates the presence of higher amounts of acids, proteinous and non proteinous compounds by *G.vittiformis* upon exposure to Cd(II), Cu(II), Pb(II), Cr(VI) and Zn(II). Analysis of each FTIR spectrum has studied thoroughly by comparing the peak values to their standard FTIR charts and determining the functional groups (Ivanova et al. 2008; Thomet et al. 1999; Paraskiewicz et al. 2011; Surewicz et al. 1993; Kalac and Svaboda 2000). From Fig. 4.45, FTIR graph of fruiting body obtained from Cr(VI) laden soil system the presence of stress related components like oxalic acid *i.e* 1253±5 and 1650±10 and thiol group *i.e* 2550±5 (Csliskan 2000) are observed. Fig. 4.46 shows peak; 2550±5 indicating presence of thiol group.

From Fig. 4.47 and Fig. 4.49 it can be observed that Cd(II) and Zn(II) FTIR shows the a peak of 2550±5 indicating the presence of Thiol group and peaks of 1253±5 and 1650±10 indicating the presence oxalic acid respectively. However, only Thiol groups (2550±5) are observed in Fig. 4.46 for Cu(II) and in Fig.4.49 for Zn(II). However Pb(II) FTIR charts did not show any characteristic peaks of oxalic acid and thiol group indicating the existence of an alternate metal tolerance mechanism. The presence of oxalic acid and thiol group in fungi and plants exposed to heavy metal stress have been reported by many researchers like Qian and Krimn (1994), Yang et al. (1999), Shi et al. (2001) who have reported that the primary stress compounds like Thiols can also wholly express the metal stress without the production of oxalic acids. In some cases both primary (thiols) and secondary (oxalic acid) stressed proteins can

be expressed to indicate the heavy metal stress. Thus, both primary (thiols) and secondary (oxalic acid) stressed proteins are detected in the fruiting body extracts of *G.vittiformis*.

Since, FTIR analysis results gives only a preliminary characterization of stress factors, the extracts of fruiting body are further subjected for LC-MS analysis for detailed characterization to determine the presence of any other stress components.

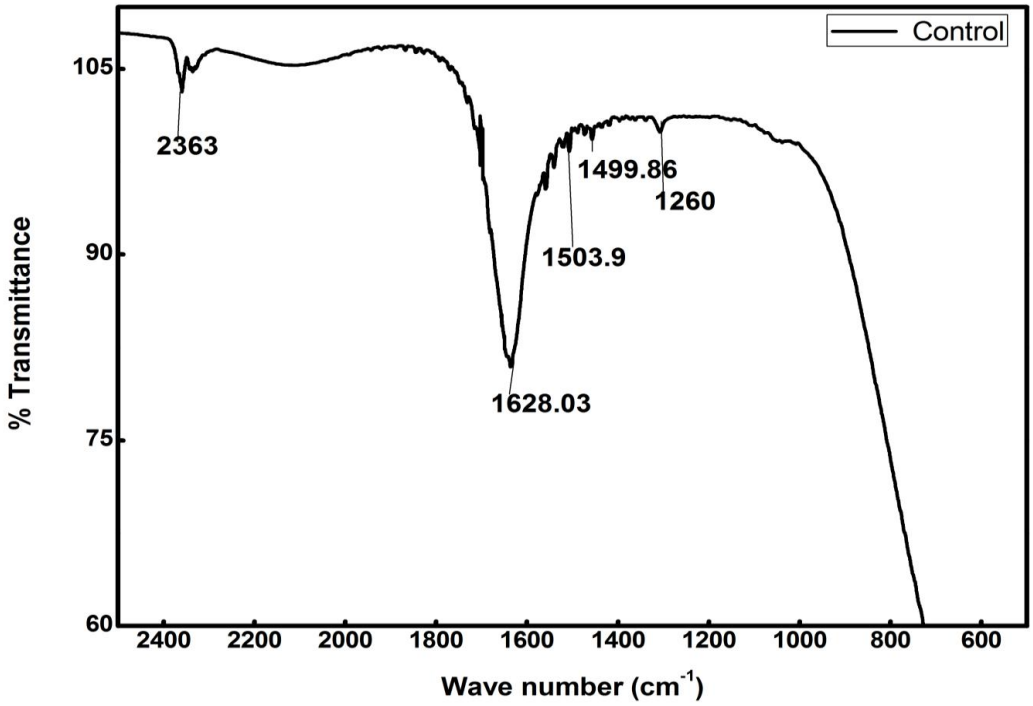


Fig. 4.44 2D-FTIR results of *G. vittiformis* from metal free environment (Control)

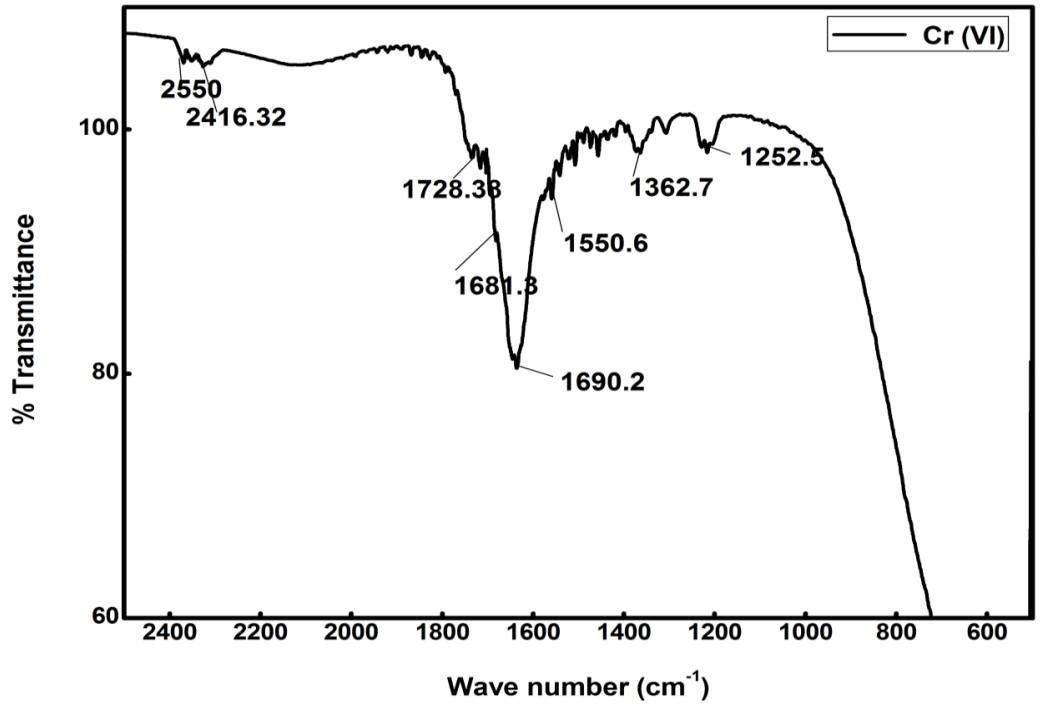


Fig. 4.45 2D-FTIR results of *G. vittiformis* from Cr(VI) laden soil system

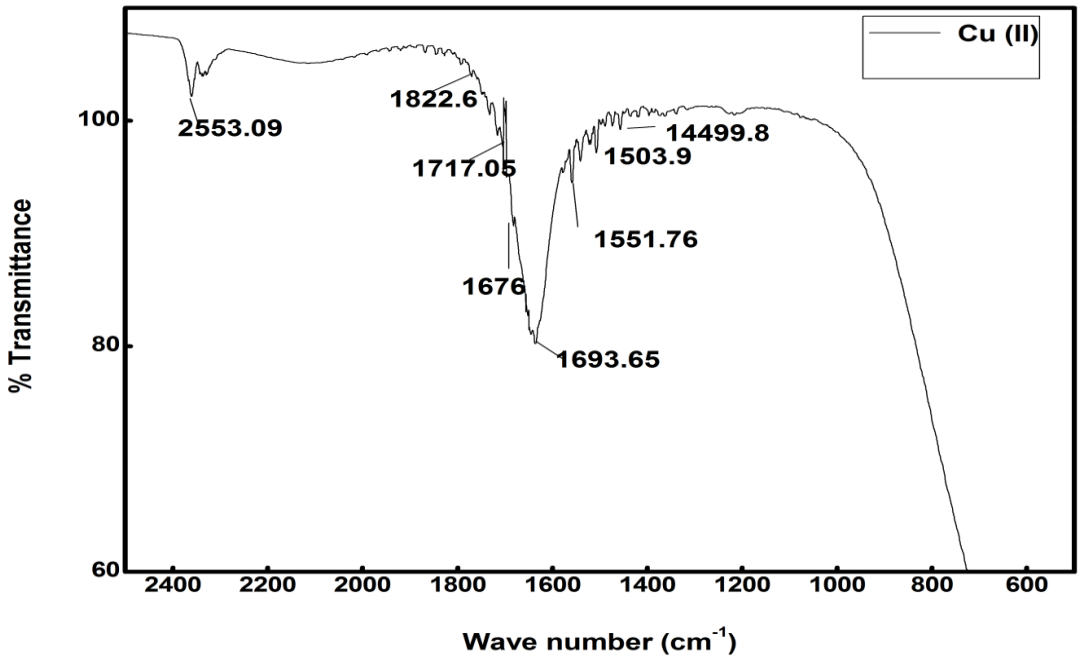


Fig. 4.46 2D-FTIR results of *G. vittiformis* from Cu (II) laden soil system.

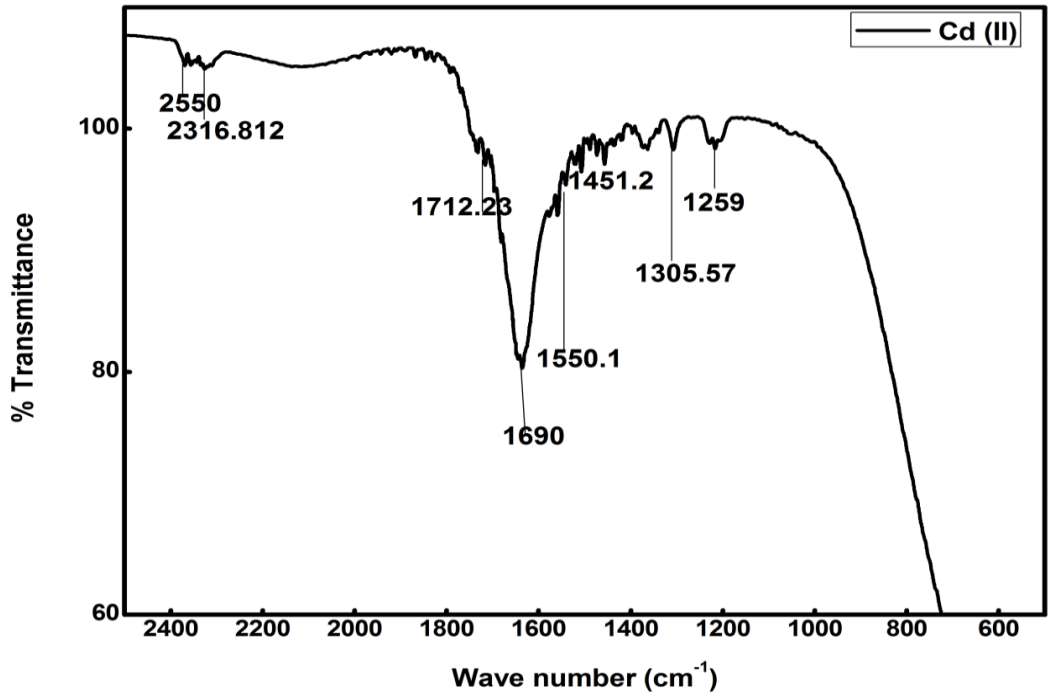


Fig. 4.47 -FTIR results of *G. vittiformis* from Cd(II) laden soil system

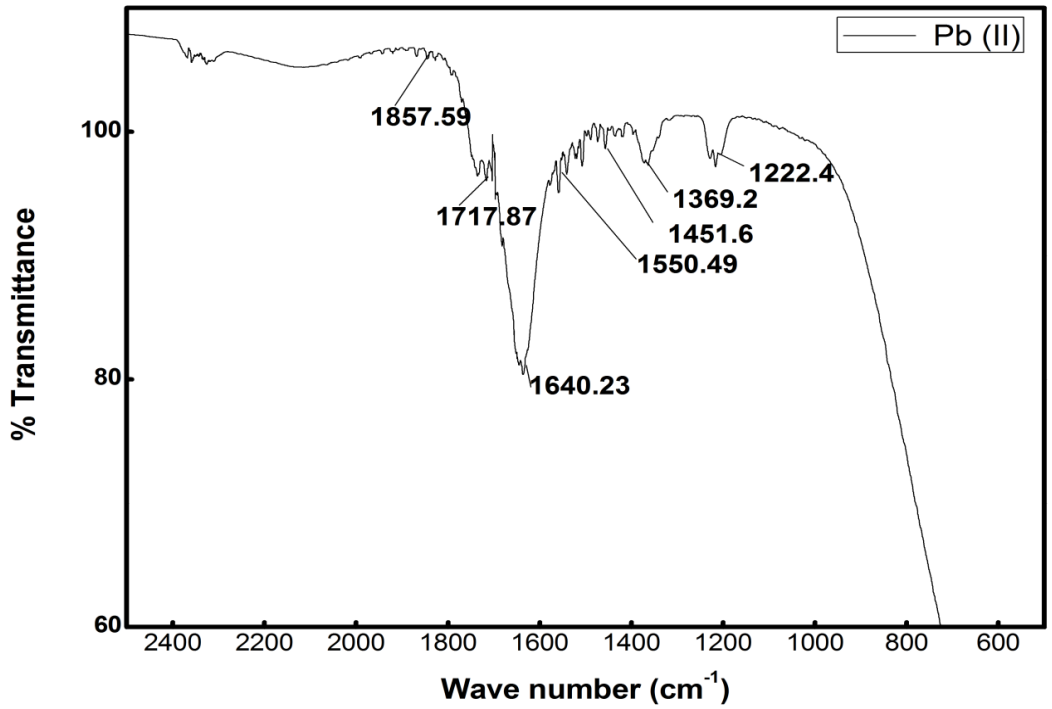


Fig. 4. 48 2D-FTIR results of *G. vittiformis* from Pb(II) laden soil system

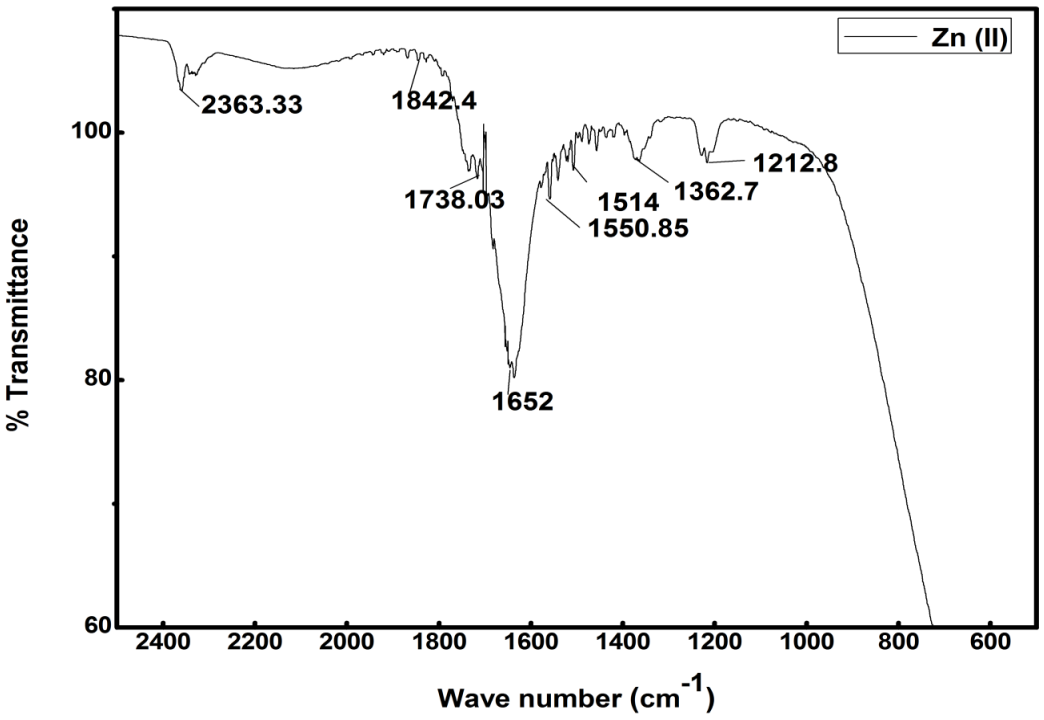


Fig. 4. 49 2D-FTIR results of *G. vittiformis* from Zn(II) laden soil system

4.8.3 LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY ANALYSIS (LC-MS)

Metal homeostasis requires intracellular complexation of metals when there is a cellular surplus and later release of metals to metal requiring apoproteins. The excess metal ions are stored in the storage sites within the cell e.g. Vacuoles (Hall 2002). LC-MS helps to identify those proteinous and non- proteinous metal ion trafficking components of *G. vittiformis* cells.

The LC-MS chromatograms of the extracts of fruiting bodies after Pb (II) accumulation are shown in Fig. 4.50 (a) and Fig. 4.50 (b). The data are obtained from Luna PFP (2) analytical column using ammonium formate and methanol as eluting buffers. Fig. 4.50 (a) shows the retention time in minutes and Fig. 4.50 (b) shows the m/z ratio of each component present in fruiting body extracts. The peaks obtained in

chromatograms were analyzed with the database to determine the components. On comparison with the literature it is observed that Fig. 4.50 (a) showed 2 peaks at 6-10 min retention *i.e* 7.4 and 8.6 indicating the presence of cysteine (Cys) and glutamine (Glu) residues which are the subunits of phytochelatins (γ-glutamylcysteine). Fig. 4.50 (a) also indicated 2 major peaks at 14-25 min retention time *i.e* 14.9 and 22.3 which indicated the presence of 2 types of phytochelatins (PC_2 and PC_3 respectively) while Fig. 4.50 (b) showed m/z peaks of Glutathione (GSH), PC_2 and PC_3 at 307, 538 and 679 respectively. Similar kinds of chromatograms obtained for both Cd (II) (Fig 4.51 (a and b)) and Cr (VI) (Fig4.52 (a and b) indicate the presence of phytochelatins (PC). Fig 4.53 (a) and (b) show the chromatogram of Cu (II) indicating the presence of both PC_2 and PC_3 (539 and 679 m/z peaks) (Shi et al. 2002; Camera et al. 2001; Odoemelam et al. 2011; Robina et al. 2011; Squellario et al. 2012).

From the studies of Grill et al. (1985), Gekeler et al. (1988), Liedschulte et al. (2010) and Gill and Tuteja (2011), it was revealed that the Phytochelatin of the general formula $(\gamma\text{-Glu- Cys})_n$ is the principal heavy metal detoxifying component in both plant and fungal kingdom. The phytochelatins can be viewed as linear polymers of the γ -glutamylcysteine (γ-Glu-Cys) portion of glutathione. These peptides could be enzymematically produced by stepwise condensation of γ-Glu-Cys moieties to a growing phytochelatin chain (PC). The PC plays a key role in maintaining cell homeostasis under heavy metal stress by binding to heavy metals like Cd, Zn, Cr etc and trafficking them to vacuoles or periplasmic space for storage Gill and Tuteja 2011).

Hence from the result of the present study, the mechanism of metal accumulation can be summarized as in Fig. 4.54. It was observed that metal uptake by *G.vittiformis* takes place through several metabolic and cytoplasmic processes; as the mushrooms comes in contact with the metal ions they are adsorbed on to the surface followed by uptake in to the periplasmic space and cytoplasm . The metal ions in the cytoplasm have various routes for detoxification. (1) The metal ions are bound

to various phytochelatins (PC_2 and PC_3) produced by *G.vittiformis.* Once they are ubiqutinized they are translocated to the fungal vacuoles by active absorbtion (ATP is utilized). (2)The metal ions in the cytoplasm trigger some amount of weak acids production which acts like heat shock proteins (HSP). These heat shock proteins bind to the metal ions and are made unavailable for any cellular activity. (3) Metal ions from the cytoplasm also move to the fungal vacuole through ionic pump. Similar heavy metal accumulation mechanism of PC has been reported in various metal resistant plant and algal species (Hall 2002; Camera et al. 2001; Grill et al. 1985; Nocito et al. 2006; Ammar et al. 2008; Yadav 2010; Tangahu et al. 2011; England and Wilkinson 2011; Scheidegger et al. 2012; Volland et al. 2013).

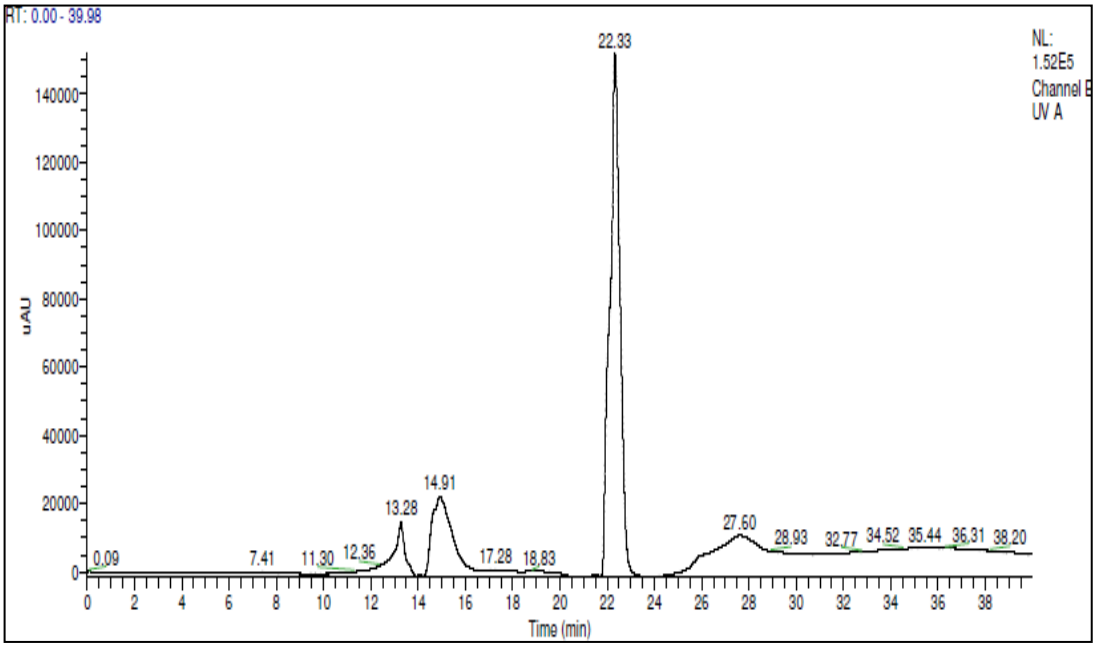


Fig. 4.50 (a) Chromatogram of LC-MS analysis for Pb(II) at various retention time (min)

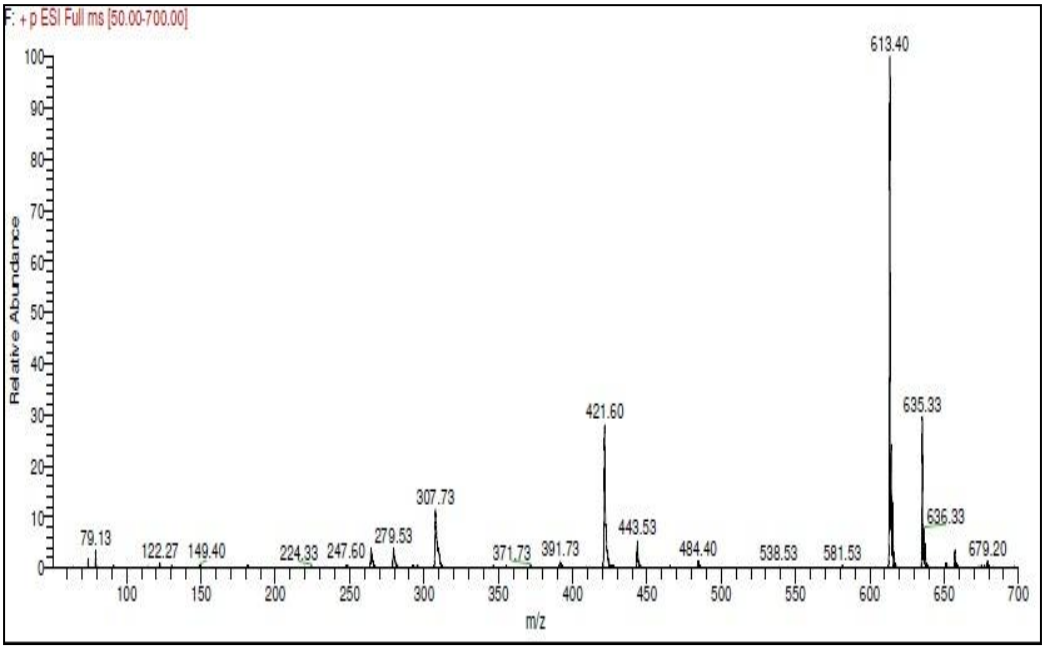


Fig 4.50 (b) Chromatogram of by LC-MS analysis for Pb(II) at various m/z ratios

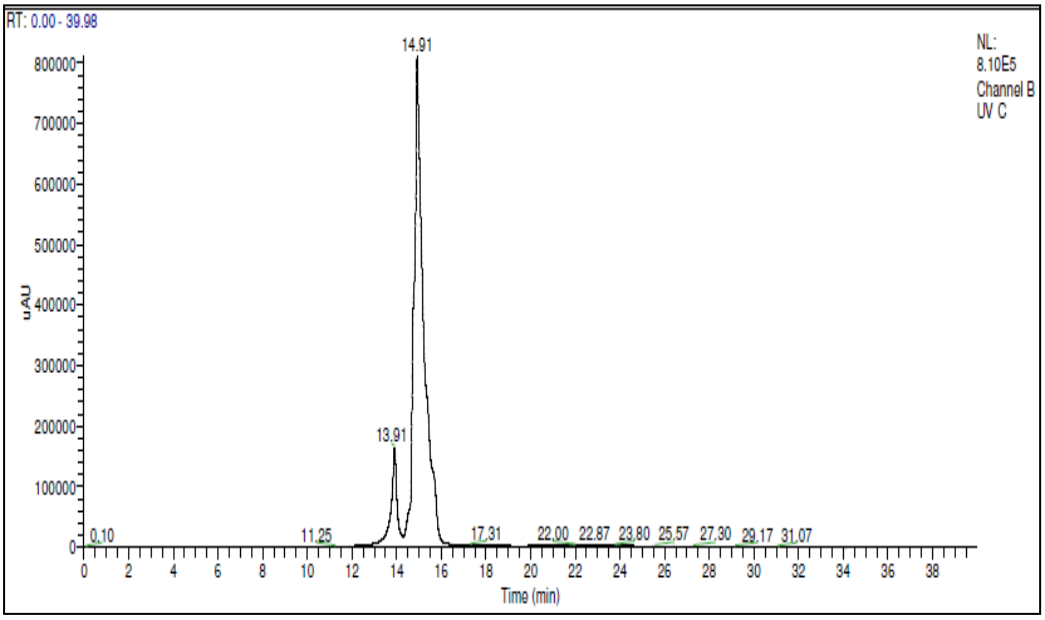


Fig. 4.51 (a) Chromatogram of LC-MS analysis for Cd (II) at various retention time (min)

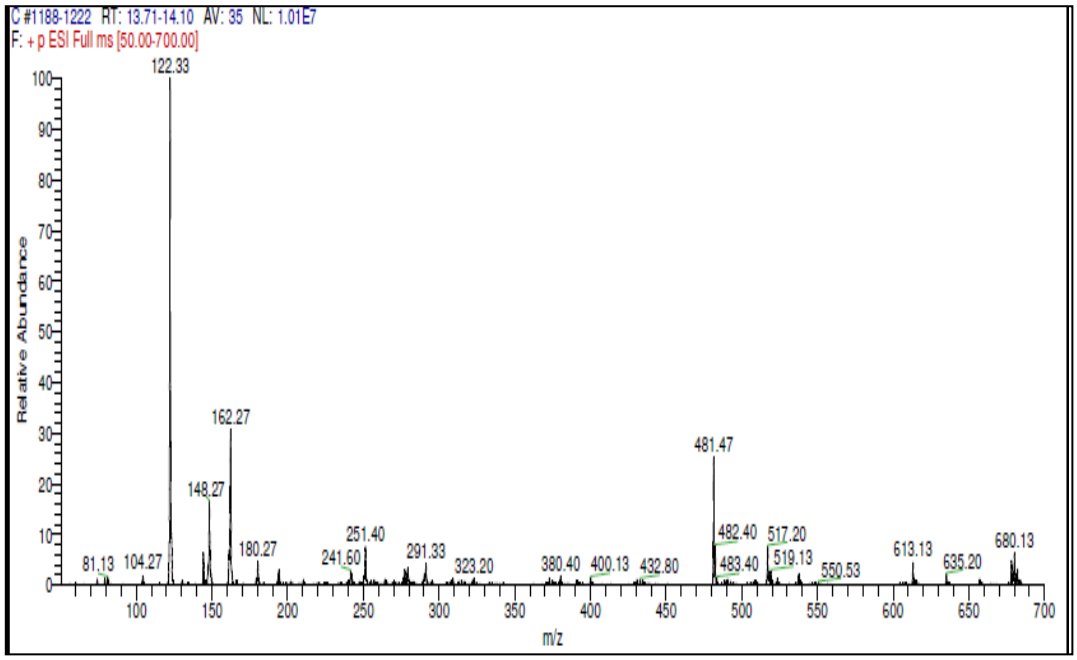


Fig. 4.51 (b) Chromatogram of LC-MS analysis for Cd (II) at various m/z ratios

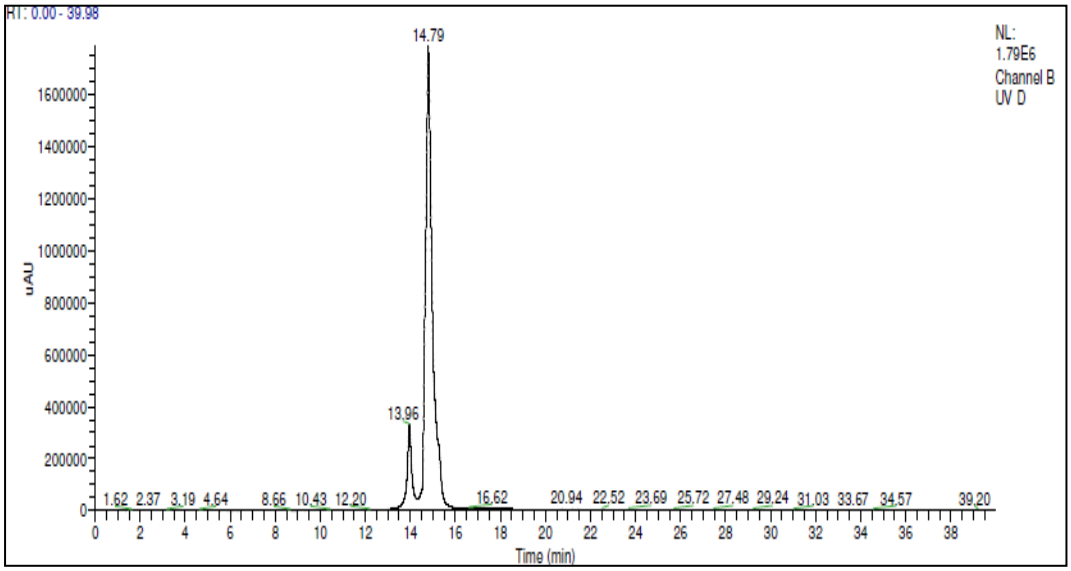


Fig. 4.52 (a) Chromatogram of LC-MS analysis for Cr (VI) at various retention time (min)

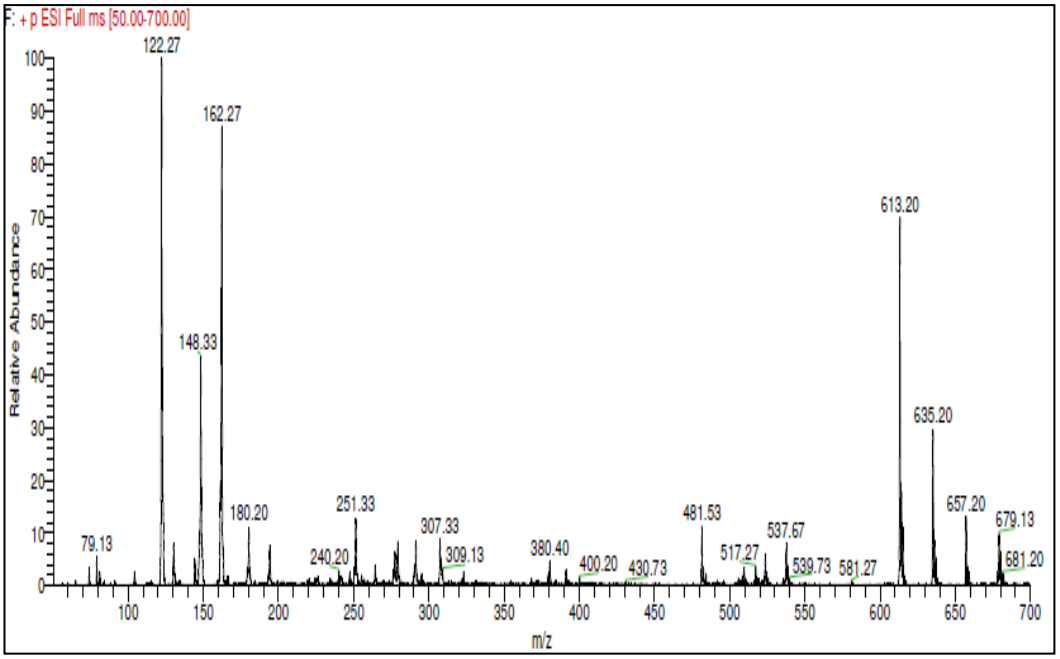


Fig. 4.52 (b) Chromatogram of LC-MS analysis for Cr (VI) at various m/z ratios

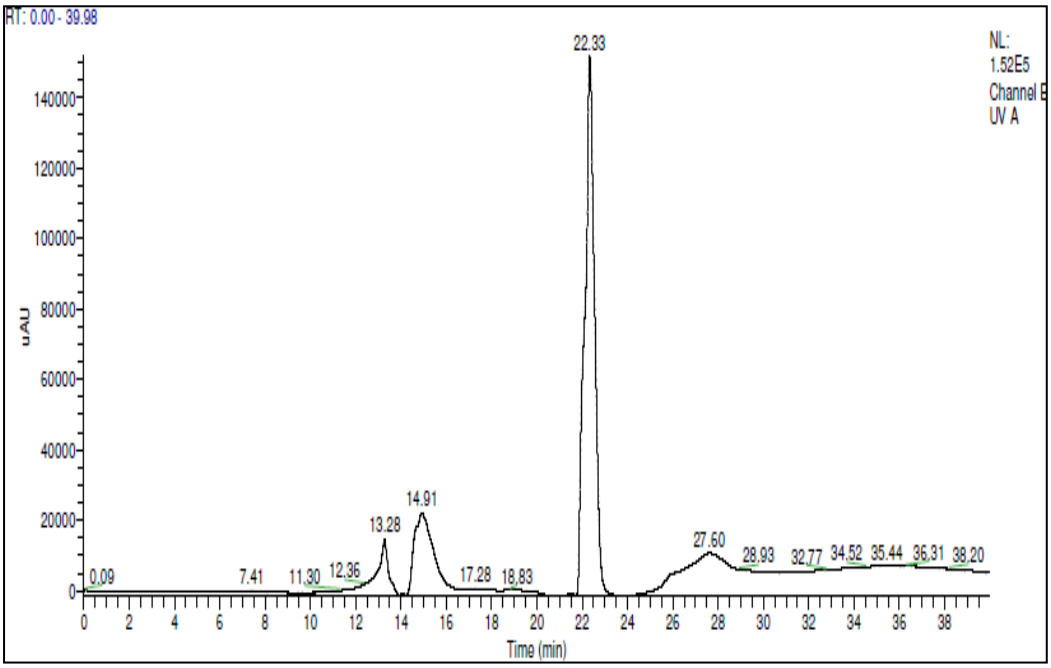


Fig. 4.53 (a) Chromatogram of LC-MS analysis for Cu (II) at various retentiontime (min)

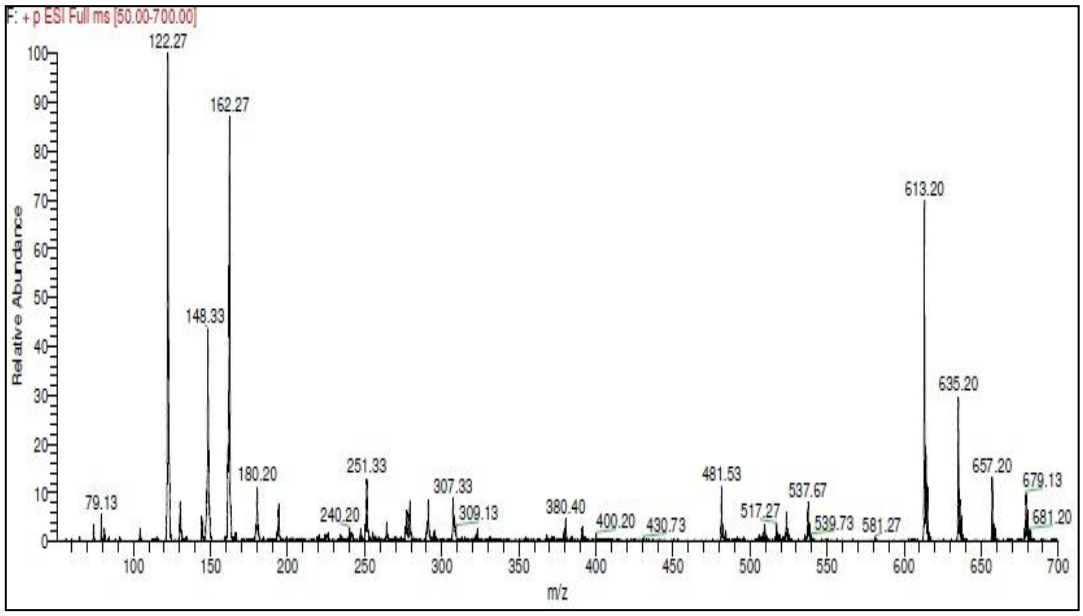


Fig. 4.53 (b) Chromatogram of LC-MS analysis for Cu(II) at various m/z ratios

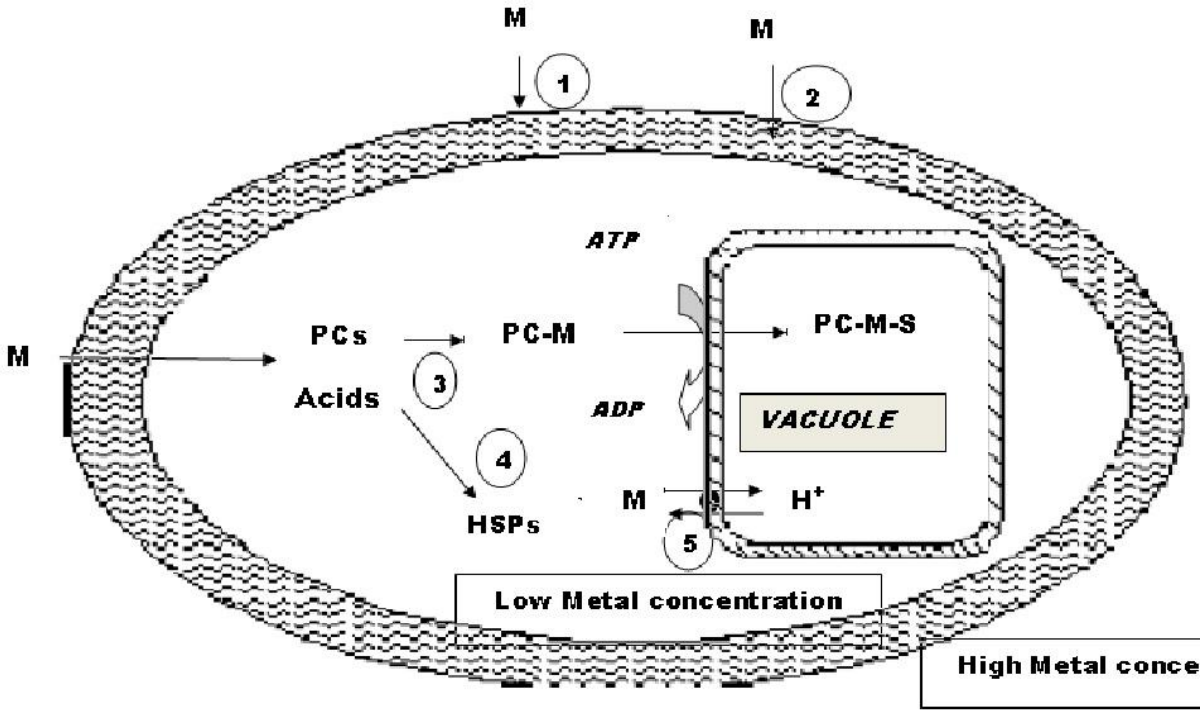


Fig. 4.54 Schematic representation of the proposed mechanism of metal uptake by *Galerina vittiformis.* (1) Metal adsorption on fungal mycelial surface which act as roots of fruiting bodies. (2) Up take and storing in periplasmic space passive absorbtion. (3) PC and acid production in response to metal stress. (4) Acids act as HSPs (heat shock proteins) bind to metal and store them to periplasmic space. (5) Transport and accumulation of metals in vacuole.

4.9 EFFECT OF CHELATING AGENTS ON HEAVY METAL BIOACCUMULATION EFFICIENCY OF *G. vittiformis*

Chelating agents play an important role in metal mobilization from soil environment. The effect of these agents can be studied by analyzing the metal content in the biomass after their addition in the metal laden environment. Metal uptake by fungi involves various processes like metal desorption from soil particles, transport of soluble metals to the stalk of the mushrooms through the mycelial surfaces *via* diffusion or mass flow and metal translocation from stalks to fruiting bodies where the metals get accumulated at higher concentration. Chelating agents are known to increase the bioavailability of heavy metals from soil (Yilmaz et al., 2003; Falandysz et al., 2007; Cayir et al., 2010; Chen, X., et al., 2009; Zhu et al., 2011).

Effect on metal bioaccumulation after the addition of organic chelating agents (Citric acid and Gallic acid) and chemical chelating agent (EDTA) into the soil were studied at 1 mmol/kg, 5 mmol/kg, 10 mmol/kg concentrations of these agents. The studies were performed at an initial metal ion concentration of 50 mg/kg and soil pH 6.5. Metal bioaccumulation in the presence of citric acid is shown in Fig. 4.55. The mushroom, *G. vittiformis* was found to have a little influence in the presence of both 1mmol/kg and 5 mmol/kg concentrations and a maximum of 10% increase was observed in the presence of 10 mmol/kg concentration. A maximum bioaccumulation of 789 mg/kg, 839 mg/kg, 28 mg/kg 889 mg/kg and 698 mg/kg for Cu(II), Cd(II), Cr(VI) Pb(II) and Zn(II) respectively were exhibited by the mushroom species when citric acid was added to the soil at concentration of 50 mg/kg of soil (Fig. 4.55).

In the case of chelating agent like gallic acid there was no influence in the presence of both 1mmol/kg and 5mmol/kg concentrations while at 10 mmol/kg concentration all the metal ions showed less than 6% increase in bioaccumulation efficiency. Fig 4.56 showed a maximum bioaccumulation of 789 mg/kg, 852 mg/kg, 40 mg/kg, 896 mg/ kg and 705 mg/ kg of Cu(II), Cd(II), Cr(VI), Pb(II) and Zn(II)

respectively are achieved with gallic acid at 10 mmol kg^{-1} concentration.

Most widely used chemical chelaters called EDTA is found to have only little influence in increasing *G. vittiformis* metal bioaccumulation efficiency. A maximum of 15% increase in bioaccumulation is observed in the presence of 5 and 10 mmol/kg concentrations of EDTA: 840 mg/kg for both Cu(II) and Cd(II), 915 mg/kg for Pb(II), 700 mg/kg for Zn(II) and 50 mg/kg for Cr(VI) as shown in Fig. 4.57. Fig.4.58 shows a comparative effect of all studied chelaters like citric acid, gallic acid and EDTA on heavy metal bioaccumulation at 10 mmol/kg concentrations and EDTA showed comparatively higher influence on bioaccumulation efficiency. Similar results were reported by various researchers like Gupta et al. (2001), Lombi et al. (2001), Kos and Lestan (2004) and Nascimento (2006), in their studies on the effect of chelating agents on bioaccumulation in bacteria and micorhizae.

From Fig. 4.58 it is also observed that the presence of studied chelating agents in the soil have no significant stimulatory effect on *G. vittiformis* bioaccumulation efficiency unlike reported for phytoremediation. Researchers like Blaylock et al. (2000), Machuca et al. (2001), Cao et al. (2007), Michael et al. (2007), Lai and Chen (2005), Mancini and Bruno (2011), Luo et al. (2006), Sun et al. (2005) and Jean et al. (2008) have reported the removal of heavy metals like Pb(II), Cu(II), Cd(II) and Zn(II) from contaminated soil in the presence of biodegradable chelaters like citric acid and gallic acid using fungi and plants. Researchers like Chen and Cutright et al. (2001), Wenger et al. (2005), Nascimento, (2006), Saifullah et al. (2009), Sun et al (2009), Sinhal et al. (2010), Zhao et al. (2010), Ullah et al. (2011), Zhu et al. (2011) and Luciano et al. (2012) have also reported the removal of Hg(II) and Li(II) from contaminated soil in the presence of various biodegradable chelaters using different kinds of fungi and plant species. The detailed recent literature on chemical chelaters and bio based chelaters are summarized in Table 4.15.

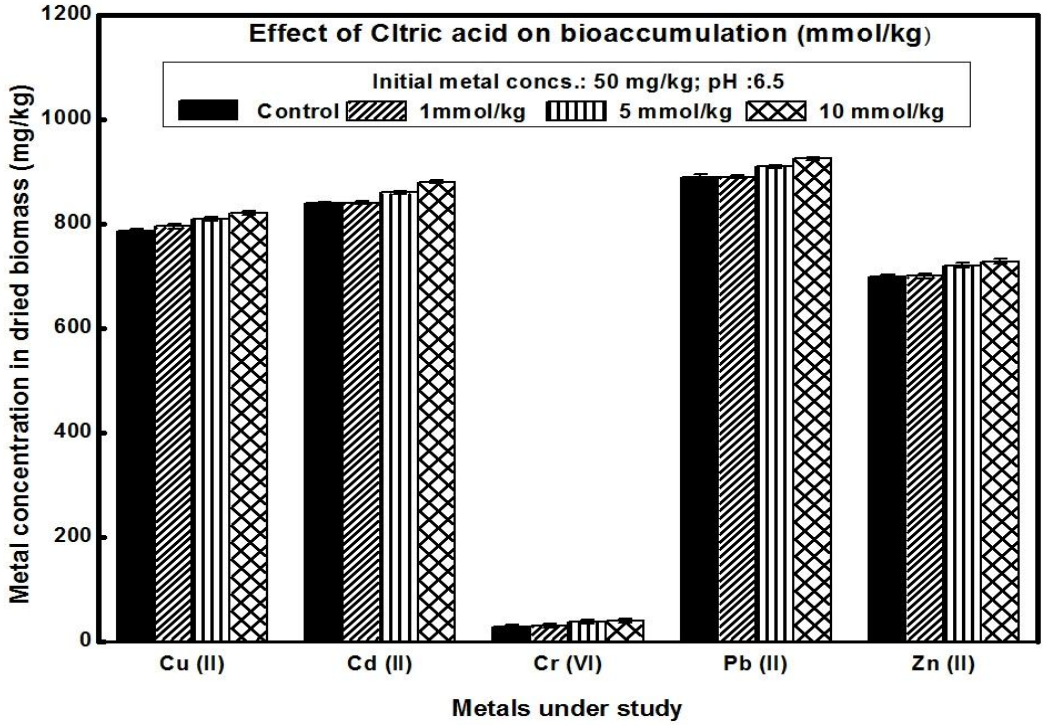


Fig.4.55 Effect of citric acid on *G.vittiformis* bioaccumulation

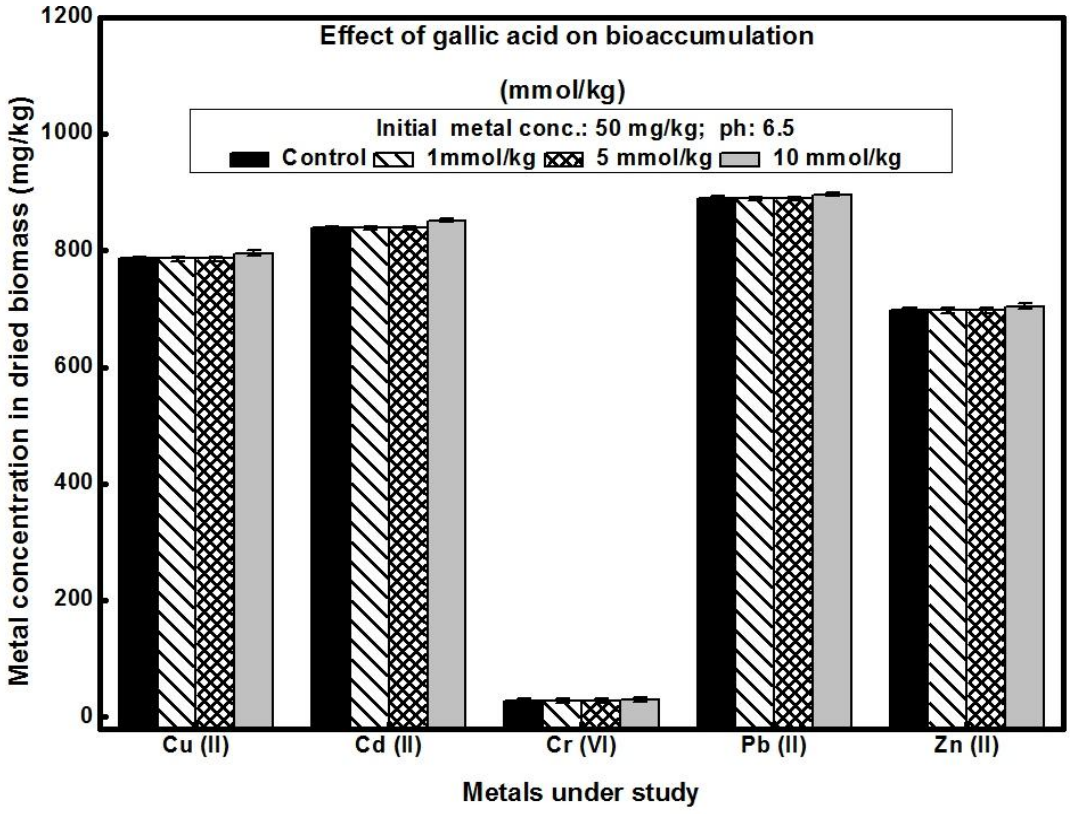


Fig.4.56 Effect of gallic acid on *G.vittiformis* bioaccumulation

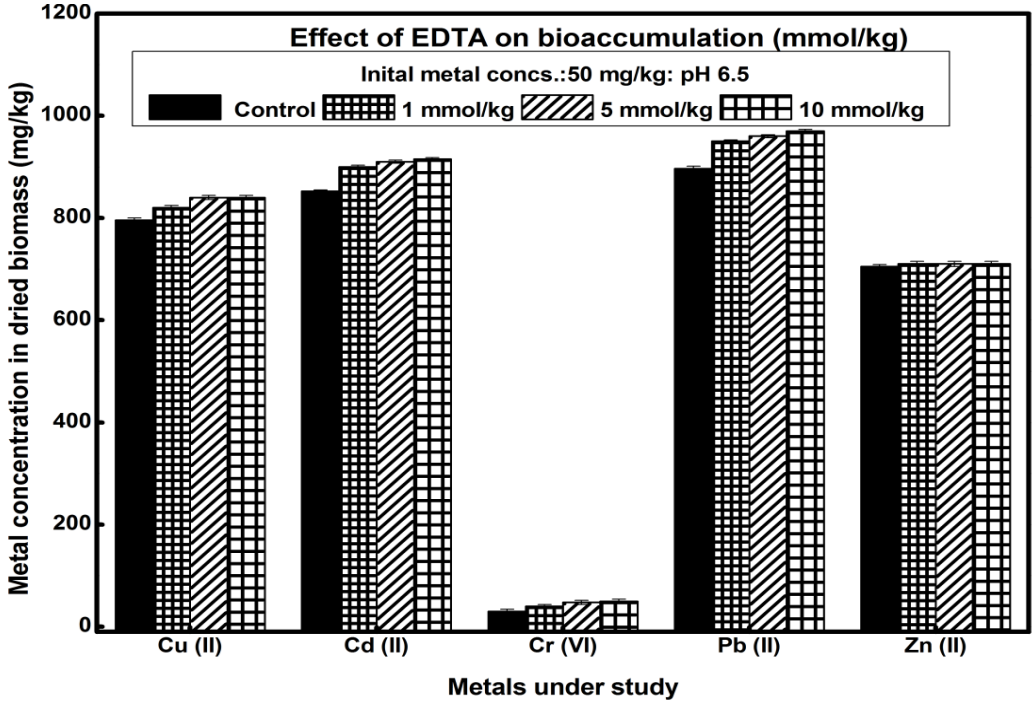


Fig. 4.57 Effect of EDTA on *G.vittiformis* bioaccumulation efficiency

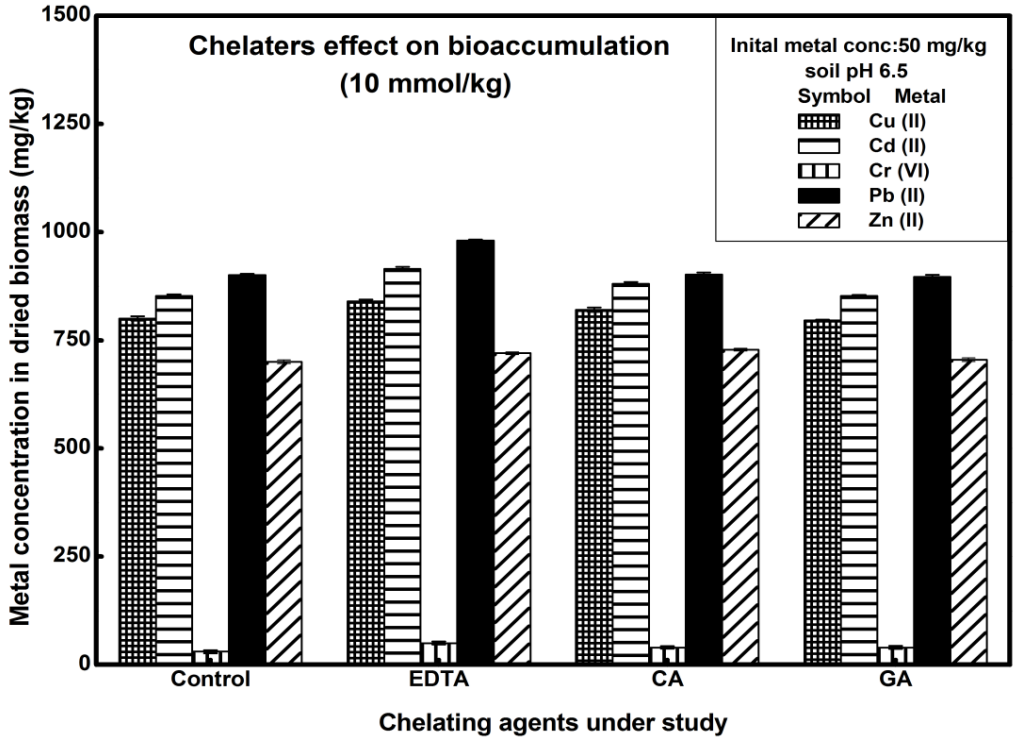


Fig. 4.58 Effect of chelaters on *G.vittiformis* bioaccumulation efficiency

Table 4.15 Chelaters affecting bioaccumulation, a literature overview.

Sl. No	Type of Chelaters used	Metals under study	References
1	**Biodegradable**		
	EDDS & MGDA	Pb(II) &Zn(II)	Cao et al. 2007
	Ethylene diamine disuccinate (EDDS)	Pb(II)	Mancini and Bruno 2011
			Zhao et al. 2010
	Citric acid, Gallic acid,	Cd(II), Pb(II), Zn(II), Cu(II) & Ni(II)	Nascimento 2006
	Vallic acid , Oxallic acid		
	Nitrilotriacetic acid		Mancini and Bruno 2011
	IDSA	Pb(II)	Zhao et al. 2010
	Citric acid	Zn(II), Cu(II), Pb(II) & Cd(II)	Sinha et al. 2010
		Cd(II), Cu(II), Pb(II) & Zn(II)	Sun et al 2009
2	**Non-Biodegradable**		
	EDTA	Pb(II) & Cr(VI)	Dipu et al. 2012, Evangelou et al. 2007
		Pb(II), Cd(II) & Ni(II)	Chen and Cutright, 2001
		Pb(II), Cu(II) & Cd(II)	Chigbo and Batty 2013,
		Hg(II)	Zhao et al. 2010, Wenger et al. 2005
		Pb(II), Cd(II) &Cr (VI)	B l a y l o c k et al. 1997

Use of chemical chelating agents like EDTA for metal removal process are not advantageous as these chemicals can bind to heavy metal ions in soil to form large sized complex. Once they form a complex its solubility increases resulting in various health problems as they can get into the food chain with much ease and can cause fatal metabolic disorders (Iranshahi et al. 2011). They also solubilise radioactive metals and increase their environmental mobility (Khan et al. 2000, Oviedo and Rodriguez 2003). The EDTA has low biodegradability hence the persistent nature of EDTA force to have other remediation techniques to avoid its toxicity (half-life of 36 years). Hence use of chelaters like EDTA is not preferred for large scale soil remediation process. Mycoremediation using *G.vittiformis* is efficient in comparison

to phytoremediation as the mushrooms exhibit higher metal bioaccumulation potential even in the absence of chelaters like EDTA.

4.10 EFFECT OF MULTI-METAL INTERACTION ON BIOACCUMULATION

In order to study the metal removal efficiency of *G.vittiformis* from multi-metal polluted environment, the mushroom was grown in multi-metal polluted soil. From the literature it is clear that the recent studies on bioaccumulation has been majorly focused on single metal system than multi- metal system, even though metal contaminated soil area often contain several metal ions dominantly (Kratochvil and Voleskey 1998; Manahan 2000; Hawari and Mulligam 2007; Kumar et al. 2008; Agarry and Oguleye 2012). When more than one metal is present, the study becomes more complicated as, the interaction of one metal accumulation in the presence of other metal ions may be synergetic, antagonistic or non-reactive. The traditional one-factor at a time experiments cannot successfully predict possible interaction between the metal ions in soil system. Thus there is a need to adopt techniques which can be used to study the interaction between the metals in multi-metal systems, which affects the removal of targeted metals. Previously different experimental design methodologies like factorial design, mixture designs (Cao et al. 2010; Agarry and Oguleye 2012) and Central composite designs were used to interpret the removal of metals from multi-metal systems (Remenarova et al. 2001). Design of experiments with RSM was also used to reduce the no of experiments in multi-metal interaction studies, instead of optimization of the bioaccumulation process (Lu et al. 2008). Cao et al. (2010) designed experiments based on Design of Experiments (DOE) to reduce the number of experiments to study metal biosorption by micro fungi in multi-metal system and used response surface methodology (RSM) to interpret the removal of three metals from multi-metal systems. The present study reports the effect of interaction of the metals in multi-metal soil system containing five metals *viz*. Pb(II), Cd(II), Cr(VI), Zn(II) and Cu(II) on removal of each of the metals through bioaccumulation by *Gallerina* sp. The experiments were conducted in a tray with soil

contaminated with the metal ions. As five metals are involved, the experiments were designed based on Design of experiments (DOE) strategy to reduce the number of experiments. Interaction effects were studied using Response surface methodology.

4.10.1 EXPERIMENTAL DESIGN AND DATA ANALYSIS

Central Composite Design (CCD) was adopted to design the experiments with the heavy metal concentration ranging from 10 to 250 mg/kg and pH 5 to 8. Heavy metal removal studies were conducted in a tray-soil system by conducting 90 sets of experiments designed as per CCD. Concentration of Pb(II), Cd(II), Cr(VI), Zn(II) and Cu(II) in the soil and pH are the factors and removal percentages of each of the metals are the responses. CCD was designed with six factors and 5 levels. The set of 90 experimental conditions designed as per CCD and the corresponding responses are presented in Table 4.16.

The results were analyzed by using response surface plots generated with MINITAB 14 software. The surface plots indicating the effect of various factors on removal percentage of the metals: Pb(II), Cd(II), Cr(VI), Zn(II) and Cu(II) are illustrated in Fig. 4.37 to 4.41, respectively. Each surface plot represents combinations of the test parameters under study, where percentage of removal of metal is correlated to a range of any two factors (metal/metal or metal/pH) along with other parameters at fixed middle/central values of CCD.

Table 4.16 List of experimental matrices and results according to response surface methodology

Design matrix							Responses; Experimental (% metal removal)				
Run Order	Metal ions under study					pH	%Cu	%Cd	%Cr	%Pb	%Zn
	Cu(II) mg/kg	Cd II) mg/kg	Cr(VI) mg/kg	Pb(II) mg/kg	Zn(II) mg/kg						
1	70	190	70	190	190	5.75	69.4	24.4	72	48.9	31.6
2	130	130	130	130	130	6.50	78.0	91.2	7.5	95.0	93.0
3	190	70	190	70	70	7.25	12.1	25.4	2.9	61.4	48.5
4	190	70	190	190	70	7.25	12.21	25.4	2.4	20.8	48.0
5	130	130	130	130	130	6.50	78.46	91.2	7.5	95.0	93.0
6	190	190	190	190	70	5.75	22.1	23.8	4.4	37.4	52.8
7	130	130	130	130	130	6.50	78.46	91.2	7.5	95.0	93.0
8	190	190	70	190	70	7.25	12.3	13.3	6.5	33.1	60.0
9	190	190	70	70	190	5.75	22.6	23.9	12.2	80.2	33.5
10	70	70	190	70	70	7.25	53.14	35.7	2.4	72.5	60.0
11	70	70	70	190	190	5.75	82.8	69.5	12	54.1	51.5
12	130	130	130	130	10	6.50	78.46	91.2	7.5	95.0	92.0
13	190	70	70	70	190	7.25	26.94	44.6	6.5	72.5	22.1
14	190	70	70	190	190	7.25	12.1	25.4	6.5	33.1	20.0
15	70	190	70	70	190	7.25	56.5	25.4	6.5	45.7	19.5
16	190	70	190	190	190	5.75	25.2	80.0	19.4	24.1	47.3
17	70	70	70	70	70	5.75	80.0	74.6	14.0	85.1	68.6
18	130	130	130	130	130	6.50	78.0	91.2	7.5	95.0	93.0
19	70	190	190	190	190	5.75	51.4	23.4	4.4	37.7	33.2
20	190	70	190	70	190	5.75	25.0	80.0	4.4	73.2	46.1
21	190	70	190	70	70	5.75	32.6	74.5	5.1	83.7	68.6
22	190	190	190	70	190	7.25	10.0	9.05	2.9	27.4	11.7
23	250	130	130	130	130	6.50	58.2	86.0	7.5	84.0	81.5
24	70	70	190	190	70	5.75	62.2	39.3	4.4	61.1	63.7
25	70	70	70	70	70	7.25	43.7	56.0	56.0	23.7	76.5
26	70	70	70	190	190	7.25	55.1	56.5	25.1	33.1	19.5
27	70	190	190	190	70	5.75	51.4	23.4	4.0	36.9	80.0
28	70	190	70	70	190	5.75	74.2	41.5	10.8	60.5	62.3
29	190	70	70	70	70	5.75	32.6	74.5	14.0	85.1	68.5
30	190	190	70	70	70	5.75	27.1	40.5	10.8	71.7	80.0
31	70	190	190	70	190	5.75	69.4	24.4	4.8	61.4	41.3
32	190	190	190	70	190	5.75	37.6	40.1	4.4	73.7	53.8
33	190	70	70	70	70	7.25	28.2	25.4	6.5	72.2	60.0
34	70	190	190	70	70	5.75	74.2	41.1	4.0	72.2	74.0
35	130	130	250	130	130	6.50	63.3	65.0	5.2	80.1	79.0
36	10	130	130	130	130	6.50	76.0	76.0	7.5	78.4	76.1
37	130	130	130	130	130	6.50	78.5	91.2	7.5	95.0	93.0
38	70	190	70	70	70	7.25	53.1	13.3	6.5	72.2	60.0
39	70	70	190	70	190	7.25	52.2	25.1	2.9	64.0	20.0
40	190	70	70	190	70	7.25	12.1	25.4	6.5	24.6	56.5

41	70	190	70	190	70	5.75	82.2	40.6	12	36.9	81.7
42	70	190	70	190	190	7.25	35.5	13.0	6.5	22.9	20.6
43	70	190	190	70	70	7.25	52.2	22.1	2.9	75.4	60.0
44	190	190	70	190	190	5.75	37.6	40.1	9.1	48.9	31.6
45	190	70	190	190	70	5.75	32.6	74.5	5.1	62.9	68.5
46	130	130	130	130	130	6.50	78.6	91.2	7.5	95.0	93.0
47	70	70	190	190	190	7.25	35.4	44.5	2.3	22.6	17.6
48	130	10	130	130	130	6.50	78.4	64.0	7.5	84.6	84.6
49	130	130	10	130	130	6.50	78.4	81.5	76	91.6	85.2
50	70	190	190	70	190	7.25	32.8	13.3	2.4	89.1	20.0
51	190	190	190	190	70	7.25	32.8	13.3	2.4	89.1	54.2
52	130	130	130	250	130	6.50	74.7	86.7	7.5	57.2	90.1
53	70	70	190	70	190	5.75	62.2	73.1	4.6	73.7	34.5
54	190	190	190	70	70	5.75	22.8	23.8	4.5	71.7	80.0
55	70	70	70	190	70	5.75	74.2	73.1	12.0	45.7	62.8
56	70	190	70	190	70	7.25	23.7	22.1	6.5	33.0	28.0
57	190	70	70	190	70	5.75	32.6	74.5	14.0	45.7	68.5
58	130	130	130	130	130	5.00	15.5	15	1.5	53.8	18.6
59	190	190	70	70	190	7.25	12.3	13.3	6.5	60.0	16.8
60	190	70	70	190	190	5.75	28.2	61.4	9.7	61.2	56.6
61	190	190	190	190	190	7.25	12.3	13.3	2.9	33.1	20.0
62	190	70	190	190	190	7.25	12.3	52	2.9	28.21	20.0
63	130	250	130	130	130	6.50	70.6	49.5	7.3	82.3	79.6
64	70	70	190	190	190	5.75	62.2	73.1	4.6	48.0	44.7
65	70	190	70	70	70	5.75	74.2	41.5	10.8	60.0	82.8
66	70	190	190	190	190	7.25	53.1	13.12	2.9	27.8	20.0
67	70	70	190	190	70	7.25	55.1	25.1	2.9	33.1	56.5
68	190	70	190	70	190	7.25	12.1	25.4	2.9	32.85	20.0
69	130	130	130	130	130	6.5	78.4	91.2	7.5	95.0	93.0
70	130	130	130	130	130	6.5	78.4	91.2	7.5	95.0	93.0
71	190	190	70	190	70	5.75	53.6	62.3	14.0	68.4	65.7
72	190	190	190	190	190	5.75	22.9	23.9	4.5	58.9	18.9
73	70	70	190	70	70	5.75	71.4	66.3	2.6	73.1	64.2
74	70	190	190	190	70	7.25	52.0	22.1	2.9	16.1	28.0
75	130	130	130	130	130	6.50	78.4	91.2	7.5	95.0	93.0
76	130	130	130	130	130	6.50	78.4	91.2	7.5	95.0	93.0
77	130	130	130	130	130	6.50	78.4	91.2	7.5	95.0	93.0
78	130	130	130	10	130	6.50	78.0	91.0	6.3	76.0	81.5
79	130	130	130	130	250	6.50	89.0	78.7	4.5	89.3	46.4
80	130	130	130	130	130	6.50	78.46	91.2	7.5	95.0	93.0
81	130	130	130	130	130	8.00	13.9	15.1	4.2	32.1	65.8
82	70	70	70	70	190	5.75	54.4	56.6	7.8	59.9	45.0
83	190	70	70	70	190	5.75	29.4	61.4	9.7	65.1	55.1
84	190	190	190	70	70	7.25	12.31	13.6	2.5	61.4	60
85	190	190	70	190	190	7.25	12.31	13.6	6.5	33.5	17.6
86	130	130	130	130	130	6.50	78.4	91.2	7.5	95..0	93.0
87	70	70	70	190	70	7.25	26.5	25.1	6.5	33.1	51.4
88	70	70	70	70	190	7.25	26.5	25.1	6.5	61.0	58.4
89	130	130	130	130	130	6.50	78.46	91.2	7.5	95.0	93.0
90	190	190	70	70	70	7.25	12.3	13.3	6.5	52	44.5

4.10.2 EFFECT OF SOIL pH AND OTHER HEAVY METALS ION CONCENTRATION ON METAL REMOVAL

The heavy metal removal efficiency of *G. vittiformis* from soil is mainly governed by certain soil conditions like pH, metal concentration, type of metal etc. (Srivastava et al. 2006). The effect of pH and concentration of other metals as individual factors and their interaction effects on the removal of target metals was studied using the response surface plots generated using MINITAB 14 software.

4.10.2.1 EFFECT OF SOIL pH AND MULTI METAL INTERACTION ON Pb(II) REMOVAL

Fig.4.59 depicts the three-dimensional plot showing the effect of different metal concentrations (Cd(II), Cr(VI), Cu(II), Zn(II) and Pb(II)) and soil pH on percentage removal of Pb(II). It is clear from Fig. 4.59 (h), (i), (k), (o) and (n) that, in Pb(II) removal the soil pH plays an important role. Percentage removal of Pb(II) is found to increase with the increase in pH in the acidic range and a maximum removal of 80% from the soil occurs at a concentration of 200 mg/kg at pH 6.5 in the presence of other metals at a hold value of 150 mg/kg soil. As the soil pH increases in the alkaline range from 6.6 to 8, the removal efficiency was found to decrease drastically for all the studied metal concentrations. Fig.4.59 (d) exhibits a saddle shaped surface plot for Pb(II) removal, which increased initially with the increase in concentrations of Cd(II) and Zn(II) whereas, beyond 150 mg/kg the removal percentage decreased from 93% to nearly 75%. Similar interaction patterns are observed in the case of Pb(II) removal for various combinations of metals like, Cu(II): Cd(II) (Fig.4.59(a)); Cr(VI): Cd(II) (Fig.4.59 (b)), Cd(II): Pb(II) (Fig.4.59 c), Pb(II): Zn (II) (Fig.4.59 (e)), Zn(II): Cu(II) (Fig.4.59 (f)); Cr(VI): Zn (II) (Fig.4.59 (g)), Cu(II):Cr(VI) (Fig.4.59 (j),Cr(VI): Pb(II) (Fig.4.59(i)) and Cu(II): Pb(II) (Fig.4.59(m)). The trend of increase in the percentage of Pb(II) removal with increase in concentration of other metals have been found upto certain concentrations and followed by a decrease with further

increase in concentration for all the metal ions under study. The maximum Pb(II) removal has been found for certain concentration of other metals under study. Hence, it can be concluded that these metal ions interact with each other in favor of Pb(II) removal.

At lower concentrations of metals, the cells may not reach their maximum bioaccumulation capacity, *i.e* the vacuoles may still have storage capacity to store the metals in chelaters bound form (Foulkes, 2000). Hence, at lower metal concentrations, percentage removal of these metals increase with the increase in metal concentrations. Whereas at higher metal concentrations, the total metals present in the environment might exceed the bioaccumulation capacity of the species. Thus, the metals present in the environmental may not be wholly transported into the cell and hence residual metal concentration in the environment remains at higher levels. In some cases higher metal removal from the soil might also increase the toxicity in mushrooms and it might become lethal to them or to certain extent the mushrooms might respond to suppress the toxicity by neutralization of metal ion ions through membrane charges (Foulkes, 2000). The input-output data from the experiments were fitted into a multiple regression model (MRA model) involving the individual effects, square effects and interaction effects, to predict the percentage removal of Pb(II) with the concentration of five metals under study and the soil pH using MINITAB-14 software. The MRA model is presented as Eq. (4.6) with the estimated parameters (coefficients) of the model in Table 4.17. The significance of the model parameters were tested by Analysis of Variance (ANOVA) using MINITAB-14. Table 4.17 presents the results of ANOVA and the coefficient of determination (R^2) .The P value for regression being less than 0.05, shows that the model is significant. The P value for linear terms and square terms are less than 0.05 but that for interaction term is greater than 0.05. Thus the linear and square terms in the model are significant, but interaction terms are less significant. The value of R^2 being 0.8121 shows that the model is reasonably good to predict the Pb(II) removal.

Table 4.17 MRA model and ANOVA results for Pb(II) removal

Metal ion under study	**Pb(II)**
P value	Regression: 0.00 Linear : 0.00 Square: 0.00 Interaction: 0.659
F value	7.26
R^2 value	0.8121
MRA model	Pb(II) removal = -1081.37+ 0.668807C_{Cu} - 0.105847 C_{Cd} + 0.325476 C_{Cr} + 0.523055C_{Pb} + 0.355528 C_{Zn}+ 340.802pH - 0.00159449 $C_{Cu*}C_{Cu}$ - 0.00144033$C_{Cd*}C_{Cd}$ - 0.00126880$C_{Cr*}C_{Cr}$ - 0.00261046 C_{Pb}*C_{Pb} - 8.36158E-04 $C_{Zn*}C_{Zn}$ - 27.2092 pH*pH+ 0.000435938 $C_{Cu*}C_{Cd}$ - 6.77517E-04 $C_{Cu*}C_{Cr}$ + 0.000508941 $C_{Cu*}C_{Pb}$ - 5.02951E-04 $C_{Cu*}C_{Zn}$ -0.0302153 C_{Cu}*pH +0.000268056 $C_{Cd*}C_{Cr}$ +0.000239931 $C_{Cd*}C_{Pb}$ - 3.03819E-06 $C_{Cd*}C_{Zn}$ + 0.0551528 C_{Cd}*pH - 2.51476E-04 $C_{Cr*}C_{Pb}$- 6.53819E-04 $C_{Cr*}C_{Zn}$+ 0.0269097 C_{Cr}*pH+ 0.000133160 $C_{Pb*}C_{Zn}$ -0.0184931C_{Pb}*pH -0.00666667C_{Zn}*pH ……..Eq. 4.6

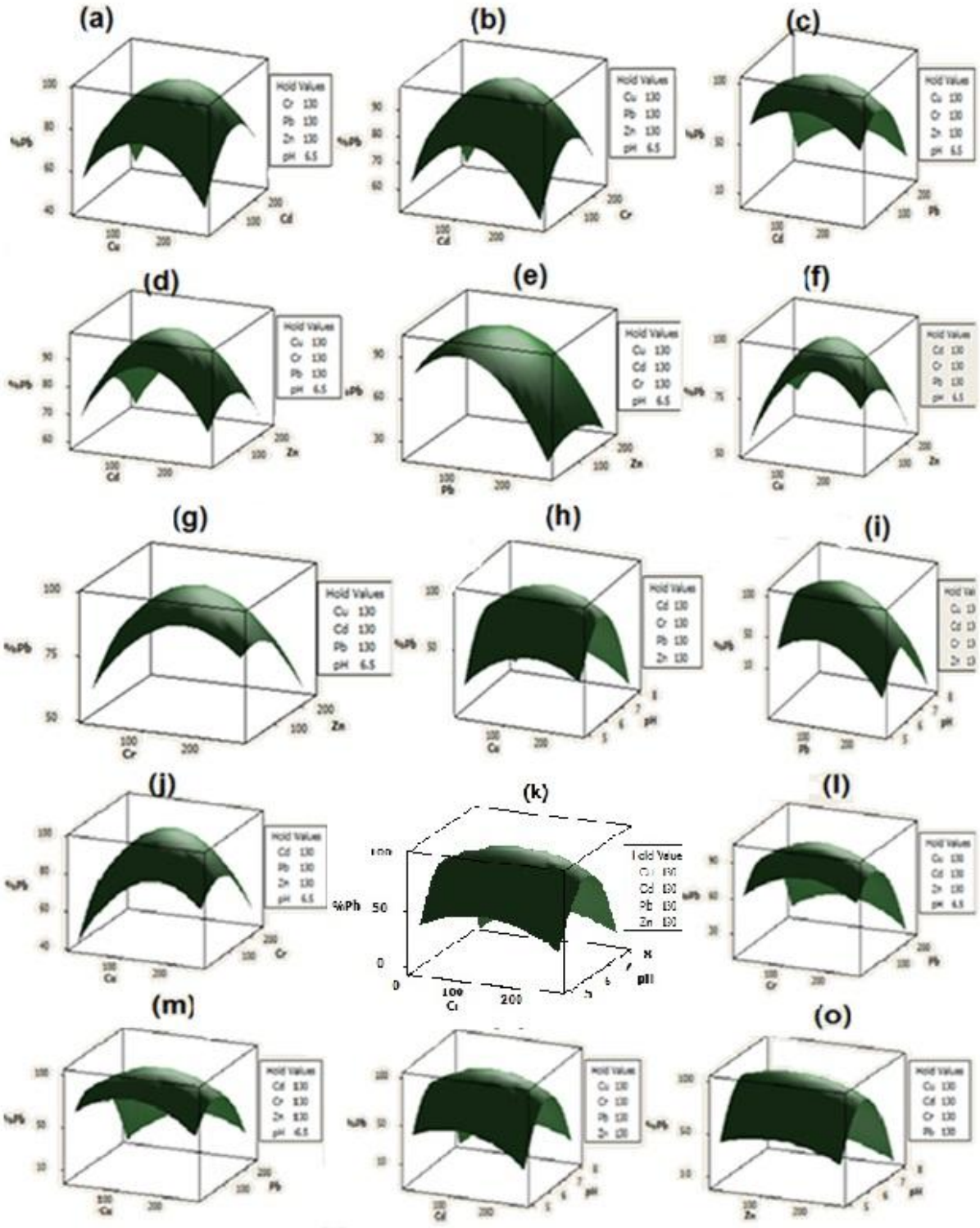


Fig.4.59 3D Surface plots showing the effect of studied factors on Pb(II)

4.10.2.2 EFFECT OF SOIL pH AND MULTI METAL INTERACTION ON Cd(II) REMOVAL

From Fig. 4.60(a to o), it is observed that, for lower pH up to 6.5, the percentage removal is found to increase for Cd(II) and as the pH increased above a pH value of 6.5, percentage removal decreased. The maximum removal percentage of Cd(II) was found at pH 6.5 and with the other interacting metal concentration range of 100 to 200mg/kg. Maximum removal percentage of Cd(II) was found at pH 6.5 and Zn(II) concentration between 100 to 200mg/kg (Fig. 4.60(j)) in the presence of other metals at middle level concentrations. Similar trend was observed with combinations such as Pb(II): pH (Fig. 4.60 (n)), Cu(II): pH (Fig. 4.60(i)), Cr(VI): pH (Fig. 4.60(k)), Cd(II): pH (Fig. 4.60(m)) and Zn(II): pH (Fig. 4.60(o). The maximum percentage removal occurred at pH of around 6.5 irrespective of the other metal concentrations, but the maximum percentage removal of Cd(II) depends on the concentration of other metals present, indicating the significance of soil pH and its interaction with the concentration of different metals on removal of metals from multi-metal contaminated soil.

The effect of Cd(II) and Zn(II) on Cd(II) removal at fixed concentrations of Pb(II), Cr(VI) and Cu(II) is shown in Fig. 4.60 (d). It is evident that maximum Cd(II) removal i.e 70% is obtained at lower Zn(II) concentrations with Cd (II) in the concentration between 100 to 200 mg/kg, above which Cd(II) removal percentage drops. The maximum percentage removal of Cd(II) increased with the increase in Zn(II) concentration up to around 150 mg/kg but dropped with further increase in concentration above this value, at any Cd(II) concentration and in the presence of other metals and pH at middle levels. It may be because of the lethality caused by higher metal concentration in the soil mixture. The trend observed is similar to that for Pb(II) removal and the reasons for the same are discussed in detail in Section 4.11.2.1. Similar trends are observed for the metal combinations like Cu(II): Cd(II) (Fig. 4.60(a), Cd(II): Cr(VI) (Fig 4.60(b)), Cd(II): Pb(II) (Fig. 4.60(c)), Cu(II): Cr(VI) (Fig.4.60(e)), Cr(VI): Pb(II) (Fig. 4.60(f)), Cu(II): Pb(II) (Fig. 4.60(g)), Cu(II): Zn(II)

(Fig 4.60(h)), Cr(VI): Zn(II) (Fig. 4.60(i)) and Pb(II): Zn(II) (Fig. 4.60(j)). The maximum percentage removal obtained at a fixed concentration of one metal varied with the concentration of second metal and vice versa, indicating significant interaction of the above mentioned metals on Cd(II) removal from multi-metal contaminated soil. Stunted growth of mushroom fruiting bodies are observed for all metal concentrations above 200mg/kg in tray studies for multi-metal interaction and it may be due to the increase in metal toxicity.

The input-output data from the experiments are fitted into a multiple regression model (MRA model) involving the individual effects, square effects and interaction effects, to predict the percentage removal of Cd(II) with the concentration of five metals under study and the soil pH using MINITAB-14 software. The MRA model is presented as Eq.4.7 with the estimated parameters (coefficients) of the model in Table 4.18. The significance of the model parameters are tested by Analysis of Variance (ANOVA) using MINITAB-14. Table 4.18 presents the results of ANOVA and the coefficient of determination (R^2). The P value for linear terms and square terms are less than 0.05 but that for interaction term is greater than 0.05. Thus the linear and square terms in the model are significant, but interaction terms are less significant. The value of R^2 being 0.8984 shows that the model is reasonably good to predict the Cd(II) removal.

Table 4.18 MRA model and ANOVA results for Cd(II) removal

Metal ion under study	**Cd(II)**
P value	Regression: 0.00 Linear : 0.00 Square: 0.00 Interaction: 0.111
F value	20.28
R^2 value	0.8984
MRA model	Cd (II) removal = -1481.80 - 0.679076 C_{Cu} +0.169691 C_{Cd} + 0.350180 C_{Cr} + 0.0656846 C_{Pb} +0.0404655 C_{Zn} + 479.597 pH - 0.00153340 $C_{Cu*}C_{Cu}$ - 0.00321743 $C_{Cd*}C_{Cd}$ - 0.00206813 $C_{Cr*}C_{Cr}$ - 9.88265E-04 $C_{Pb*}C_{Pb}$ - 0.00125910 $C_{Zn*}C_{Zn}$ - 39.1271 pH*pH - 1.53516E-04 $C_{Cu*}C_{Cd}$+ 0.000337891 $C_{Cu*}C_{Cr}$ + 0.000348741 $C_{Cu*}C_{Pb}$+ 9.91753E-05 $C_{Cu*}C_{Zn}$- 0.0555104 C_{Cu*}pH - 3.85026E-04 $C_{Cd*}C_{Cr}$ - 6.63628E-05 $C_{Cd*}C_{Pb}$ - 5.75477E-04 $C_{Cd*}C_{Zn}$ +0.0944340 C_{Cd*}pH - 1.37630E-04 $C_{Cr*}C_{Pb}$ + 0.000628602 $C_{Cr*}C_{Zn}$ + 0.0149826 C_{Cr*}pH + 0.000443793 $C_{Pb*}C_{Zn}$ + 0.0168785 C_{Pb*}pH + 0.0307604 C_{Zn*}pH ……Eq. 4.7

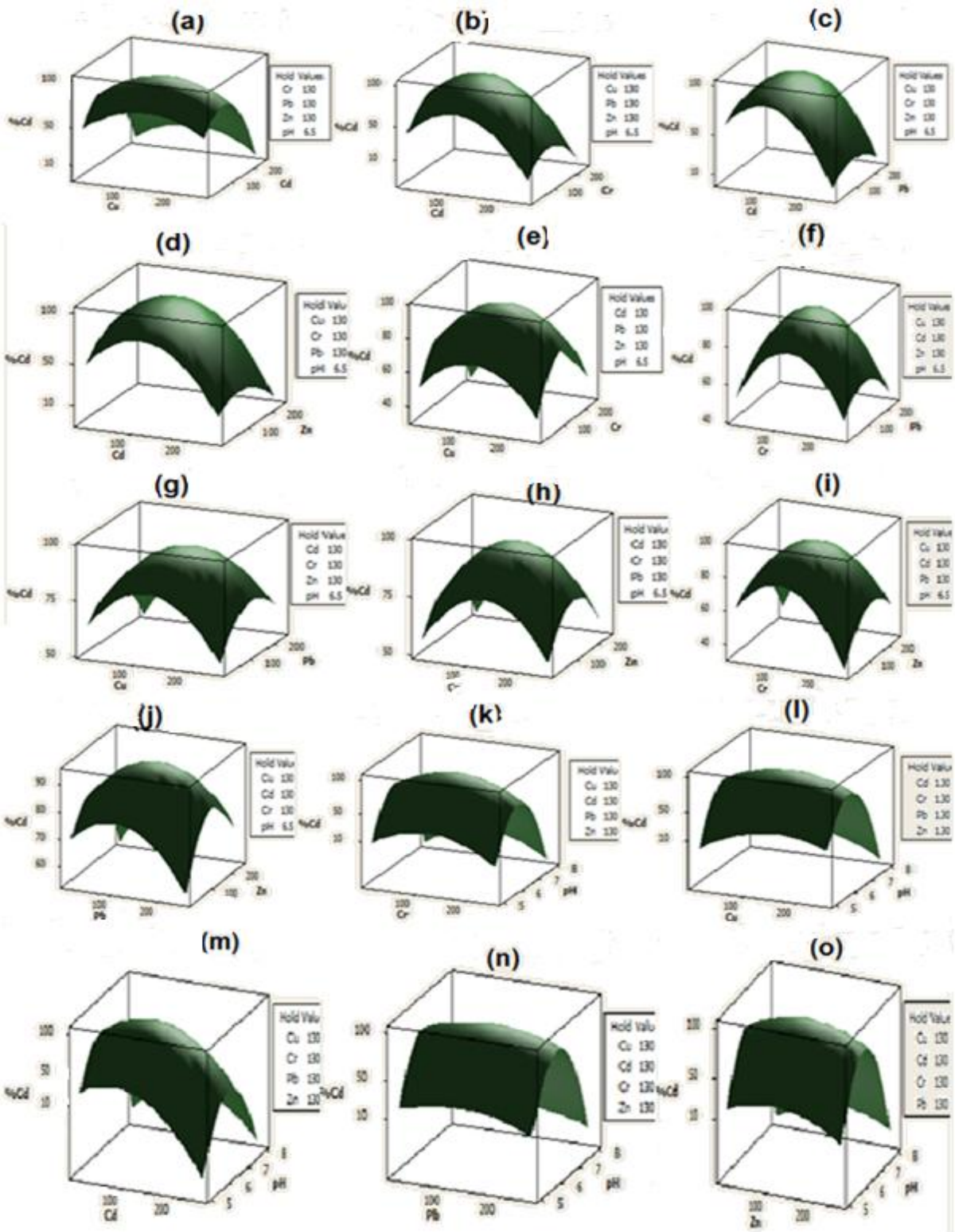


Fig. 4.60 The 3D Surface plot showing the effect of studied factors on Cd(II) removal

4.10.2.3 EFFECT OF SOIL pH AND MULTI METAL INTERACTION ON Zn(II) REMOVAL

From Fig. 4.61(i, k, m, n and o) it was observed that up to pH 6.5, the Zn(II) removal percentage increases. But as the pH increased above 6.5, percentage removal dropped. The maximum removal percentage of Zn(II) was found at pH 6.5 (Fig. 4.62(o)) with the other interacting metal concentration range between 100 to 200 mg/kg of soil containing all the metals under study at middle level. Similarly for all the combinations as Pb(II): pH (Fig.4.61 (n)), Cu(II): pH(Fig.4.61 (m)), Cr(VI): pH (Fig.4.61 (i)) and Cd(II):pH (Fig.4.61 (k)), the maximum percentage removal occurred at pH of around 6.5 irrespective of the other metal concentrations, but the maximum percentage removal of Zn(II) depended on the concentration of other metals present, indicating the significance of soil pH and its interaction with the concentration of different metals on removal of Zn(II) from multi-metal contaminated soil.

The effect of Cd(II) and Zn(II) on Zn(II) removal at fixed concentrations of Pb(II), Cr(VI) and Cu(II) is shown in Fig.4.61 (d). The maximum Zn(II) removal was obtained with Cd(II) in the concentration between 100 to 200 mg/kg, above which Cd(II) removal percentage dropped. The maximum percentage removal of Zn(II) increased with the increase in Zn(II) concentration up to around 150 mg/kg but dropped with further increase in concentration above this value, at any Cd(II) concentration and in the presence of other metals and pH at middle levels. It may be because of the lethality caused by higher metal concentration in the soil mixture. The trend observed is similar to that for Pb(II) and Cd(II) removal and the reasons for the same are discussed in detail in Section 4.12.2.1. Similar trends are observed for the metal combinations like Cu(II): Zn(II) (Fig. 4.61(d)), Cu(II): Pb(II), (Fig. 4.61(c)), Cu(II): Cr(VI) (Fig. 4.61(e)), Cr(VI): Zn(II) (Fig. 4.61(j)), Cr(VI): Pb(II), (Fig. 4.61(f)), Cd(II): Pb(II) (Fig. 4.61(c)), Cd(II): Cr(VI) (Fig. 4.61(b)) and Pb(II): Zn(II) (Fig. 4.61(h)). The maximum percentage removal obtained at a fixed concentration of one metal varied with the concentration of the second metal,

indicating synergetic effect of the metals and significant interaction of the above mentioned metals on Zn(II) removal from multi-metal contaminated soil. Stunted growth of mushroom fruiting bodies were observed for all metal concentrations above 200 mg/kg in tray studies for multi-metal interaction and it may be due to the increase in metal toxicity. The input-output data from the experiments were fitted into a multiple regressi-on model (MRA model) involving the individual effects, square effects and interaction effects, to predict the percentage removal of Zn(II) with the concentration of five metals under study and the soil pH using MINITAB-14 Software. The MRA model is presented as Eq.4.8 with the estimated parameters of the model. The significance of the model parameters were tested by Analysis of Variance (ANOVA) using MINITAB. Table 4.19 presents the results of ANOVA and the coefficient of determination (R^2). The P value for regression, linear and square term being less than 0.05 indicating significance of the model. Mean while the interaction terms are less significant. The value of R^2 being 0.8430 shows that the model is reasonably good to predict the Zn(II) removal.

Table 4.19 MRA model and ANOVA table for Zn(II) removal

Metal ion under study	**Zn(II)**
P value	Regression: 0.00 Linear : 0.00 Square: 0.00 Interaction: 0.10
F value	15.63
R^2 value	0.8430
MRA model	Zn(II) removal = -1173.99+0.423054 C_{Cu} +0.713540 C_{Cd} +0.275366 C_{Cr}+0.299579 C_{Pb}+ 0.640612 C_{Zn}+360.723 pH - 0.00180735 $C_{Cu*}C_{Cu}$ -0.00163687 $C_{Cd*}C_{Cd}$ - 0.00157472 $C_{Cr*}C_{Cr}$ -0.00132020 $C_{Pb*}C_{Pb}$- 0.00247402 $C_{Zn*}C_{Zn}$ -27.8337 pH*pH -9.35764E-05 $C_{Cu*}C_{Cd}$ + 0.000280208 $C_{Cu*}C_{Cr}$ + 0.000405990 $C_{Cu*}C_{Pb}$ -1.52604E-04 $C_{Cu*}C_{Zn}$ -0.00343750 C_{Cu*} pH + 0.000368750 $C_{Cd*}C_{Cr}$ -4.05469E-04 $C_{Cd*}C_{Pb}$ -5.51042E-04 $C_{Cd*}C_{Zn}$ -0.0356319 C_{Cd*}pH + 0.000173872 $C_{Cr*}C_{Pb}$ -1.89757E-04 $C_{Cr*}C_{Zn}$ + 0.00249306 C_{Cr*} pH + 0.000177170 $C_{Pb*}C_{Zn}$ -0.00748611C_{Pb*} pH -0.0224097C_{Zn*} pH ……**Eq. 4.8**

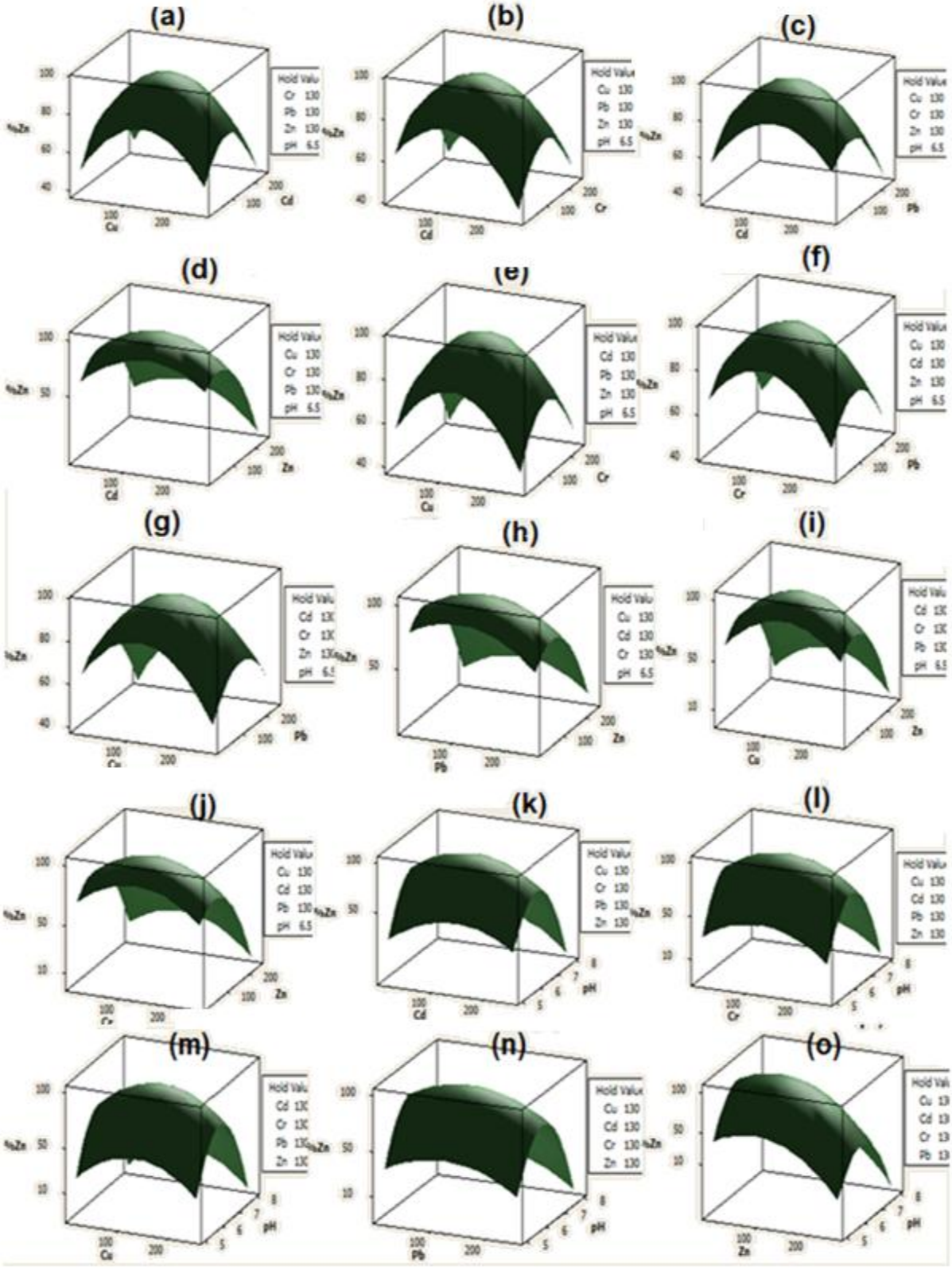


Fig.4.61 The 3D Surface plots showing the effect of studied factors on Zn(II) removal

4.10.2.4 EFFECT OF SOIL pH AND MULTI METAL INTERACTION ON Cu(II) REMOVAL

The three dimensional plots, Fig. 4.62 (i, m, n, o) on percentage removal of Cu(II) is found to increase at lower pHs and higher metal concentrations. The maximum removal percentage of Cu(II) is observed at pH 6.5 (Fig. 4.62 (n)) with the other interacting metal concentrations ranging between 100 to 200mg/kg of soil containing all the metals under study at middle level. Similarly for all the combinations as Pb(II): pH (Fig. 4.62 (o)), Cu(II): pH (Fig. 4.62 (n)), Cr(VI): pH (Fig.4.62 (m)) and Cd(II): pH (Fig.4.62 (l)) respectively.

The Cu(II) removal percentage increases with increase in Cd(II) at lower Zn(II) levels, but as the concentration of Cd(II) and Zn(II) in soil increased the Cu (II) accumulation was found to decrease significantly as shown in Fig 4.62 (e). The maximum percentage removal of Cu(II) increased with the increase in Cu(II) concentration up to around 150mg/kg but dropped with further increase in concentration above- this value, at any Cd(II) concentration and in the presence of other metals and pH at middle levels. The reasons for the same are discussed in detail in Section 4.10.2.1. Similar trend was observed for the metal combinations like Cr(VI) : Cu(II) (Fig. 4.62(i)), Cu(II) : Pb(II) (Fig. 4.62(h)), Cu(II) : Zn(II) (Fig. 4.62(f)), Cr(VI) : Zn(II) (Fig. 4.62(g)), Cr(VI) : Pb(II) (Fig. 4.62(k)), Cd(II) : Pb(II) (Fig. 4.62(c)), Cd(II) : Cr(VI) (Fig. 4.62(b)) and Pb(II) : Zn(II) (Fig. 4.62(d)).

The three dimensional plot showing the interaction of Cu(II) removal with varying factors like Cu(II) and Zn(II). Fig. 4.62(f) showed a decrease in Cu(II) removal with increase in concentration of Cu(II) irrespective of the presence of other metal ions in the soil. Similar trend was observed for multi-metal combinations namely Cu(II) : Cr(VI) (Fig. 4.62(i)), Cu(II) : Pb(II) (Fig. 4.62(h)), Cu(II) : Zn(II) (Fig. 4.62(f)) on Cu(II) removal. Cu being an essential micronutrient has more evolved protein pathways that regulated their uptake (Turkekuel et al. 2003; Tuzen 2003). The input-output data from the experiments are fitted into a multiple regression

model (MRA model) involving the individual effects, square effects and interaction effects, to predict the percentage removal of Cu(II) with the concentration of five metals under study and the soil pH using MINITAB-14 software. The MRA model is presented as Eq. 4.9 with the estimated parameters of the model. The significance of the model parameters were tested by Analysis of Variance (ANOVA) using MINITAB. Table 4.20 presents the results of ANOVA and the coefficient of determination (R^2) .The P value for regression being less than 0.05, show that the model is significant. The P value for linear terms and square terms are less than 0.05 but for the interaction terms it is greater than 0.05. Thus linear and the square terms in the model are significant, but interaction terms are less significant. The value of R^2 being 0.8640 shows that the model is reasonably good to predict the Cu(II) removal.

Table 4.20 MRA model and ANOVA table for Cu(II) removal

Metal ion under study	**Cu(II)**
P value	Regression: 0.00 Linear : 0.00 Square: 0.00 Interaction: 0.869
F value	14.61
R^2 value	0.8640
MRA model	Cu(II)removal = -1250.74 -0.172124 C_{Cu} + 0.327494 C_{Cd} + 0.00959901 C_{Cr} + 0.306199 C_{Pb} + 0.0301965 C_{Zn} +417.852 pH - 0.00169620 $C_{Cu*}C_{Cu}$ -0.00118161 $C_{Cd*}C_{Cd}$-0.00143231 $C_{Cr*}C_{Cr}$ - 0.00105314 $C_{Pb*}C_{Pb}$ -5.42724E-04 $C_{Zn*}C_{Zn}$ -34.1534 C_{pH*} pH - 5.02604E-05 $C_{Cu*}C_{Cd}$ -1.46354E-04 $C_{Cu*}C_{Cr}$ + 0.000326302 $C_{Cu*}C_{Pb}$ + 2.09201E-05 $C_{Cu*}C_{Zn}$ + 0.0518333 C_{Cu*}pH -9.54861E-05 $C_{Cd*}C_{Cr}$ + 1.17188E-05 $C_{Cd*}C_{Pb}$ + 4.76562E-05 $C_{Cd*}C_{Zn}$ - 0.00177778 C_{Cd*} pH -1.45139E-04 $C_{Cr*}C_{Pb}$-1.84896E-04 $C_{Cr*}C_{Zn}$ + 0.0634375 C_{Cr*} pH + 0.000208073 $C_{Pb*}C_{Zn}$ -0.0146806 C_{Pb*} pH +0.0121528C_{Zn*}pH ……..Eq.4.9

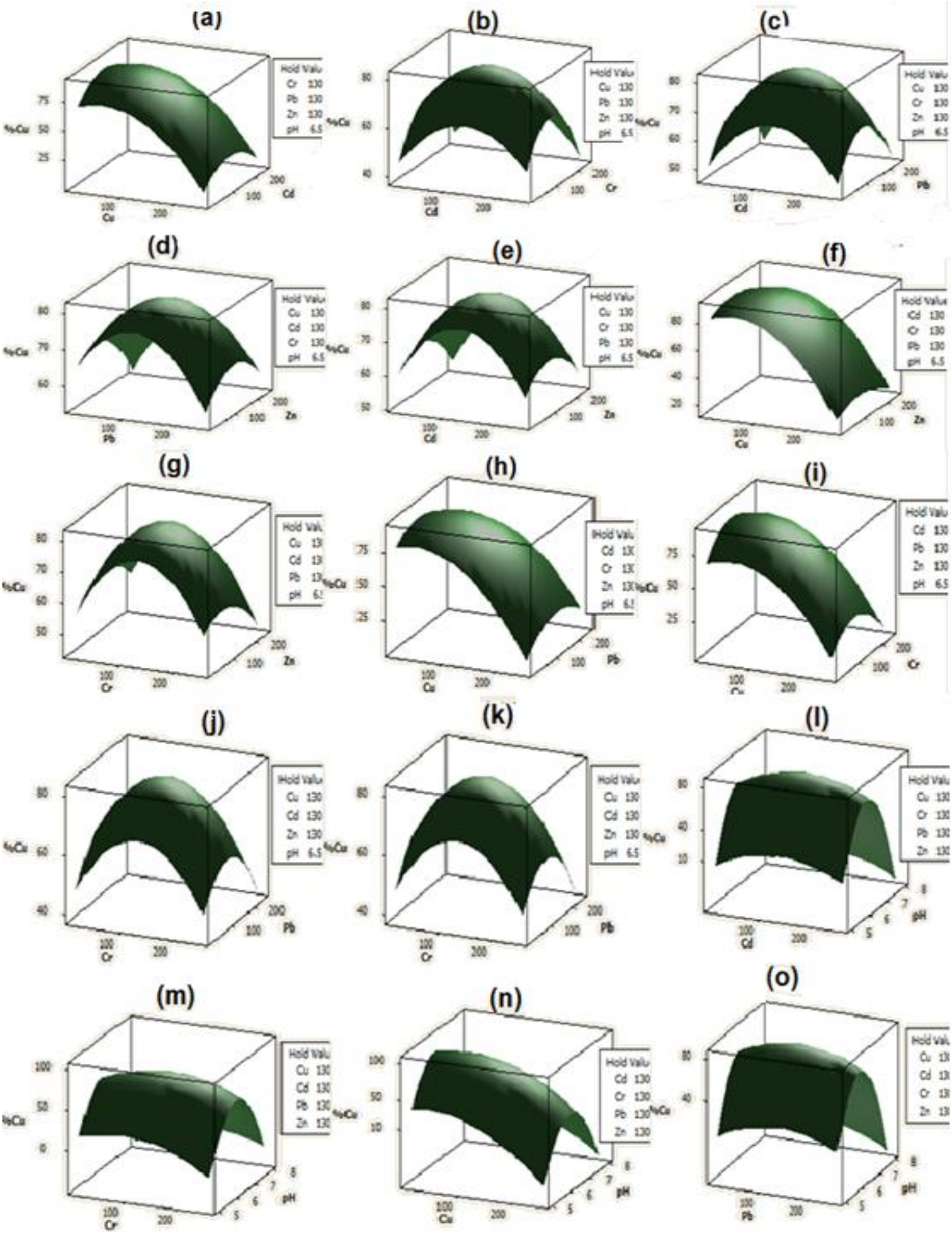


Fig.4.62 The 3D Surface plots showing the effect of studied factors on Cu(II) removal

4.10.2.5 EFFECT OF SOIL pH AND MULTI METAL INTERACTION ON Cr(VI) REMOVAL

From Table 4.21 Cr(VI) removal percentage is found to be very low for all the experiments conducted as per CCD. The results obtained for removal of Cr(VI) by RSM, revealed that the methodology adapted for analyzing multi metal interaction for Cr(VI) removal using *Galerina vittiformis* may not favour the removal process as the values are found to be unrealistic. The bioaccumulation results discussed in Section 4.6 also indicated that the accumulation potential of Cr(VI) by these selected mushroom species were low, which indicates that Cr(VI) metal ions exhibit reduced movement in mushrooms from the soil due to their physical and chemical characteristics. The process of Cr(VI) removal was found to show a highly nonlinear and stochastic behavior owing to lower accumulation potential by these mushrooms.

The input-output data from the experiments were fitted into a multiple regression model (MRA model) involving the individual effects, square effects and interaction effects, to predict the percentage removal of Cr(VI) with the concentration of five metals under study and the soil pH using MINITAB-14 software. The MRA model is represented as Eq.4.10 with the estimated parameters of the model. The coefficient of determination (R^2) being 0.5010 reveals that the model fits the data very poorly for the prediction of the Cr(VI) removal which may be due to the stochastic nature of the process. Hence further analysis on multi metal and pH interaction effects on Cr(VI) removal through RSM was not carried out.

Upon comparing the effects of all the metal combinations on metal removal indicates that maximum removal of metals by *G. vittiformis* for each metal concentration is in the range of around 150 mg/kg soil. It can be understood that the maximum activity of *G. vittiformis* may be found for metal concentrations of around 150 mg/kg of soil for each of these metals under study in a multi-metal system and the metals at concentrations above this value, may be lethal or have inhibitory effect on

the metal uptake by the mushrooms. Similar results were observed in the studies of Lu et al., 2008, Azila et al., 2008 and Cao et al., 2010 on multi metal interaction on metal uptake by mushrooms. *G. vittiformis* has been found to be effective in the removal of Pb(II), Cd(II), Cu(II) and Zn(II) at an optimal pH of 6.5. However, it was found to be ineffective for the removal of Cr(VI). Thus it may be concluded that *G. vittiformis* may be used effectively for the remediation of multi metal contaminated soil even though it may not be successful in case of Cr(VI) removal.

Table 4.21 MRA model and ANOVA table for Cr (VI) removal

Metal ion under study	**Cr (VI)**
P value	Regression: 0.00 Linear : 0.00 Square: 0.00 Interaction: 0.497
F value	2.06
R^2 value	0.5010
MRA model	Cr (VI) removal = -1250.74 -0.172124 C_{Cu} + 0.327494 C_{Cd} + 0.00959901 C_{Cr} + 0.306199 C_{Pb} + 0.0301965 C_{Zn} +417.852 pH - 0.00169620 $C_{Cu*}C_{Cu}$ -0.00118161 $C_{Cd*}C_{Cd}$-0.00143231 $C_{Cr*}C_{Cr}$ - 0.00105314 $C_{Pb*}C_{Pb}$ -5.42724E-04 $C_{Zn*}C_{Zn}$ -34.1534 CpH*pH - 5.02604E-05 $C_{Cu*}C_{Cd}$ -1.46354E-04 $C_{Cu*}C_{Cr}$ + 0.000326302 $C_{Cu*}C_{Pb}$ + 2.09201E-05 $C_{Cu*}C_{Zn}$ + 0.0518333 C_{Cu*}pH -9.54861E-05 $C_{Cd*}C_{Cr}$ + 1.17188E-05 $C_{Cd*}C_{Pb}$ + 4.76562E-05 $C_{Cd*}C_{Zn}$ - 0.00177778 C_{Cd*}pH -1.45139E-04 $C_{Cr*}C_{Pb}$-1.84896E-04 $C_{Cr*}C_{Zn}$ + 0.0634375C_{Cr*}pH + 0.000208073 C_{Pb*Zn} -0.0146806 C_{Pb*}pH + 0.0121528 C_{Zn*}pH ……..……..Eq. 4.10

CHAPTER – 5

SUMMARY AND CONCLUSION

In the current study, 10 species of mushrooms were collected from municipal waste dump yards. Isolation and screening were carried out to select a potential mushroom species for mycoremediation of metal contaminated soil. Three isolates were selected after screening based on their tolerance towards metals such as: Cu(II), Cd(II), Cr(VI), Pb(II) and Zn(II). The organisms were identified as *G. vittiformis* (M6), *Pleurotus* Sp. (M5) and *Polyporus* Sp. (M9). The mushroom species *G. vittiformis* (M6) was chosen among the three isolates based on its capability to form fruiting bodies and high metal bioaccumulation potential. The site of metal bioaccumulation in the mushroom was identified. The kinetics of metal removal from soil by *Galerina vittiformis* was studied and rate limiting step in the process of metal removal was identified. Effect on addition of chelaters in to the soil environment on metal bioaccumulation by the mushroom species was analysed. The mechanism of metal bioaccumulation was proposed. Effect of presence of multi metal ion concentrations and pH on removal of each of the target metals under study were analyzed based on response surface methodology through experiments designed as per central composite design. These studies were conducted to investigate the suitability of the selected mushroom species for metal removal from multi metal contaminated environment.

Critical findings of the research:

- The heavy metal bioaccumulation potential of *Galerina vittiformis* belonging to *Basidiomycetes* class has been reported for the first time.
- *Galerina vittiformis*, has been found to have higher tolerance and bioaccumulation potential for the heavy metals, Cu(II), Cd (II), Pb(II) and Zn(II) compared to the mushrooms reported in the literature.
- Bioaccumulation of heavy metals from contaminated soil using live mushrooms for its entire life span including fruiting body stage has been first time investigated.

- Among the studied heavy metals the mushroom; *G. vittiformis* has been found to accumulate 670 mg/kg, concentration of Pb(II), the highest concentration ever reported by any mushrooms in the literature.

- First time an attempt has been made to investigate the mechanism of bioaccumulation by mushrooms.

Based on these investigations following conclusions are drawn:

- Ten different species of mushrooms were identified in the municipal dump yards of Mangalore.

- Three isolates, identified as *Pleurotus* Sp. (M5), *Galerina vittiformis* and *Polyporus* Sp. (M9) were found to have high metal tolerance, healthy growth and good bioaccumulation potential of heavy metals Cu(II), Cd (II), Pb(II), Zn(II) and Cr(VI).

- Parameters like, initial metal concentration, soil pH and incubation time were found to have significant effect on the bioaccumulation of metals from the soil slurry during the mycelial stage of the mushrooms.

- The mushroom *G. vittiformis* (M6) has been found to have high metal bioaccumulation potential in mycelial stage of its life cycle when compared to *Pleurotus* Sp. (M5) and *Polyporus* Sp. (M9) and thus considered as a potent mushroom species for heavy metal removal from soil by mycoremediation.

- The mushroom, *G. vittiformis* (M6) has been found to be with the potential to yield fruiting bodies at high metal concentrations considered in the study.

- Higher BAF values in the presence of fruiting bodies have been observed compared to the mycelial stage of the mushroom which indicates the

significance of the fruiting bodies in the bioaccumulation process, owing to the possession of larger biomass. These mushrooms have significant features as bioremediating agents, such ease of separation of the metals from the soil and the possibility of removal of mushrooms from the soil following remediation.

- Heavy metals accumulated by the fruiting bodies were found to get stored in their pileus than the stalk of the mushroom.

- In metal removal from soil using *G. vittiformis,* intra-particle diffusion is not the sole rate limiting step. The metal removal kinetics for metal ions like Cu(II), Pb(II) and Zn(II) follows pseudo- second order equation and for Cd(II), removal kinetics follow pseudo-first order equation indicating that the removal rate is governed mainly by the surface reaction and biosorption on the surface is the rate controlling step.

- *G.vittiformis* was found to produce two types of Phytochelatins, namely PC_2 and PC_3 in response to Cu(II), Cd(II), Cr(VI), Pb(II) and Zn(II) metal stress. These Phytochelatins are known to transfer the excess metal ions into the vacuoles of the cell and thereby reducing the metal toxicity in the cell. Oxalic acids and thiols were also detected through FTIR results of the pileus. Phytochelatin and acid mediated *Mechanism of bioaccumulation* has been postulated.

- Organic chelating agents (Citric acid and Gallic acid) and chemical chelating agent (EDTA) were found to enhance the metal accumulation potential of *G.vittiformis* marginally. EDTA has been found to be better chelating agent for metal removal as compared to the organic chelating agents. However, owing to only marginal enhancement in metal bioaccumulation potential by the addition of EDTA and low

biodegradability of EDTA, EDTA may not favor for large scale mycoremediation of soil.

- The maximum activity of *G. vittiformis* in terms of metal bioaccumulation was found to occur at metal concentrations around 150 mg/kg of soil for each of the metals under study in a multi-metal interaction system. *G. vittiformis* was found to be effective in removal of Pb (II), Cd (II), Cu (II) and Zn (II) from multi metal contaminated soil except in removal of Cr (VI). The soil pH of around 6.5 was found to be favorable for metal removal. Thus it may be suggested that *G. vittiformis* can be used effectively to remediate soil under multi-metal contaminated condition.

Based on the above mentioned results, it can be concluded that the wild non edible mushroom species, *G. vittiformis* is efficient in removal of heavy metals, Cu(II), Cd(II), Pb(II) and Zn(II) from soil under both single and multi-metal contamination situations. Wild non-edible mushroom species, *G. vittiformis* has been found to be more efficient in accumulating the heavy metals from soil compared to certain edible species like *Pleurotus* Sp. and *Agaricus* Sp., reported in literature, thus making it a favorable bioremediation agent. Taking into consideration, hyperaccumulating plants like *Brassica junceae, Phaseolous vulgaris, Triticum aestivum etc*. which might take longer duration (3 or 6 months) for removing metals from soil (Long et al 2010; Wuyep et al. 2007), mycoremediation using *G. vittiformis* can be considered as an effective and ecofriendly alternate method for remediation of soil contaminated with heavy metals, thus enhancing the potential of establishment of mycoremediation in large scale as a better, easy and potentially economical bioremediation technique.

SCOPE OF FUTURE- WORK

- Field trials on heavy metal removal using *Galerina vittiformis* can be conducted to analyze its efficiency in real situations and optimze the parameters or establish the favoral condition.
- Sequencing, engineering and cloning of gene/s responsible for Phytochelatin production could be an interesting preposition in an effort to increase efficiency of the same and other known agents.
- Mushroom species can be used as indicators /biosensors for soil contamination by heavy metal through florescence response.
- A detailed and accurate biochemical pathway of metal tolerance and accumulation by *Galerina vittiformis* can be studied which may be helpful to understand and enhance the accumulation process.

APPENDIX-I

MINERAL BROTH MEDIUM

The mineral broth medium of the following composition was used in the study:

Components	g/L
Peptone	5.0
Yeast extract	1.0
Ferric citrate	0.1
Sodium chloride	19.45
Magnesium chloride	5.9
Magnesium sulphate	3.24
Calcium chloride	1.8
Potassium chloride	0.55
Sodium bicarbonate	0.16
Boric acid	0.0022
Sodium silicate	0.0004
Sodium fluoride	0.0024
Ammonium nitrate	0.0016
Disodium hydrogen phosphate	0.008

The components of the medium were dissolved in 1 litre of water and the pH of the medium was set to 7.6. The medium was sterilized by autoclaving at 15 psi pressure for 20 minutes at 121°C. The mineral agar medium of pH 7.6 was prepared by adding 1.5% (w/v) of agar to the liquid medium before sterilization.

APPENDIX-II

DIRECT SEQUENCING OF ITS REGIONS

Sample preparation:

One must have a pure culture for this technique. Grow culture in V8 or potato dextrose broth (or some other suitable liquid medium) at room temperature in the dark for 48-72 hr. Tear off approximately 100 mg mycelium, rinse well in sterile Nanopure water, drain off excess water aseptically, and add to a bead-beater tube containing two 5-mm diameter beads. Hold on ice until ready for lysis. Add Buffer AP1 and RNase A, beat tube for 30 sec at 2500 rpm, incubate at 65°C as indicated in instructions for DNEasy Plant Mini Kit, and follow remaining kit instructions.

PCR Master Mix (per 25 µl reaction; prepare sufficient mix for 12 reactions):

Nanopure water	sufficient to bring to 12.5 µl
Primer ITS1 or Primer ITS5, 5 µM	2.5 µl
Primer ITS4, 5 µM	2.5 µl
DNA polymerase	0.5 µl

Sample genomic DNA volume necessary to add 10 to 20 ng DNA

Mix 12.5 µl of Master Mix with 12.5 of each Epicentre Failsafe® PCR 2X Premix (designated Premix A through L).

Primers (600 to 650 bp)

Prepare to a concentration of 5 µM (5 pmol/µl) in TNE buffer:

ITS1 (White et al, 1990)	5'- TCCGTAGGTGAACCTGCGG-3'
ITS5 (White et al., 1990)	5'-GGAAGTAAAAGTCGTAACAAGG-3'
ITS4 (White et al, 1990)	5'- TCCTCCGCTTATTGATATGC-3'

Cycling Protocol (2 hr 3 min):

Name of Protocol (GeneAmp 9700, max ramp rate): ITS1-ITS4

1. 95ºC for 120 sec
2. 35 cycles of: 95ºC for 30 sec; 55ºC for 30 sec; 72ºC for 60 sec
3. 72ºC for 600 sec

Sequencing Amplicon:

Pool amplicon from 2-3 successful reactions, and run 40-50 µl of reaction mixture in three lanes joined together in a 1.5% agarose gel. Excise band and clean it using the QiaQuick gel extraction kit or equivalent, paying special attention to instructions in the kit which relate to direct sequencing. Elute in 30 µl Buffer EB, quantify, and submit for sequencing.

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List of publications based on this research work

JOURNAL PAPERS:-

Published:

1. **Dilna Damodaran,** Vidya Shetty, K., Raj Mohan, B. (2014). "Remediation of Cd (II), Cr (VI), Cu (II), Pb (II) and Zn (II) contaminated soil using mushroom- Galerina vittiformis". Ecotox Environ Safe. dii: S0147-6513(13)00478-8. doi: 10.1016/j.ecoenv.2013.10.033. (Impact factor: 2.203)

2. **Dilna Damodaran.,** Vidya Shetty, K, Raj Mohan, B., (2013)."Effect of Chelaters on bioaccumulation of Cd (II), Cu (II), Cr (VI), Pb (II) and Zn (II) in *Galerina vittiformis* from soil". *Int Biodeter Biodegr.* 85,182-188. doi.org/10.1016/j.ibiod. 2013.05.031. (Impact factor: 2.05)

3. **Dilna Damodaran**, Raj Mohan, B., Vidya Shetty, K., (2013). "The Uptake Mechanism of Cd(II), Cr(VI), Cu(II), Pb(II), and Zn(II) by Mycelia and Fruiting Bodies of Galerinavittiformis," BioMed Reser International(formerly titled Journal of Biomedicine and Biotechnology)- Hindawi publications. Article ID 149120, 11 pages, 2013. doi:10.1155/2013/149120. (Impact factor: 2.88)

4. **Dilna Damodaran**, Raj Mohan, B., Vidya Shetty, K., (2011). **"**Review onMushrooms in the Remediation of Heavy Metals from Soil"**.** *Int J Environ Pollut.Mangmt,* 3 (1): 89- 101.(Impact factor: 0.8)

5. **Dilna Damodaran**, Raj Mohan, B and Vidya Shetty, K. Studies on the Interaction of Heavy Metals in Multi-metal Biosorption Process by *Galerina vittiformis* from Soil." *Bioremediation Journal*- Article ID 14546, 13 pages, 2014. doi:10.1267/2014/14989. (Impact factor: 0.9)

Paper published in conference proceedings:

1. **Dilna Damodaran**, Raj Mohan, B., Suresh Gummadi., (2011). **"**Bioremediation of soil by removing heavy metals using *Saccharomycis cereviseae"*. Proceedingss of 2nd International Conference on Environmental Science and Technology- IPCBEE,6 (2) IACSIT Press, Singapore.

PRESENTATION AT INTERNATIONAL CONFERENCES

1. **Dilna Damodaran**, Raj Mohan B., Vidya Shetty, K., (2012). **"**Remediation of heavy metals like Cu (II), Cd (II), Cr (VI), Pb (II) and Zn (II) from soil using macro fungi". International conference on Recent Developments in Environmental Impact Assessment and Integrated Approach for Carbon Management- Solutions, Technology Development and Pollution Abatement (RDEIA- IACM 2012) on March 28-30.

2. Madushree, M., **Dilna Damodaran**, Raj Mohan, B. (2012). **"**Biosorption of Heavy Metals like Cd (II), Zn (II), Cr (VI) from Contaminated Water by *Trichodermasp.*". International conference on Recent Developments in Environmental Impact Assessment and Integrated Approach for Carbon Management- Solutions, Technology Development and Pollution Abatement (RDEIA- IACM 2012) on March 28-30, 2012.

3. **Dilna Damodaran**, Raj Mohan, B., Suresh Gummadi. (2011). "Bioremediation of Soil by Removing Heavy Metals Using *Saccharomyces cerevisiae*" ICEST 2011 26-28, February 2011, Singapore.