ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

MYCOREMEDIATION OF HEAVY METAL CONTAMINATED SOIL USING OYSTER MUSHROOM: *Pleurotus ostreatus*

M.Sc. THESIS

Dicle ÜRÜNAY

Department of Advanced Technologies

Molecular Biology - Genetics and Biotechnology Programme

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MAY 2015

Dicle Ürünay, a **M.Sc.** student of ITU **Institute of Graduate School of Molecular Biology Genetics and Biotechnology Programme,** student ID **521111130**, successfully defended the **thesis** entitled "**MYCOREMEDIATION OF HEAVY METAL CONTAMINATED SOIL USING OYSTER MUSHROOM: *Pleurotus ostreatus* "**, which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Whenever the working class, LGBT individuals, women, Kurds, Turks, Palestinian, Alawites, Syrian, Circassians, Zazas, Armenians, Pomaks, Africans, Rums and others; people whose language, labor, culture, religious denomination, skin color, gender, sexual orientation is exploited; realize that they are in the same boat with

cows in the dairy industry,

chickens in poultry farms,

sheeps taken to the slaughterhouse,

stray cats and stray dogs,

snakes who have been skinned,

rhinos whose horn has been ripped off,

trees which have been torn down,

forests which have been burned,

streams which have been hand-cuffed,

rats in a vivisection lab,

fish whose lips were pierced by a hook,

donkeys whose skin is worn down from carrying excess loads,

bulls whose back have been punctured,

racoons who have been skinned alive,

elephants whose tusks have been ripped off,

bees whose honey is stolen,

orangutans who were forced into prostitution,

and all share the same fate because of the policies of the governing power; when they remember that cleavers, gas chambers, shackles, torture chambers, abuse tactics had been once used on them and they never forget that they might be targeted again, then we can have hope for nature. Only then this struggle will go beyond flags, borders, armies, genders, species, and become the struggle for the Mother Earth. This hope has to be for all earthlings, not only for humankind.

As the saying goes, "The world is still between the horns of the ox".

Dicle Ürünay

In memory of Panda, the cat (April, 2011-June 8, 2015)

Special thanks to Özmen, for translation…

FOREWORD

Firstly, I thank to my super cats with their full energy and dogs who live peacefully in I.T.U Ayazağa Campus. It is such a pleasure to be under their supervision during this thesis.

Secondly, I would like to thank to my co-adviser Prof. Dr. Mustafa Sait Yazgan for his support and taking great pains to cheer me up and my advisor Asocc. Prof. Dr. Gizem Dinler Doğanay to open her door to me.

Looking back, I remain immensely grateful to Prof. Dr. Şeminur Topal whom i started my graduate study with, in Yıldız Technical University, never left me alone even after she retired and Prof. Dr. İsmail Toröz who guided me through the process of researching, organizing, polishing, submitting, publishing my student work and Asocc. Prof. Selma Ünlü for her help in my analysis.

It was an opportunity for me to have four legged, two legged, three legged, finned, hoofed friends. Thank you for your patience Ceyda, in spite of my clumsiness. Thank you my beloved friends who taught me and showed me anything, and the ones who encouraged me, whose names in my memory and in my heart. And my special thank you to Buse Şener and her family who were with me when I was hard up and when i was in a difficult situation.

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In memory of Panda, the cat (April, 2011-June 8, 2015)

May 2015

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

MYCOREMEDIATION OF HEAVY METAL CONTAMINATED SOIL USING OYSTER MUSHROOM: *Pleurotus ostreatus*

SUMMARY

According to fossil records, fungi diverged from other life around 1.5 billion years ago and probably fungi colonised on Earth during the Cambrien, which is long before human beings evolved on it.

The term "mycology" is derived from Greek word "mykes", meaning mushroom which is a branch of life science that refers the study of fungi. In general, mushrooms are responsible for decomposing of organic molecules to provide continuance of life by recycling organic wastes and returning of nutrients back into the ecosystem. These features of fungi reveal a term called as "*mycoremediation*".

Increasing human population and supporting unconscious consumerism by a politics of capitalist world economy caused the pollution of the environment with synthetic compounds which has become a major problem all around the world. These synthetic compounds are called as xenobiotics which do not occur naturally in the biosphere so are not easily degraded by the natural microflora and fauna. Biological approaches based on the environmental biotechnology are focusing on the development of "environmentalist technologies". Further, these clean technologies focus on the use of metabolic pathways of organisms for the remediation of waste. One such biological method is mycoremediation (fungal remediation). The mushrooms and other fungi act as enzymatic machinery for degradation of a wide variety of waste/pollutant. However mushrooms, basidiomycetous fungi, are becoming more popular nowadays for remediation purposes as a bioremediation tool.

The white rot fungi in all the fungi species, are known to degrade polyaromatic hydrocarbons (PAHs), chlorinated aromatic hydrocarbons (CAHs), polycyclic aromatics, polychlorinated biphenyls, polychlorinated dibenzo(p)dioxins, the pesticides and some azo dyes. White-rot fungi are also used in bioremediation of polluted soils and accumulation of heavy metals and involved in mineralization, biodeterioration, biodegradation, transformation and co-metabolism. *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Pleurotus tuber-regium*, *Lentinus squarrosulus* etc. are white-rot fungi, so far used in bioremediation researches.

Heavy metals are natural constituents of the earth crust. Their non- biodegradable, non-thermodegradable features contribute to environmental pollution mainly and show toxic effects on organisms. The most common heavy metals found at contaminated sites, in order of abundance are Pb, Cr, As, Zn, Cd, Cu and Hg. Those metals are important since they are capable of decreasing crop production due to the risk of bioaccumulation and biomagnification in the food chain. There is also the risk of superficial and groundwater contamination.

Cadmium, mercury and lead can describe as three big poisinous metals which are not known essential role for living organisms. Cadmiums chemical similarity to Zn which is an essential micronutrient for plants and animals, probably responsible of its toxicity. Mercury, Hg, is the only liquid metal at standard temperature, and major source of Hg contamination is caused by coal combustion. Mostly Pb contamination in nature arises during the improper disposal of Pb storage batteries.

The aim of this study is to remediate contaminated soils from three most toxic heavy metals using *Pleurotus ostreatus* mycelium. Soil and mycelium heavy metal analysis were performed by Bureau Veritas Mineral Laboratories, in Canada. Soil Pb and Cd analyses were performed using ICP-MS analysis. In this study, bioaccumulation percentage of was studied with three different concentration of Pb, Cd and Hg for two different incubation period. The results showed that bioaccumulation percentage increased by incubation period for lead, cadmium and mercury. While SET I mycelium analyses showed that increasing concentration of lead caused a decrease for its bioaccumulation, in SET II bioaccumulated concentration of Pb did not showed a significant difference for all concentrations. According to bioaccumulation percentage calculations for Pb, it showed a decrease in order of 2.5 ppm, 5 ppm and 10 ppm. Cadmium showed the lowest bioaccumulation percentage in Content 2, comparison to Content 1 and Content 3, both SET I and SET II.

Heavy metal analyses of mycelium samples showed that the most bioaccumulated heavy metal by *Pleurotus ostreatus* was mercury. The mycelium samples belonged to 10 ppm mercury contained soil samples showed 54 % Hg bioaccumulation and 85 %, in SET I and SET II, respectively. The heavy metals which were used in this experiment in order of bioaccumulation percentages were Hg > Cd> Pb.

İSTİRİDYE MANTARI *Pleurotus ostreatus* KULLANILARAK MİKOREMEDİASYON YÖNTEMİ İLE TOPRAKTAN AĞIR METAL GİDERİMİ

ÖZET

Fosil kayıtlarına göre Funguslar 1.5 milyar yıl önce diğer yaşam formlarından ayrılmış ve büyük ihtimal ile Kambriyen Dönemi'nden beri Dünya üzerinde kolonize olmuş durumdadırlar. Fungusların Dünya üzerindeki varoluşları insan türünün yeryüzü üzerindeki evriminden çok daha uzun bir döneme işaret etmektedir.

"Mikoloji" terimi kökenini Yunanca mantar anlamına gelen "mykes" kelimesinden alır ve funguslar üzerine çalışan yaşam bilimini niteler. Genel olarak mantarlar, organik moleküllerin ayrıştırılmasını ve besinlerin ekosisteme geri dönüşümünü sağlayarak yaşamın sürekliliğini sağlamaktadırlar. Fungusların ayrıştırıcı ve geri dönüştürücü özelliği "Mikoremediasyon" terimini ortaya çıkarmıştır.

Artan insan popülasyonu ve kapitalist dünya ekonomisinin bilinçsiz tüketimi desteklemesi, yaşadığımız çevrenin ve doğal kaynakların sentetik bileşiklerle kirlenmesine ve bunun gün geçtikçe dünya çapında büyük bir probleme dönüşmesine sebep olmaktadır. Biosferde doğal olarak meydana gelmeyen bu sentetik bileşikler "ksenobiyotik" olarak adlandırılır. Sentetik bileşiklerin büyük bir kısmı doğal mikroflora ve fauna tarafından kolayca parçalanamazlar. Endüstriyel ve çevre biyoteknolojisindeki biyolojik yaklaşımlar; atık üretimin azaltılması, atıkların temizlenmesi, daha az zararlı, kullanılabilir ve kolay bozunur formlara dönüştürülmesi gibi "çevreci teknolojiler"i temel alır. Bahsi geçen çevreci yaklaşımlar canlılardaki metabolik yollarının kullanımına odaklanır. Bu biyolojik yöntemlerden biri de fungal remediasyon olarak bilinen mikoremediasyon yöntemidir.

Mantarlar ve diğer funguslar çok sayıda atığın/kirleticinin ayrıştırılmasında ya da parçalanmasında görev alan enzimatik mekanizmalardır. Bazitli fungusların biyoremediasyon aracı olarak kullanımı ise gün geçtikte popülerlik kazanmaktadır.

Filamentli funguslar bir ağacın dallarını andıran yapıları ile üzerinde büyüyüp geliştikleri substratı salgıladıkları hücre dışı sindirim enzimleri ile sindiren, potensiyel olarak iyi birer ayrıştırma ajanıdır. Odunsu dokuların hücre duvarlarında bulunan lignini parçalayabilme özelliğine sahip enzimatik özellikleri ile beyaz çürükçül mantarlar tüm fungus türleri arasında odunsu yapıları parçalayan ve/veya çürüten funguslar olarak bilinirler.

Beyaz çürükçül mantarların çoklu aromatik hidrokarbonları, klorlu aromatik hidrokarbonları, polisiklik aromatikleri, poliklorlu bifenilleri, poliklorlu dibenzo(p)dioksinleri, pestisitleri, insektitistleri ve bazı azo boyalarını parçaladığı bilinmekte ve bu fungus türleri aynı zamanda toksik organik bileşiklerin parçalanması sonucu ortaya çıkan ağır metallerin biyolojik birikimlerinde ve kirlenmiş toprakların mineralleştirilmesi ya da nitratlaştırılması amacıyla da kullanılmaktadır. *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Pleurotus tuber-regium*, *Lentinus squarrosulus* ve benzeri beyaz çürükçül funguslar bu zamana dek biyoremediasyon araştırmalarında kullanılmış türlerdir. Beyaz çürükçül mantarlar kirlenmiş toprakların biyoremediasyonunda;

madenleştirmeyi, biyolojik bozunumu, dönüştürümü, eş metabolizmayı içeren ve biyoakümülasyon olarak bilinen ağır metallerin biyolojik birikiminde kullanılır.

Ağır metaller yer kabuğunu doğal bileşenleridir. Biyolojik ve ısı etkisiyle bozunur olmayan yapıları çevrede birikmelerine ve ağır metal kirliliğe sebep olur. Ağır metaller genellikle yoğunluğu 5'ten büyük, organizmalar üzerinde toksik etki gösteren yaklaşık olarak 65 metalik elementi kapsayan bir grup olarak tanımlanır.

Çevrede bulunan ağır metaller, fungal hücre dışı enzimlerle direkt olarak etkileşim halindedir. Çevreden alınan ağır metaler beyaz çürükçül mantarların miselerinde birikir. Ağır metaller genel olarak enzimatik reaksiyonların inhibitörleridir. Metal iyonları hücre içerisine girdiğinde hücre dışı enzimlerin üretimini transkripsiyonel (yazılım) ve tranlasyonel (çevirimsel) düzeylerde etkiler. Esansiyel ağır metallerin düşük konsantrasyonları aynı zamanda lignolitik (lignin parçalayıcı) enzim sistemlerinin gelişimi için gereklidir.

1996 yılında U.S EPA tarafından yayınlanan raporda kirlenmiş alanlarda bulunan ağır metallerin bulunma miktarını Pb, Cr, As, Zn, Cd, Cu and Hg şeklinde sıralanmıştır. Biyolojik birikim ve biyoartış ile ürün veriminin azalmasına sebep olduğundan topraktaki metallerin miktarı önem taşımaktadır. Toprakta bulunan ağır metaller aynı zamanda yer üstü ve yeraltı su kaynakları için risk arz etmektedir.

Kadmiyum, civa ve kurşun, organizmalar üzerinde tanımlanmış herhangi bir esansiyel rolü olmayan en zehirli üç ağır metaldir. Kadmiyumun toksisitesi, bitkiler ve hayvanlar için esansiyel bir mikroelement olan çinkoya olan benzerliğinden kaynaklanmaktadır. Kadmiyum ve kurşun genellikle Cd/Pb içeren pillerin, çeşitli elektronik eşyaların, kabloların ve benzeri ürünlerin üretiminde kullanılan ağır metallerdir ve bu ürünlerin geri dönüşümlerinin/geri kazanaımlarının sağlanmaması, evsel atıklarla birlikte çöplerde bulunması kadmiyum ve kurşun kirliliğine sebep olur. Civa kirliği ise daha çok kömür yanması sonucu ortaya çıkmaktadır.

Bu çalışma; kurşun , kadmiyum ve civa ile üç farklı konsantrasyonda yapay olarak kirletilmiş topraktan, istiridye mantarı olarak bilinen *Pleurotus ostreatus*'u kullanılarak iki farklı inkübasyon dilimi için, ağır metallerin farklı zaman dilimlerindeki biyolojik birikimlerini araştırmak üzere planlanmıştır. Deney sonunda toprak ve misel örnekleri ayrı ayrı paketlenerek Bureau Veritas Mineral Laboratuvarları'na gönderilmiş ve analizleri yaptırılmıştır. Ağır metaller ICP-MS cihazı ile tayin edilmiştir. Toprakta hali hazırda kalan ve miseller tarafından biriktirilen ağır metal konsantrasyonları belirlenmiştir.

Misele akümüle olan Pb, Cd, Hg inkübasyon süresinin uzamasıyla artmıştır. SET II'ye ait misellere akümüle olan kurşun, kadmiyum ve civa konsantrasyonları SET I'e ait misel örneklerine nazaran daha yüksektir.

SET I misellerinde kurşunun artan konsantrasyonla birlikte miselde biriken Pb konsantrasyonu azalma gösterirken, SET II misellerinde kurşunun misele akümüle olan konsantrasyonu, artan kurşun konsantrasyonu ile birlikte önemli bir değişiklik göstermemiştir. SET I ve SET II içerisinde 2.5, 5 ve 10 ppm olarak artan Pb konsantrasyonları ile kurşunun bioakümülayon yüzdesinin azaldığı gözlemlenmiştir.

Kadmiyumun SET I ve SET II de artan kadmiyum konsantrasyonlarına karşı cevabı ilginç bir tablo sergilemiştir. SET I ve SET II içersinde artan konsantrasyonla birlikte kadmiyumun miselde tayin edilen konsantrasyonu artarken, hem SET I hem de SET

II için 2.5, 5 ve 10 ppm konsantrasyonlarında Cd bioakümülasyon yüzdesi İçerik I> İçerik III> İçerik II şeklindedir.

Bioakümülasyon yüzdeleri incelenen ağır metaller içerisinde civa SET I ve SET II için en yüksek bioakümülasyon yüzdesini göstermiştir. 10 ppmlik civa konsansantrasyonu için SET I'de 54 % ve SET II'de 85 % bioakümülasyon yüzdesi hesaplanmıştır.

Buna göre her iki inkübasyon süresi için misel tarafından en çok akümüle edilen ağır metalin civa olduğu, kurşunun ise en az akümüle edildiği belirlenmiştir. Misel örneklerine akümüle olan kurşun, kadmiyum ve civa konsantrasyonları çoktan aza sırasıyla Hg> Cd> Pb şeklindedir.

1. INTRODUCTION

The pollution of the environment with synthetic organic chemical compounds has become a main problem world wide. Large quantities of organic and inorganic compounds are released into the environment every day as a result of techno-industrial consumption activities of civilized human being. Soil, water and atmosphere contamination is a typical prior effect of industrial activities.

Many of synthetic chemical compounds introduced to the nature called as xenobiotics that do not occur naturally in the biosphere and many of which are not easily degraded by the natural microflora and fauna (Sullia, 2004).

Industrial activities, electroplating units, mining, metal processing, disposal of high metal wastes, leaded gasoline and paints, batteries, fertilizers, animal manures, pesticides, coal combustion residues, fossil fuel, spillage of petrochemicals, and atmospheric deposition are caused contamination of soils by the accumulation of heavy metals and metalloids (Khan et al, 2008; Zhang et al., 2010). Domestic wastes, batteries, exhausts from auto's and emissions from electroplating industries are the biggest anthropogenic pollution sources for heavy metal accumulation (Olade, 1987).

Pollution of the biosphere by heavy metals due to industrial, agricultural and domestic activities has been threaten of safe, fair and rational utilization of soil. Because of the metals can not be degraded like organic pollutants, they persist in ecosystem by accumulating in food chains.

Environmental biotechnology is focusing on the development of "clean-environmentalist and sustainable technologies" which emphasizes on reducing waste generation, treatment and conversion of waste in some useful or non-virulent, easily degradable chemical forms. Further, these clean technologies focus on the use of metabolic pathways of living organisms for the remediation of waste. One such biological method is mycoremediation is based on the use of fungi for the removal of waste from the environment, ability of fungal enzymes, extracellular lignin-degrading enzymes such as manganese peroxidase, laccase and lignin peroxidase.

The mushrooms and other fungi possess enzymatic machinery for a wide variety of waste/pollutant degradation (Purnomo et al. 2013; Kulshreshtha et al. 2013).

1.1 Purpose of Thesis

The purpose of this study is mycoremediation of cadmium, mercury and lead from soil using ligninolytic fungus *Pleurotus ostreatus* inoculum, via a process known as bioaccumulation.

The specific aims of this study were,

- mycoremediation of heavy metal contaminated soils which were enriched by wheat straw and
- show bioaccumulation level in fruiting body of *Pleurotus ostreatus* and soil in factor of incubation time.

For these purposes; three different concentrations of cadmium, mercury and lead were used to contaminate soil artificially and *Pleurotus ostreatus* was chosen as mycoremediation tool for its one and two month cultivation period in lab scale, were studied.

1.2 Literature Review

The filamentous fungi are enable to act as good potential agents for degradation by growing branch-like structures on the substratum and digesting it through the secretion of extracellular enzymes. Filamentous fungi are composed of very thin threads called as *hyphae*. Hyphae grow at the tip and divide repeatedly to create long and branching chains (Shoji et al., 2006). The hyphae continue to grow and crisscross until they form a network of threads called as *mycelium*. Digestive enzymes are secreted from tip of the hyphae. These extracellular enzymes break down the organic matter found in the environment into smaller molecules which are used by the fungi as a nutrient source.

The branching filamentous manner of fungal growth allows more efficient colonization and exploration into the contaminated soil (Hamman, 2004). As fungi grow on agricultural wastes like corncobs and sawdust and quite a number of wastes, expected that using fungi in a bioremediation process much more economical and ecological friendly way to clean up the environment.

The white rot fungi in all the fungi species, are known as wood-degrading and/or wood-decaying fungi because of their ability to degrade lignin by the activities of enzymes they have. White-rot fungi are known to degrade polyaromatic hydrocarbons (PAHs), chlorinated aromatic hydrocarbons (CAHs), polycyclic aromatics, polychlorinated biphenyls, polychlorinated dibenzo(p)dioxins, the pesticides (DDT) and lindane, and some azo dyes as well as have been used for bioaccumulation of heavy metals by biodegrading of toxic organic compounds and mineralizing or nitrifying of contaminated soils (Cookson, 1995). *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Pleurotus tuber-regium*, *Lentinus squarrosulus* etc. are white-rot fungi, so far used in bioremediation researches (Adenipekun and Lawal, 2012).

Many mushrooms can also accumulate radioactivity and some of them are called as *hyperaccumulators* because of their high accumulation ability. Often, a toxic waste has multiple contaminats and if heavy metals are present, some mushroom will concentrate them and become too toxic to eat (Stamets, 2005).

The process of hyperaccumulation of heavy metals by fungi suggest a new, simple strategy for remediating heavy metal contaminated sites (**Table1.1**): harvesting metal-laden mushrooms reduce gradually cadmium, mercury, arsenic, lead and radioactive elements such as cesium-134 and cesium-137 by products of nuclear technologies (Stamets, 2005).

Fruiting body forming fungi have an advantage over other fungi, since the heavy mass of compacted hyphea composing the mushroom's body makes it easy to collect bioconcentrated toxins in solid form (Stamets, 2005).

Arica and follow researchers (2003) used turkey tail mushroom (*Trametes versicolor*) and *Pleurotus* sajor-*caju* mycelia to remediate mercuric ions form wastewater. In this research, they noticed that dead mycelium removed 73 to 81 percent of mercuric ions from the wastewater samples, a process called as mycofiltration. The live mycelium of some fungi produce organomercury lyases which break down organomercury into HgO by mercuric reductase. Using this types of reducing enzymes, fungi can precipitate many metals around their mycelia such as cadmium, lead, silver, selenium etc. Several factors can affect bioaccumulation of heavy metals so using mushroom as a bioaccumulation factor or how much concentrate a metal is compared to the background level (Stamets, 2005).

Table 1.1: Removal of pollutants by biomass of mushroom using biosorption process.

Mushroom spp.	Pollutants	Remarks	References
Agaricus bisporus, Lactarius piperatus	Cadmium (II) ions	Wild *L. piperatus* showed higher removal efficiency on Cd (II) ions compared to the cultivated *A. bisporus.*	Nagy et al. (2013).
Fomes fasciatus	Copper (II)	Mushroom is efficient in biosorption of Cu (II) ions and hot-alkali treatment increased their affinity for Cu (II) ions.	Sutherland and Venkobachar (2013).
Pleurotus platypus, Agaricus bisporus, Calocybe indica	Copper, Zinc, Iron, Cadmium, Lead, Nickel	Mushrooms are efficient biosorbent for the removal these ions from aqueous solutions.	Lamrood and Ralegankar (2013).
Flammulina velutipes	Copper	Mushroom compost used as biosorbent for removing copper ions from aqueous solution.	Luo et al. (2013).
Pleurotus tuber-regium	Heavy metals	*Pleurotus tuber-regium* biosorb the pollutant heavy metals from the soil artificially contaminated with some heavy metals.	Oyetayo et al. (2012).
Pleurotus ostreatus	Cadmium	Mushroom possess biosorption capacity and mechanism of biosorption was observed.	Tayet al.(2011).
Pleurotus sajor-caju	Zinc	Mushrooms biosorb the heavy metals.	Jibran and Milsee Mol (2011).

Source: Adapted from Kulshreshtha et al., 2014.

Saprophytic fungi which live on dead organisms, may be more useful for cleaning up the deposit or surface contaminats, while mycorrhizal species which is a symbiotic

relation between fungus-vascular plant root, offer a transport system from areas deeper underground (Stamets, 2005).

Once the mycelia accumulate heavy metal into the fruiting body, they can be picked and transported out of the area. If the metal-laden mushrooms ar not removed, then bacteria and other fungi will cause them to decompose and return to the soil (Stamets, 2005).

The mushrooms' affinity for absorbing metals promise a new research area. When understanding hyperaccumulation rates and selectively factors, we can use mushrooms more afficiently in heavy metal remediation (Stamets, 2005).

It was reported that biodegradation of petroleum products, crude oil and their spillage and bioaccumulation of heavy metals from contaminated soil were carried out by white-rot fungi (Adenipekun, 2008; Adenipekun and Omoruyi; 2008; Adenipekun and Fasidi, 2005; Isikhuemhen et al., 2003; Ogbo and Okhuoya, 2009; Ogbo, 2006). In fungal bioremediation, fungi use different methods to decontaminate polluted areas.

These methods are; (i) Biodegradation, (ii) Bioconversion, (iii) Biosorption (Kulshreshtha et al., 2014).

(i) Biodegradation

Biodegradation is the breakdown process of complex organic materials which achieved by living organisms. Biodegradation leads to complete mineralization of the complex moleules into simpler compounds like CO_2, H_2O, NO_3 and other inorganic compounds (Kulshreshtha et al., 2014).

Mushroom extracellular enzymes like peroxidases, ligninase (lignin peroxidase, manganese dependent peroxidase and laccase), cellulases, pectinases, xylanases and oxidases take roles during this process (Nyanhongo et al. 2007) and are induced by their substrates.

(ii) Bioconversion

Bioconversion is a biologic process to transform biomass materials from one form to another and also known as biotransformation. One of the most important bioconversion product is mushrooms as a result of cultivating on industrial and agro-industrial wastes.

Substrates are used for fungal cultivation preferentially determined by regional availability (Kulshreshtha et al., 2014). For example lignocellulosic wastes, like sawdust, straw and wood chips can be used for cultivation of mushroom which can be further use as a product, like food.

Using mushrooms as mycoremediation tool in the process of industrial waste bioconversion, result as protein rich fruiting bodies of mushroom (Kulshreshtha et al., 2014). On one hand this process producing mushrooms as a food, on the other hand it helps in solving pollution problems.

(iii) Biosorption

Biosorption is a physico-chemical and metabolically-independent process which includes absorption, adsorption, ion exchange, surface complexation and precipitation. Biosorption has been adressed the removal or recovery of metallic ions/ pollutants/ xenobiotics from solution by living or dead microorganisms and their components, plant materials, industrial and agricultural wastes and natural residues (Fomina and Gadd, 2014; Gavrilescu 2004). Biomass exhibits as an ion exchanger of biological origin. Mushroom mycelium and spend mushroom compost is cheap and easy way to prepare a biosorbent.

Opposite to biosorption, bioaccumulation process carried out by living cells. Biosorption and bioaccumulation participate in elemental cycling in the environment (Chojnacka, 2010). Living organisms by its very nature bioaccumulate chemical substances, pollutants, toxicants in biomass and this processes occur permanently. The uptake of pollutants by mushrooms connect with combination of two processes, one is an active metabolism-dependent process termed as *bioaccumulation* and the other one is not requiring metabolic energy termed as *biosorption*.

There are some difference between biosorption and bioaccumulation processes (**Table 1.2**). Whereas bioaccumulation is carried out by living cells an active metabolism-dependent processes, which includes both transport into the cell and partitioning into intracellular components, biosorption is a process of the binding of pollutants to the biomass without requiring metabolic energy which carried out by dead biomass. Unlike living mushroom biomass, dead mushroom biomass is not sensitive to concentrations of toxicants and adverse operating conditions like pH, temperature, nutrient supply, initial metal ion concentration, and the concentration of cells.

Table 1.2 : The comparison between biosorption and bioaccumulation.

Biosorption	Bioaccumulation
Passive process	Active process
Biomass is not alive	Biomass is alive
Metals are bound with cellular surface	Metals are bound with cellular surface and interior
Adsorption	Absorption
Reversible process	Partially reversible process
Nutrients are not required	Nutrients are required
Single-stage process	Double-stage process
The rate is quick	The rate is slow
Not controlled by metabolism	Controlled by metabolism
No danger of toxic effect	Danger of toxic effects caused by contaminants
No cellular growth	Cellular growth occurs
Intermediate equilibrium concentration of metal ions	Very low equilibrium concentration of metal ions

Source: Adapted from Chojnacka, 2010.

2. THE KINGDOM OF FUNGI

The Fungi Kingdom show a range of morphologies from unicellular yeasts to polymorphic and filamentous fungi, many of which have complex macroscopic fruiting bodies (Gadd, 1992). Ecological mission of fungi are decomposing of organic materials with concomitant nutrient cycling, provide continuance of natural selection and population adjustment by their pathogenic activity and being symbionts of animals and plants.

It is esstimated that there may exist from 1 to 5 million species fungus in addition to currently described 100 000 fungal species (Myllys et al., 2013). Fungi were classified as plants until 1960s, but at present according to DNA and RNA analysis in 1990s genomic researches, it was revealed that fungi are actually more closely related to animals than plants (Myllys et al., 2013).

The classification of fungi updates. Based on the current knowledge, the kingdom of Fungi composed of seven phyla which are called as Microsporidia, Blastocladiomycota, Neocallimastigomycota, Chytridiomycota, Glomeromycota, Ascomycota, and Basidiomycota (Hibbett et al., 2007).

Fungi can be defined as non-photosynthetic hyphal eukaryotes and related forms (Carlile et al., 2004). The majority of fungi are composed of vegetative structures called as *mycelia* that comprises a mass system of branching, threadlike *hyphae* (**Figure 2.1**). The hypha is divided by cross-walls into compartments which are equivalent to cells of other organisms. Such compartments may have several nuclei (Singh, 2006). In other words hyphal growth forms branches and this network is called *mycelium* (**Figure 2.2**). This branching threads thrive through the soil, compost, wood or other lignocellulosic material on which the fungus is growing (Chang and Miles, 2004). The fungal cell wall is composed of chitin which is composed of glucosamine. Chitin is a rigid heteropolymer matrix. Under favorable conditions after growth period, matured mycelium produces the fruiting structure, which we call it as *mushroom* (Chang and Miles., 2004).

Figure 2.1: *Pleurotus ostreatus* mycelium in petri dish on coffeee grounds.
Source: Adapted from Tobi Keller, 2012.

Fungi obtain energy from chemicals and use organic compounds as carbon source which are called chemotrophic heterotrophs. Fungi secrete extracellular enzymes to achieve extracellular digestion by degrading polymers outside of the cell and then absorb nutrients into the cell through the cell membrane (Carlile et al., 2004).

According to growth modes, fungi generally display either yeast-like or filamentous. Yeasts are unicellular organisms a single nucleus per cell bounded by a cell wall. Filamentous fungi display several distinct morphologies.

For fungal cultivation, optimizing environmental conditions are important for fungal growth and compost yield. Almost all fungi are aerobes, oxygen absolutely necessary for growing. In absence of oxygen fungi grow poorly or not. Due to the fact that fungal cell mass composed of 70 – 90 % water and all biochemical reactions take place inside the cell, in cytoplasm, water is essential for fungi like all life forms too.

For most fungi maximum temperature for growth is between 30 – 40 ^{o}C and if compost temperature rise over 40 ^{o}C some thermophilic fungi can also grow in composts (Carlile et al., 2004).

Generally fungi prefer acidic growth conditions, between pH 4-7. In fungal cultivation, compost pH is usually keep as low as possible while it encourages the fungal growth, does not allow bacterial contamination (Carlile et al., 2004).

Furthermore *Pleurotus ostreatus* forms the most primordia in a response to light, was reported. Continuous, optimal light stimulation during the primordia formation resulted in the largest population of primordia. Jang et al. (2013) also noted that *Pleurotus ostreatus* require light to develop fruiting body properly.

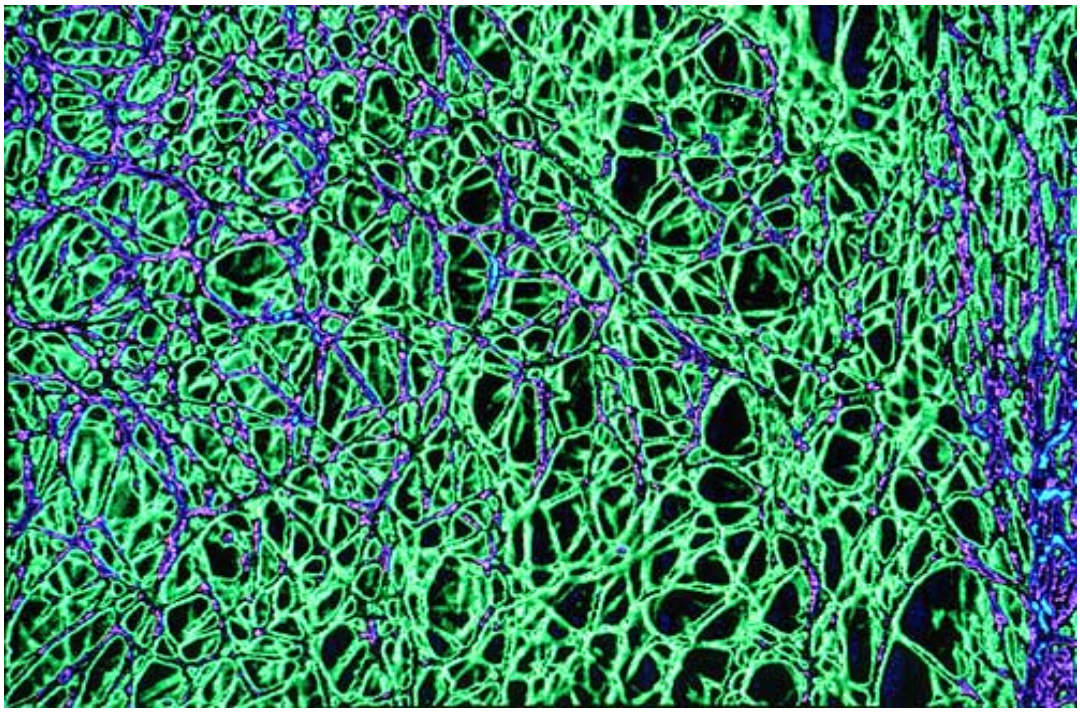


Figure 2.2 : Scanning Electron Micrograph of Mycelium (Magnified 500 X).
Source: Adapted from Stamets, 2005.

This study focus on the ligninolytic activity of wood-degrading, white-rot fungus, *Pleurotus ostreatus.*

2.1 Wood-Degrading Fungi

Basidiomycota is a phylum of Kingdom of Fungi which compose of 32 % of the described fungi including most wood-decaying species, as well as pathogens and mutualistic symbionts (Riley et al., 2014).

Based on the ability on degrading wood component, wood-decaying basidiomycetes have been classified as white-rot and brown-rot fungi (Riley et al., 2014). The biodegradation of wood constituents into small molecules carry out with multi-enzymatic cooperation. The white rot fungi attack directly the “lignin barrier” by a

versatile machinery of enzymes. This multienzyme system which include “feed back” type enzymes, allows simultaneous transformation of both lignin and cellulose (Leonowicz et al.,1999) . Lignocellulose is combination of lignin and cellulose made up of densly packed polysaccharide components in lignin layer called as microfibers, which protect them against the activity of hydrolytic enzymes and other external factors (Fengel, 1971). The structure of lignocellulose in plant cell wall is presented in **Figure 2.3** (URL-1).

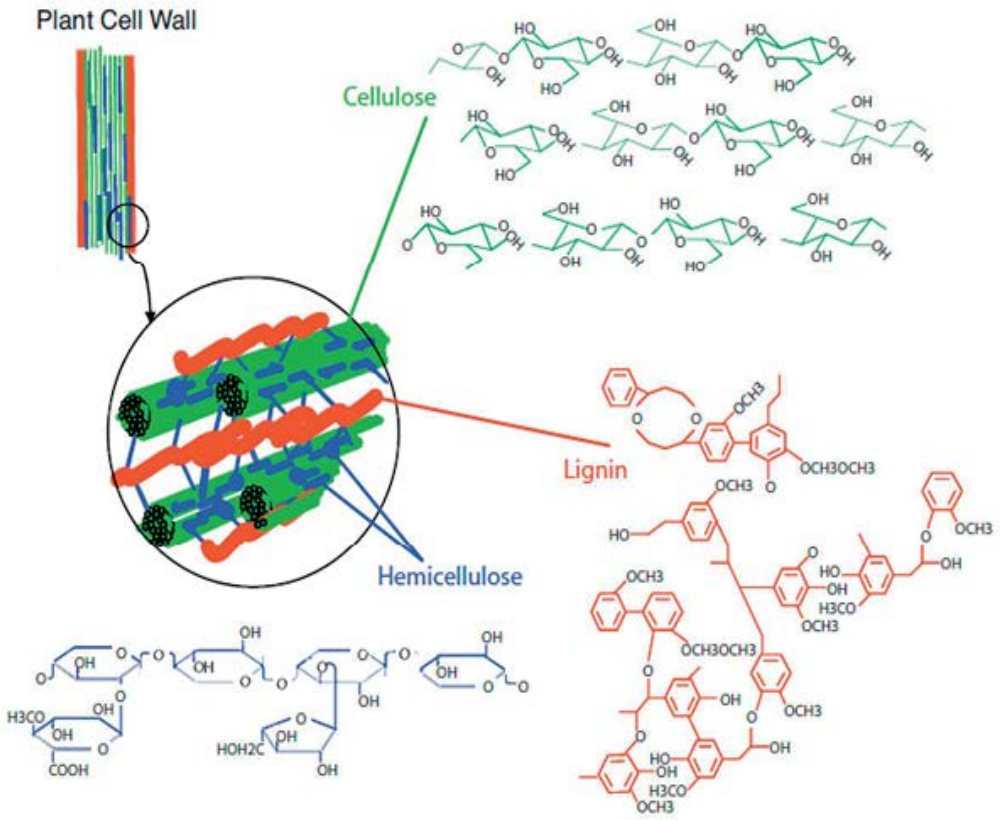


Figure 2.3: Structure of lignocellulose in plant cell wall.

White rot fungal strategy is decomposing the lignin in wood and gain access to the cellulose and hemicelluloses that are embedded in the lignin matrix. The brown rot fungi preferentially decay the cellulose in wood and do not degrade the lignin extensively (Hammel, 1997). The two groups that have an ability of mineralizing lignin called *ligninolytic fungi*. Some white-rot fungi removing lignin first, use hemicellulose and cellulose as energy source (Winquist, 2014). They can simplify energy exchange between the aboveground and belowground systems (Singh, 2006).

Since removal of dark colored of lignin, white-rotted wood residue turned into white, and gains soft and fibrous structure. Because of the reason brown-rot fungi degrade

cellulose and hemicellulose, and modify lignin brown-rotted wood residue is dark and shrink. The colour of wood residue indicates the presence of lignin modification (Hatakka, 2001).

Most of wood-degrading species belong to members of the genera Phanerochaete, Pleurotus and Trametes. These species also have an ability to survive in soil when suitable substrates are available (Baldrian, 2008). When some of them are able to grow in living trees called as *pathogenic fungi* but mostly grow on dead trees called as saprotrophic fungi (Winquist, 2014). The roles of wood-degrading fungi in ecosystem are decomposing wood and recycling the nutrients. Moreover, fungi modify soil permeability and soil ion exchange capacity and detoxify contaminated soil (Singh, 2006).

2.1.1 White-rot fungi

Lignin degradation of white-rot fungi have been examined more than fifty years. For the first time in 1985 Bumpus et al. proposed the use of this fungus for bioremediation following the discovery of the extracellular oxidative ligninolytic enzymes in a white-rot fungus *Phanerochaete chrysosporium*. The white-rot fungus *P. chrysosporium* has emerged as an archetypal model system for mycoremediation. During the last decade fungi have been used in the treatment of a wide variety of wastes like phenolic and chlorinated phenolic compounds, petroleum hydrocarbons, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, heavy metals, chlorinated insecticides and pesticides, dyes, biopolymers and wastewaters (Singh, 2006).

2.1.2 *Pleurotus ostreatus* (Jacquin ex Fries) Kummer

Pleurotus ostreatus the prototypic oyster mushroom, has long been a favorite of mushroom hunters for food, especially find in the spring time in lowland, hardwood forests and widespread in many temperate and subtropical forests throughout the world. It is a saprotroph that acts as a primary decomposer of wood, especially deciduous trees, and beech trees in particular as well as is one of the few known carnivorous mushrooms. Its mycelia can kill and digest nematodes, which is believed to be a way in which the mushroom obtains nitrogen.

While this mushroom is often seen growing on dying hardwood trees, it only appears to be acting saprophytically, rather than parasitically. As the tree dies of other causes, *P. ostreatus* grows on the rapidly increasing mass of dead and dying wood. They

actually benefit the forest by decomposing the dead wood, returning vital elements and minerals to the ecosystem in a form usable to other plants and organisms (Stamets, 2000).

A US company, *Ecovative Design*, has proposed using the mycelium along with the growing substrate as a substitute for petroleum derived expanded polystyrene packing material or as an insulating material (URL-1). The oyster mushroom can be consider as a medicinal mushroom, since it contains lovastatin which work to reduce cholesterol (URL-2). *Pleurotus ostreatus* common names are; The Oyster Mushroom, Oyster Shelf, Tree Oyster, Straw Mushroom, Hiratake (Japanese for "Flat Mushroom"), Tamogitake (Japanese). For Genus Pleurotus, *Pleurotus ostreatus* is the type species which represents a huge complex of subspecies, varieties and strains. *Pleurotus ostreatus* dominates in river-bottoms and common from the spring through late fall (Stamets, 2000). Scientific classifiation of Pleurotus ostreatus shown in **Table 2.1**.

Pleurotus ostreatus present some advantages for its cultivation. First of all it can be grown on wide range of substrates, it is cheap to produce, easy to grow and taste in good.

Table 2.1: Scientific classification of *Pleurotus ostreatus.*

KINGDOM:	Fungi
DIVISION:	Basidiomycota
CLASS:	Hymenomycetes
ORDER:	Agaricales
FAMILY:	Tricholomataceae
GENUS:	Pleurotus
SPECIES:	*Pleurotus ostreatus*

2.2 White-Rot Fungi in Heavy Metal Bioremediation

Based on the literature, total research of fungal bioremediation have been on white-rot fungi. A wide range of substrates like agricultural wastes such as wood chips, wheat straw, corncobs, sawdust, bark, rice, annual plant stems and wood, alfalfa, spent mushroom compost, sugarcane bagasse, coffee pulp, sugar beet pulp, okra,

canola meal can be activated in inoculum production off-site or on-site or mixed with contaminated soils to enhance degradation (Singh, 2006).

Together with the ability to degrade a wide range of pollutants, capability to colonize in soil matrix, resist at high concentrations of organic or inorganic compounds, survive in restrictive conditions make microorganisms useful for soil bioremediation. The specific characteristics of different groups of fungi make an organism a useful bioremediator (Anastasi et al., 2013) (**Figure 2.4**).

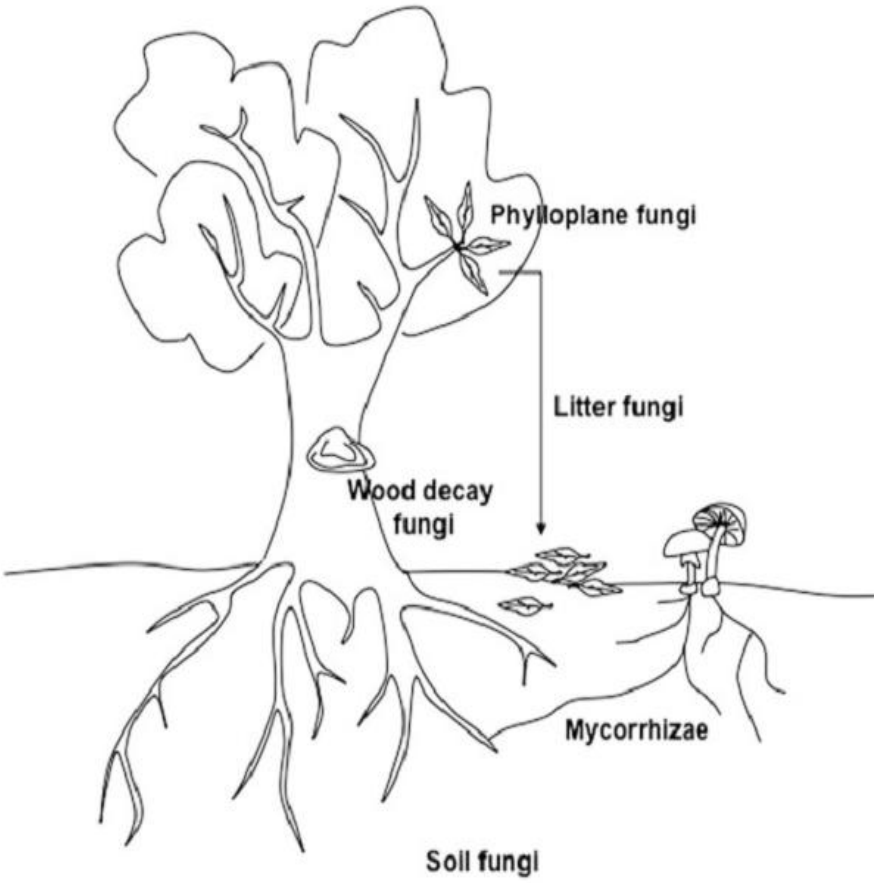


Figure 2.4: Different ecophysiological groups of fungi potentially involved in bioremediation. *Source:* Adapted from Anastasi et al., 2013.

The easiest method of treating contaminated soil is, adding organic matter to contaminated area and allow organic matter to form compounds with contaminant (i.g heavy metals) (Kellogg and Pettigrew, 2004). This process called as composting and it is applicable to waste conversion into mulching and treatment of hazardous waste (Adenipekun and Lawal, 2012).

Until the present time, using fungal approaches to degrade petroleum hydrocarbons, mycoremediation has grown out of prior research area (Atlas and Bartha, 1992; Cerniglia et al., 1992). White-rot fungi are also used in bioremediation of polluted soils and accumulation of heavy metals and involved in mineralization, biodeterioration, biodegradation, transformation and co-metabolism (Bennet et al., 2002).

Heavy metals that found in the environment directly interact with fungal extracellular enzymes (Adenipekun and Lawal, 2012). Heavy metals which are taken up by the fungus cause a physiological response. Metal responsive elements (MRE) were identified in the promoters of *Pleurotus ostreatus* laccase genes poxc and poxa1b which interact with Cu-responsive transcription factors (Sannia et. al, 2001).

The metals are taken up environment concentrated in white-rot fungi mycelia. White-rot fungi growing on wood in nature while accumulating Cd, Fe, Zn and Cu from wood to their fruit bodies, exclude Mn and Pb (Baldrian, 2002).

The researches on white-rot fungi and heavy metal interactions have been generally focus on fungal extracellular enzymes production as a response to heavy metal presence in environment. These enzymes have crucial role in both mycoremediation processes and industrial enzyme technology and its applications. Lignin-modifying enzymes (LMEs) are extracellular non-specific oxidative enzymes which are used for several applications in pulp and paper processing industries, enzymatic hydrolysis of lignocellulosic substrates and bioremediation of contaminated soils and effluents.

Addition of 1–5 mM Cd increased the laccase activity in *Pleurotus ostreatus* (Baldrian, 2002). When extract was added from straw culture, copper increased the activity of extracellular laccase production, addition of 1 mM Ag, Hg, Pb, Zn, and H_2O_2 decreased activity of laccase (Baldrian, 2002).

It was noted that during the growth of *Daedalea quercina* in equimolar metals containing media, metal ions accumulating decreased in the order Zn>Cu>Pb>Al (Gabriel et al., 1996). The preference for individual heavy metals is specific to species (Baldrian, 2002). During 8-day cultivation of four white-rot fungi *Daedalea quercina*, *Ganoderma applanatum*, *Stereum hirsutum* and *Schizophyllum commune,* it was reported that in 1 mM solutions of Al, Cd, Pb, and Ca each, most Pb was found in *Stereum hirsutum*, most Cd, Al, and Ca in *Ganoderma applanatum*. From equimolar solution (1 mM), Pb was accumulated by all fungi except *Ganoderma*

applanatum but *Ganoderma applanatum* was accumulated more Al than other white-rot fungus (Gabriel et al., 1994).

There are three phases for the successful implementation in mycoremediation process. Using techniques to prepare inoculum and their improvements lead to success in the first phase of the use of white-rot fungi in mycoremediation process. The second phase associated with engineering processes which includes clarifying technical protocols for final design. Monitoring, adjustment, continuity, and maintenance of the engineering system guide to success for the final phase of the mycoremediation process (Singh, 2006).

For soil bioremediation, processes using white-rot fungi have been patented by a few companies, like EarthFax Development Corporation in Utah and Gebruder Huber Bodenrecycling in Germany. These companies employ white-rot fungi for soil bioremediation, but at the present time there is not known a broader use (Singh, 2006).

3. INTERACTIONS OF HEAVY METALS WITH WHITE-ROT FUNGI

Heavy metals are natural constituents of the earth crust. Their non-biodegradable, non-thermodegradable features contribute to environmental pollution mainly.

The study of the interaction between toxic metals and fungi has long been of scientific interest which basis of many fungicidal preparations used in the control of plant pathogens (Horsfall, 1956; Ross, 1975).

Following observations on the ability of fungi to resist and adapt to toxic metals, stimulated further research on physiological, biochemical and genetic explanations for these phenomena (Ashida, 1965; Ross, 1975; Gadd, 1986a).

Environmental pollution speeding up by toxic metals, metalloids, radionuclides and organometal(loid)s has also led to increased interest to fungi which are sometimes presence in metal-polluted habitats. It is also point at the uptake and translocation of toxic metals and radionuclides to fruit bodies of edible fungi, and the significance of mycorrhizal fungi in polluted habitats (Gadd, 1986a; Brown and Hall, 1990).

3.1 Classification of Metals

Metals are directly and/or indirectly affect fungal growth, metabolism and differentiation. While many metals such as K, Na, Mg, Ca, Mn, Fe, Cu, Zn, Co and Ni are essential for fungal metabolism, many others like Rb, Cs, Al, Cd, Ag, Au, Hg and Pb have no apparent essential function (Gadd, 1992). Anyhow, all these elements can interact with fungal cells and be accumulated by physico-chemical mechanisms and transport systems (Gadd, 1988).

Most essential and non-essential metals display toxicity above a certain concentration. The concentration of toxicity depends on the organism, the physico-chemical properties of the metal and environmental factors (Gadd, 1992). This situation may require expression of a detoxification mechanism in living organisms. (**Figure 3.1**).

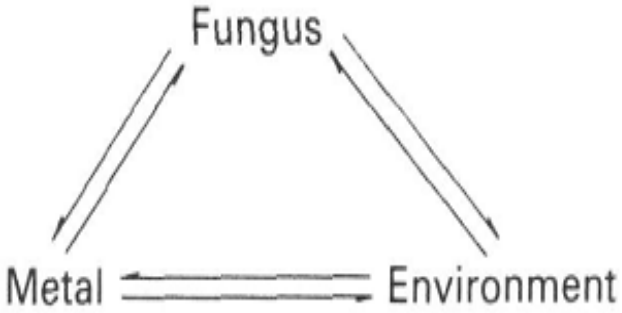


Figure 3.1: Schematic representation of metal-fungal interactions. *Source: Adapted from* Gadd, 1992.

3.1.1 Heavy metals

The term of "heavy metals" are often defined as a group of approximately 65 metallic elements, of which density greater than 5, with the general ability to show toxic effects on organisms (Gadd and Griffiths, 1978; Nieboer and Richardson, 1980).

3.1.2 The most poisonous heavy metals in the environment

The most common heavy metals found at contaminated sites, in order of abundance are Pb, Cr, As, Zn, Cd, Cu and Hg (U.S. EPA, 1996). Those metals are important since they are capable of decreasing crop production due to the risk of bioaccumulation and biomagnification in the food chain. There's also the risk of superficial and groundwater contamination. Moreover cadmium, mercury and lead can describe as three big poisinous metals which are not known essential role for living organisms (Wuana and Okieimen, 2011).

Knowledge of the basic chemistry, environmental and associated health effects of these heavy metals is necessary in understanding their speciation, bioavailability, and remedial options.

3.1.2.1 Cadmium

Cadmium is a transition element with atomic number 48, atomic weight 112.4, density 8.65 g cm^{-3}, melting point 320.9 ˚C, and boiling point 765 ˚C which directly below Zinc in the periodic table.

In compounds, Cd occurs divalent Cd(II) ion. Cadmiums chemical similarity to Zn which is an essential micronutrient for plants and animals, probably responsible of its toxicity (Wuana and Okieimen, 2011).

Cadmium mostly uses in Cd/Ni batteries. Other useage areas of Cd are pigmentation and polyvinyl chloride (PVC) stabilizator, in alloys and electronic compounds. Cadmium is also present in phosphate fertilizers, detergents and refined petroleum products. The application of agricultural fertilizers, pesticides, and sewage sludge and the disposal of industrial wastes or the deposition of atmospheric contaminants increases the total concentration of Cd in soils. Cadmium is very biopersistent and once absorbed by an organism, remains resident for many years (Wuana and Okieimen, 2011).

3.1.2.2 Mercury

In the periodic table, mercury belongs to same group with zinc and cadmium. Mercury is the only liquid metal at standard temperature and pressure which has atomic number 80, atomic weight 200.6, density 13.6 g cm^{-3}, melting point −13.6 ˚C, and boiling point 357 ˚C.

Mercury is usually recovered as a byproduct of ore processing (Smith et al., 1995). Major source of Hg contamination is coal combustion. Hg occurs in naturally and exists in mercuric (Hg^{2+}), mercurous (Hg_2^{2+}), elemental (Hg^o), or alkylated form (methyl/ethyl mercury) (Wuana and Okieimen, 2011).

3.1.2.3 Lead

In periodic table, lead belogs to group IV and period 6. Its atomic number 82, atomic mass 207.2, density 11.4 g cm^{-3}, melting point 327.4 ˚C, and boiling point 1725 ˚C. It is a naturally occurring, blue-gray metal usually found as a mineral combined with other elements, such as sulphur (i.e., PbS, $PbSO_4$), or oxygen ($PbCO_3$), and ranges from 10 to 30 mg kg^{-1} in the earth's crust.

Mostly Pb is used in manufacturing of Pb storage batteries. Other uses include solders, bearings, cable covers, ammunition, plumbing, pigments, and caulking (Wuana and Okieimen, 2011).

3.2 Fungitoxicity of Metals

3.2.1 Uptake of heavy metals

Heavy metals present in the environment directly interact with fungal extracellular enzymes and cause a physiological response. White-rot fungi can accumulate metals in their mycelia (**Figure 3.3**).

The metal uptake partially depends on the membrane potential which is linked with Ca transport. The fruiting bodies of most white-rot fungi contain high amounts of Ca which play a role in cotransportation of non-essential bivalent metals (Tyler, 1980). The heavy metals accumulation mechanism in molecular level have not been studied in white-rot fungi.

It was reported that *Pleurotus ostreatus* was able to accumulate 20 mg g^{-1} dry weight Cd from 150 ppm Cd containing liquid medium and at least 20 % of accumulated Cd deposited intracellularly (Favero et al., 1991).

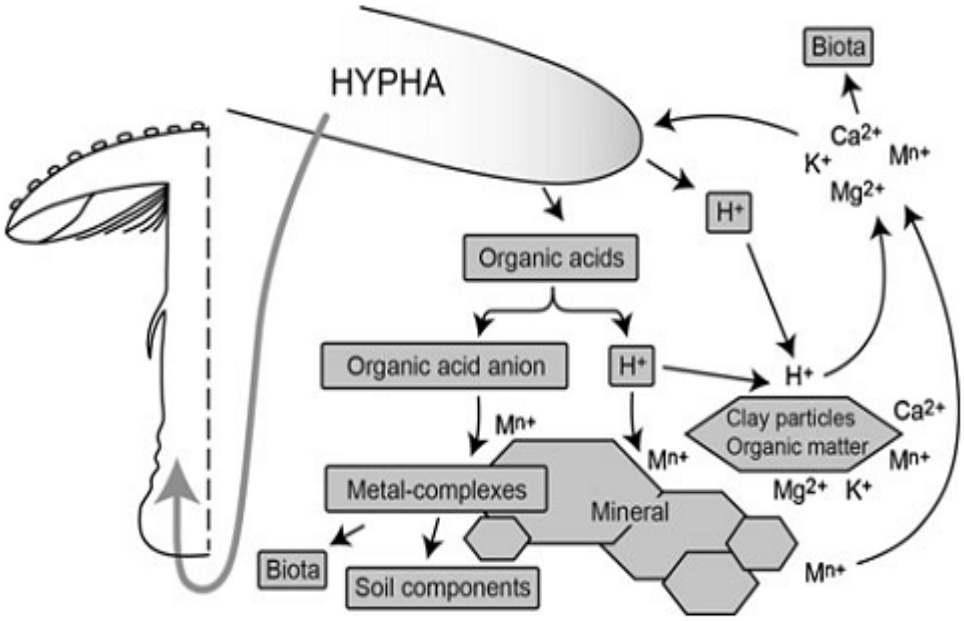


Figure 3.2: Dissolution of metals in soil and their transfer to fruiting body. *Source:* Falandysz and Borovička, 2013 (Modified from Gadd, 2004).

3.2.2 Mechanisms of toxicity

Toxic metals can cause harmful effects in many ways that include the blocking of functional groups of biologically important molecules (e.g. enzymes and transport systems for essential nutrients and ions), the replacement and/or substitution of essential metal ions from biomolecules and functional cellular units, conformational modification, denaturation and inactivation of enzymes and disruption of cellular and organellar membrane stability, as a result of their strong coordinating abilities (Ochiai,1987) (**Fig. 3.2**).

The metal species is represented in cationic form. All the interactions shown maybe involved in fungal survival and/or metal detoxification.

M^{n+}

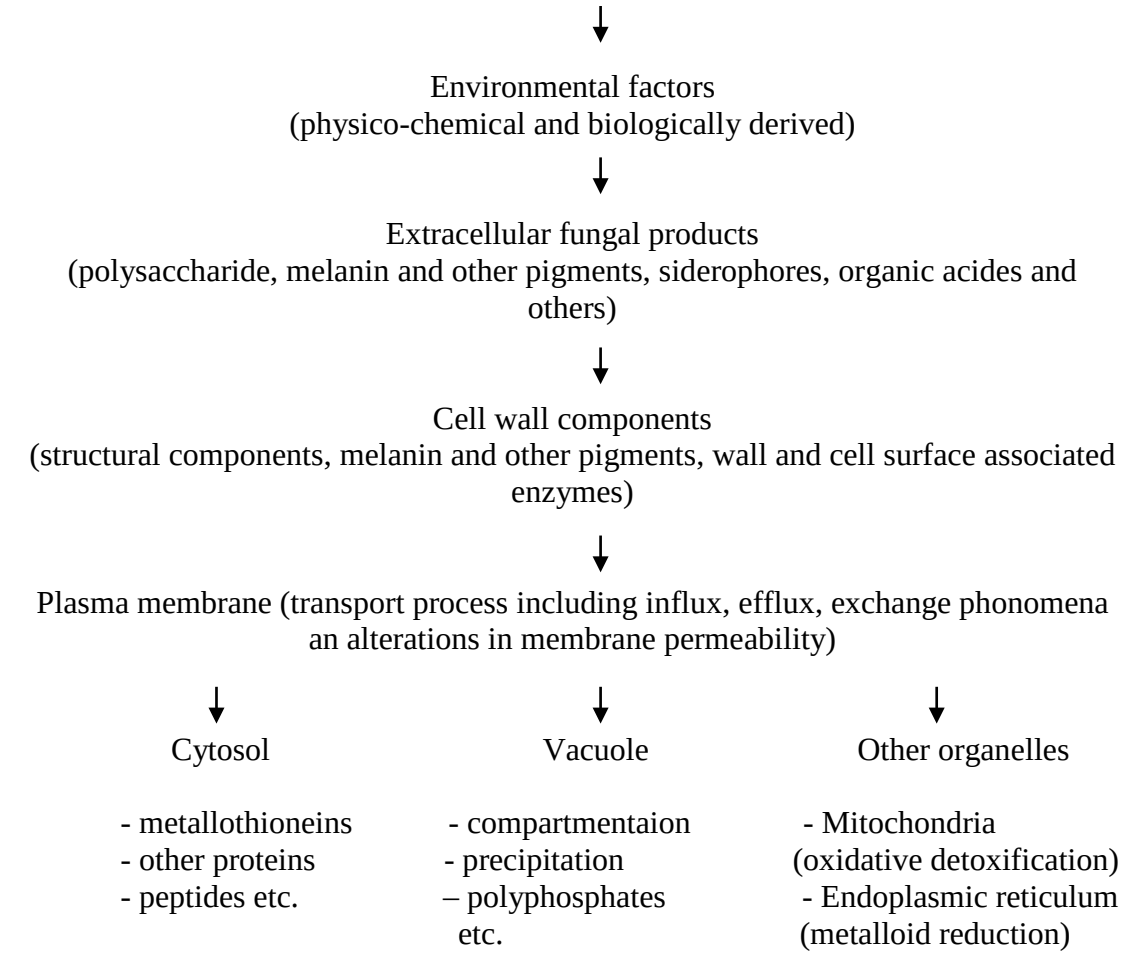


Figure 3.3: Diagrammatic representation of metal interactions with fungi.
Source: Adapted from Gadd, 1992.

3.2.3 Environmental influence on heavy metal toxicity toward fungi

Metal speciation, biological availability and toxicity depends on the physico-chemical properties of a given environment, or growth medium (Gadd, 1992). These properties determine essential and non-essential metal-organism interactions. Metal-fungal responses can be affected by pH. The pH affects metal speciation and mobility and influencing other aspects of cell physiology and metabolism indirectly. Increasing pH can result of metal hydroxides or oxides formation and precipitation. (Collins and Stotzky, 1989; Hughes and Poole, 1991).

External pH may also affect the speciation of metal-binding ligands. Most of toxic interactions between heavy metals and fungal cells can be interpreted in terms of pH effects (Newby and Gadd, 1986; Gadd, 1986a). For example fungitoxicity of Cd may increase with increasing pH (pH 5-9), possibly as a result of formation of $CdOH^+$ which may enter cells more easily than Cd^{2+} (Gadd, 1992). The fungal cell wall is the first cellular site which interact with metal species. Metal removal from solutions

depends on type and concentration of metal ion(s), biomass and environmental factors (Gadd, 1992).

The fungal cell wall act as a barrier which control the uptake of solutes into the cell that include toxic metal species (Gadd, 1986 a,b).

3.2.4 Resistance and tolerance

Inner biochemical and structural properties, physiological and including morphological changes genetic adaptations and environmental modification of metal speciation, metal ions availability and toxicity determine fungal survival in the presence of toxic metals (Gadd and Griffiths, 1978; Gadd, 1990b, 1992b).

Whereas *metal resistance* is defined as the ability of an organism to survive metal toxicity by means of a mechanism produced indirect response to the metal ions such as synthesizing metallothioneins or γ-glutamyl peptides (Mehra and Winge, 1991), *metal tolerance* is defined as the ability of an organism to survive metal toxicity by means of inner properties and/or environmental modification of toxicity (Gadd, 1992 b,c).

3.2.5 Defense mechanism

The interaction of fungi with heavy metals can cause even killing the mycelium. For this reason, fungi evolved active defense mechanisms to ease off the toxicity of metals (Baldrian, 2002).

The defense mechanism is usually based on using extracellular and intracellular chelating compounds to immobilize the concerned heavy metals. Oxalate is one of the typical metal chelators produced by both white-rot and brown-rot fungi. Oxalic acid production by fungi immobilize soluble metal ions and complexes as insoluble oxalates. It was reported that among white-rot fungi, *Pleurotus ostreatus* and *Pleurotus chrysosporium* and produce higher amounts of oxalate (Shimada et al., 1997).

3.3 Effects of Heavy Metals on White-Rot Fungi

Metal species may positively or negatively affect fungal growth, metabolism and differentiation depending on the element(s) concerned. Fungi may remove metals from solution by physico-chemical and biological processes and/or transform them

into other forms, including organometal(loid)s. Fungi may alter the environment by metabolic activities and growth (Gadd, 1992).

3.3.1 Growth and metabolic activity

Growth is a complex phenomenon when consider the viewpoint of heavy metals toxicity. For all white-rot fungi, Cd and Hg are the most toxic metals in general (Baldrian, 2002) and the essential metals are less toxic than others.

In *Phanerochaete chrysosporium* growth rate was limited in presence of 50 ppm Ni, Cd, and Pb whereas it was decreased presence of 150 ppm Co and Cu and Mn in 300 ppm (Falih, 1998). At the same time, it was noted that low concentrations of the essential metals like Co, Cu and Mn increased the growth rate partially (Falih, 1997). In *Pleurotus ostreatus,* the highest substrate decomposition was observed at 0.5–1 mM Cd (Gabriel et al., 1996). Colonization in soil interferes with presence of metals (Baldrian et al, 2000).

3.3.2 Mycelial morphology

The decrease of fungal growth rate which caused by heavy metals is often accompanied by morphological changes on growing mycelium. Heavy metals induce morphological changes among all groups of fungi. For instance presence of heavy metals in growing cultures of fungi, caused to discoloration (Baldrian, 2002).

3.3.3 Fungal extracellular enzymes

In general, according to reaction they catalyse, enzymes divided into six classes: oxidoreductases (EC 1), transferases (EC 2), hydrolases (EC 3), lyases (EC 4), isomerases (EC 5) and ligases (EC 6) (URL-2).

Most fungal extracellular enzymes belong to oxidoreductases and hydrolases enzyme groups (Lundell and Mäkelä, 2013). Oxidoreductases catalyse the oxidation or reduction reactions, i.e. transfer of hydrogen or oxygen atoms or electrons from one substrate to another. Hydrolases catalyse hydrolysis reactions, i.e. the breaking chemical bonds by the addition of water (URL-3).

There are two heavy metals act the part of the ligninolytic reactions which catalyzed by certain enzymes. In lignin degradation, role of manganese (Mn) has been studied in detailed. Manganese is directly roled in the Mn-dependent peroxidase (MnP) catalytic cycle. It was also reported that Mn plays a regulatory role in the expression of lignin peroxidase, laccase and in the degradation of lignin (Périé and Gold, 1991;

Perez and Jeffries, 1992). The other heavy metal copper (Cu) is the cofactor of the laccase, an enzyme involved in lignin degradation. It has been known for long time that copper presences the catalytic center of the laccase but the important regulative role of copper in laccase production has only recently been addressed. (Baldrian, 2002).

In general heavy metals are inhibitors of enzymatic reactions (Vallee and Ulmer, 1972). In white-rot fungi, the extracellular enzymes often face high concentrations of metals, since they are not protected by the cell-associated metal-detoxification mechanisms. When metals enter the cell, affect the production of extracellular enzymes on the levels of transcriptional and translational regulation (Baldrian, 2002).

Mercury exhibits its toxicity by binding to SH groups present in the active or regulation sites of enzymes on which caused irreversible inactivation. Copper and cadmium bind to aromatic amino acid residues of enzymes and also cause oxidative damage of proteins by inducing oxidative stress which associated with the production of reactive oxygen species like hydroxyl or superoxide radicals (Stohs and Bagchi, 1995). It seems that low concentration of essential heavy metals are required for development of the ligninolytic enzyme system. Also the metals increased solubilization and mineralization of lignin (Singhal and Rathore, 2001).

3.3.4 Fungal reproduction

It was found that heavy metals are harmful for fungal reproduction in different taxonomic groups. In white-rot fungi, the sensitivity of fruiting show differences species to species. For example *Agrocybe perfecta* failed to produce fruit bodies during growth on 0.05–1 mM Cd containing straw, *Pleurotus ostreatus* was much less sensitive (Gabriel et al., 1996). In another strains of same species, sporocarps formation unaffected by up to 285 ppm Cd in the fungal substrate (Favero et al., 1990) According to accumulation amounts, it is probably said that Cd accumulated preferentially in caps by accumulated 56 ppm Cd whereas stems accumulate 36 ppm (Gabriel et al., 1996).

4. MATERIALS AND METHODS

4.1 Materials and Equipments

In this study first of all, oyster mushroom *Pleurotus ostreatus* was cultivated. *P. ostreatus* commercial straw spawn and a bale of straw which was used for this study supplied from Marmara Mantar, Çatalca/Istanbul.

During the fungal cultivation process, for fungal tissue culture malt-extract agar was used. Malt extract agar and agar-agar were supplied from Merck and barley grains which were used for generating mother spawn bought from a local market in Üsküdar.

Soil samples were taken from Istanbul Technical University Ayazaga Campus, Maslak/Istanbul. The heavy metal solutions were delivered from Merck.

The equipments, chemicals, solutions, results of heavy metal analyses in soil and in mycelium were listed in Appendix A, Appendix B, Appendix C, Appendix D and Appendix E, respectively at page 91-99.

4.1.1 *Pleurotus ostreatus* commercial spawn bag

Pleurotus ostreatus commercial spawn bag delivered from Marmara Mantar Compost Production Factory, Çatalca in June, 2014.

During one month it was stored in a dark room with the temperature between 25-30^{o}C. At the end of thirty days, when the compost completely turned to white and the first fruiting bodies appeared, it was moved to a brighter place which protected from direct sunlight and watered everyday (**Figure 4.1**).

Growing fruiting bodies collected to get pure culture in petri dishes.

Figure 4.1: *Pleurotus ostreatus* commercial spawn bag.

4.1.2 Experimental soil

Approximately 20 kilograms of soil was taken from Istanbul Technical University Ayazağa Campus in July, 2014 in 5 cm depth (**Figure 4.2**). Because of compact structure of soil firstly it was sieved with Mesh No.7 which is equal to 2.83 mm, then with Mesh No.10 which is equal to 2 mm particule size. Soil was stored in a covered plastic container at room temperature until using for experiments. In all experiments sterilized soil was used.

Figure 4.2: Experimental soil sampling area.

4.1.3 Heavy metals

Heavy metals which used in this experiments supplied from Merck. Heavy metals as standard solutions which used in this study were lead (Pb), cadmium (Cd) and mercury (Hg). The stock solutions are in 1 g/l concentration (**Figure 4.3**).

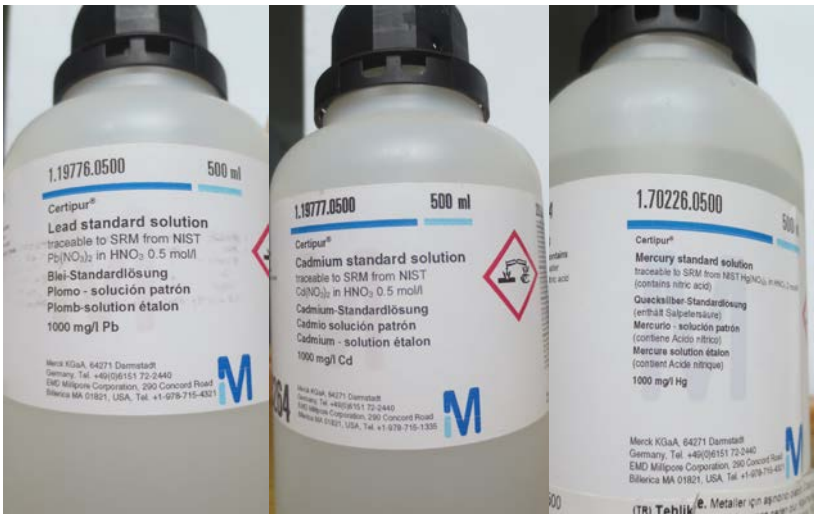


Figure 4.3: Lead, cadmium and mercury standard solutions.

4.2 Methods

4.2.1 *Pleurotus ostreatus* cultivation procedure

Pleurotus ostreatus cultivation includes three inoculation stage. In first stage fungal tissues which taken out from fruiting body under sterile conditions inoculated on solid state medium. It is called as *tissue culture*. In this experiment 2 % Malt Extract Agar was used as solid medium. Secondly, growing mycelium on agar used for generating mother spawn by inoculation of already sterilized grains. In this experiment barley grains were used. In third stage pasteurized wheat straw inoculate with mother spawn for mushroom spawn preparation. In this experiment wheat straw used as bulk substrate.

Spawn is the vegetative mycelium of a selected mushroom which is grown on a convenient medium like wheat straw, sawdust, wood chips, etc for raising mushroom crop. It essentially involves in pure culture preparation of mushroom from tissues on any agar medium, followed by culturing on sterilized grains and further multiplied on grains. The spawn composed of mycelium of the mushroom and a supporting medium which provides nutrition to the fungus during its growth (Kumar, 1995).

The spawn prepared using pure culture mycelium on agar medium in petri plates as inocculant is called as *mother spawn*. Two or three weeks after inoculation fully

colonized mother spawn bottles can be used for inoculating commercial spawn bags (Kumar, 1995).

4.2.1.1 Malt extract agar

Malt extract agar is used as a general purpose growth media to isolate and cultivate yeasts and molds from clinical samples, as well as a wide range of environmental sources.

The medium prepared by 4 g malt extract and 4 g agar agar dissolved in 200 ml distilled water in a 500 mL glass bottles before sterilization in autoclave for 121^{o}C, 15 minutes. *Sterilization* procedure was used for killing bacteria, viruses, fungi and their spore forms present on a surface, in a fluid, in medication, or culture media. After sterilization process media left to get warm but not to allow solidified and poured into 20 mL in per petri dish.

In the first step of pure culture preperation, collecting fruting bodies of *Pleurotus ostreatus* was taken to laminar flow cabin. Biustri and pens were sterilized by beck flame and fruiting bodies divided into two parts from root area. Sterile mushroom tissues took out with pens and replaced in agar plugs (**Figure 4.4 a, b**).

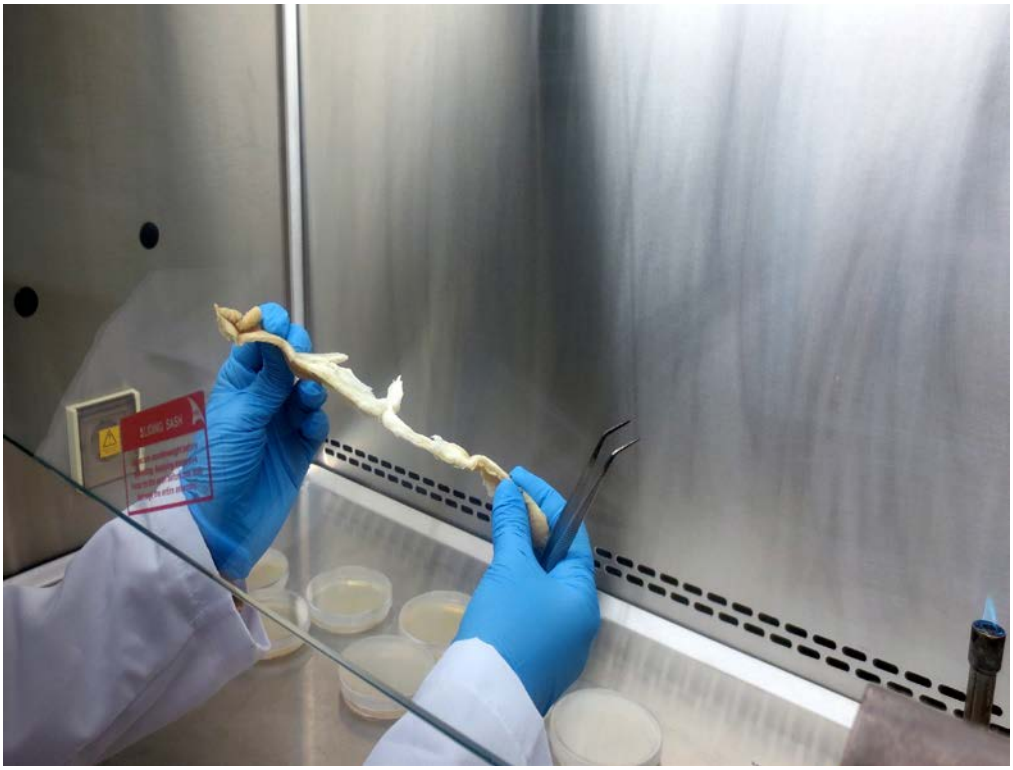


Figure 4.4 : (a) Fruting bodies were divided into two parts from root area.

Figure 4.4 : (b) Sterile mushroom tissues were replaced in agar plugs.

After first inoculation to petri dishes, it was observed that the mycelium spread all around the petri surface in one week under 25-30^{o}C. Then it was subcultured up to 5-6 times to obtain pure culture. Some of them stored in +4^{o}C for future use (**Figure 4.5**).

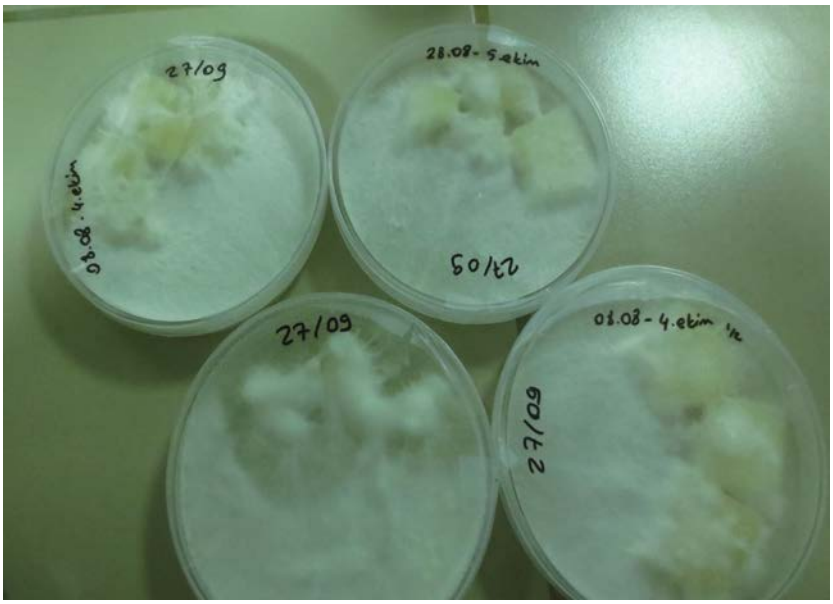


Figure 4.5: Growing *Pleurotus ostreatus* mycelium on agar plate.

4.2.1.2 Generating mother spawn

The next step in the cultivation process is to increase culture size by growing mycelium on grain, often known as *mother spawn*. Mushroom cultures grown on grain are easily proliferated by shaking and allow to transfer by pouring from one container to another. As an example, one bottle of grain spawn can be used to inoculate kilograms of the final substrate. Grain is fairly inexpensive and readily available at stores. It is readily colonized by many saprophytes, including *Pleurotus*

species. Spawn substrate i.e. cereal grains should not be broken, old, and insect damaged. Most of the cereal grains are good substrate for spawn production of oyster mushroom (Kumar, 1995).

In this experiment barley grains were used. Appoximately 600 g of barley grains were weighted. Before used in inoculation, barley grains were washed with tap water a few times to remove soil debris, straw particles and undesirable seed of grasses, weeds, etc. and soak in gypsium ($CaSO_4$) contained distilled water for 12-24 hours.

It was reported that the addition of calcium sulfate (gypsum) stimulated mycelial growth of Shiitake in a liquid media supplemented with sawdust (Raaska, 1990). Calcium sulfate affects pH and its pH-altering ability is minor until the sulphur evolves into sulfuric acid. Calcium and sulphur are important for mushroom metabolism (Stamets, 2000). If the substrate lacks of these elements, yields are adversely affected. A beneficial effect of calcium sulfate on the growth rate of other wood decomposers is strongly suspected (Stamets, 2000).

According to local mushroom cultivators, they also recommend using calcium sulfate in mushroom compost preparation. Calcium sulfate forms an equilibrium matrix with the water and prevents the compost turning to greasy structure. It also helps the colloids to flocculate producing a compost with a more granular structure with increased water holding capacity. Gypsum provides Ca^{++} ions which helps to prevent the loss of nitrogen (from the breakdown of proteins during the act of composting) by chelating the ammonia, a mineral essential to mushroom growth (URL-4, URL-5, URL-6). Gypsum is added at the outset of composting at ~20 kg (40 lb) per ton of dry ingredients it means 2 % calcium sulfate addition (Royse and Beelman, 2013).

After barley grains were soaked in gypsum contained water for 12-24 hours, wihtout changing water boiled on a stove for 5 minutes and extra water removed. For the right consistancy, grains were shaked several time until no vapor left. Then grains were left till they reached the room temperature.

All glass jars used in this experiment were designed and modifed for this experiment. Drilled jar lids were stucked with poly fill which allows oxygen exhange that mycelium needs while it prevents the access of foreign substances and microorganisms.

300 g boiled barley grains were weighted in each of custom glass jars and the taps were closed. Taps were covered by aluminium foil and sterilized for 15 minutes,

121ºC (Stamets, 2000) in pressure cooker and the sterilization was controled with autoclave tape (**Figure 4.6**).

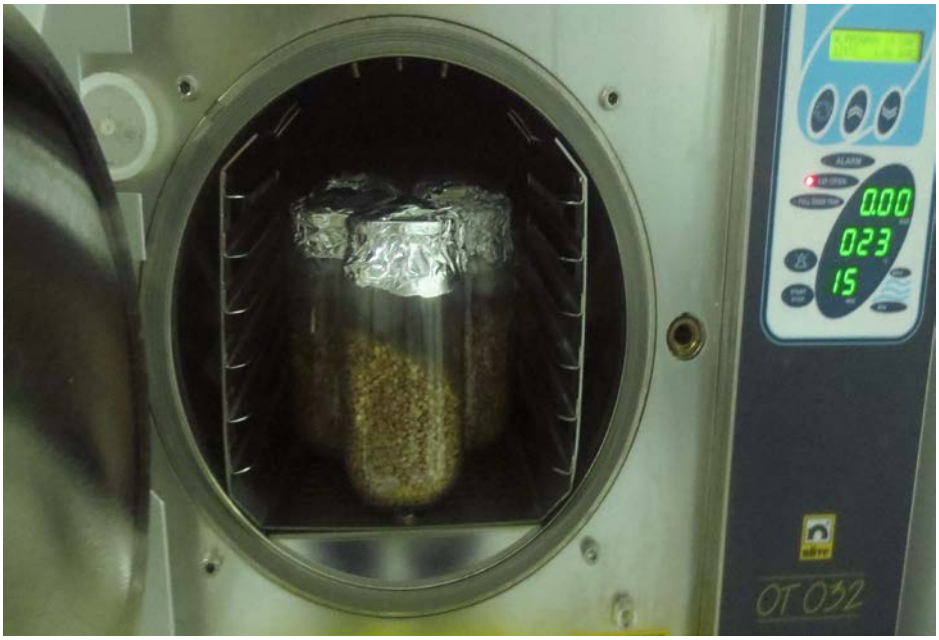


Figure 4.6: Sterilization of barley grains.

After sterilization, jars were left to cool because high temperature may even cause mycelium to die and cautiously inoculated with previously prepared pure culture of *Pleurotus ostreatus* agar plates under the laminar flow cabinet. Small pieces were cut from agar and place onto sterilized barley grains (**Figure 4.7**).

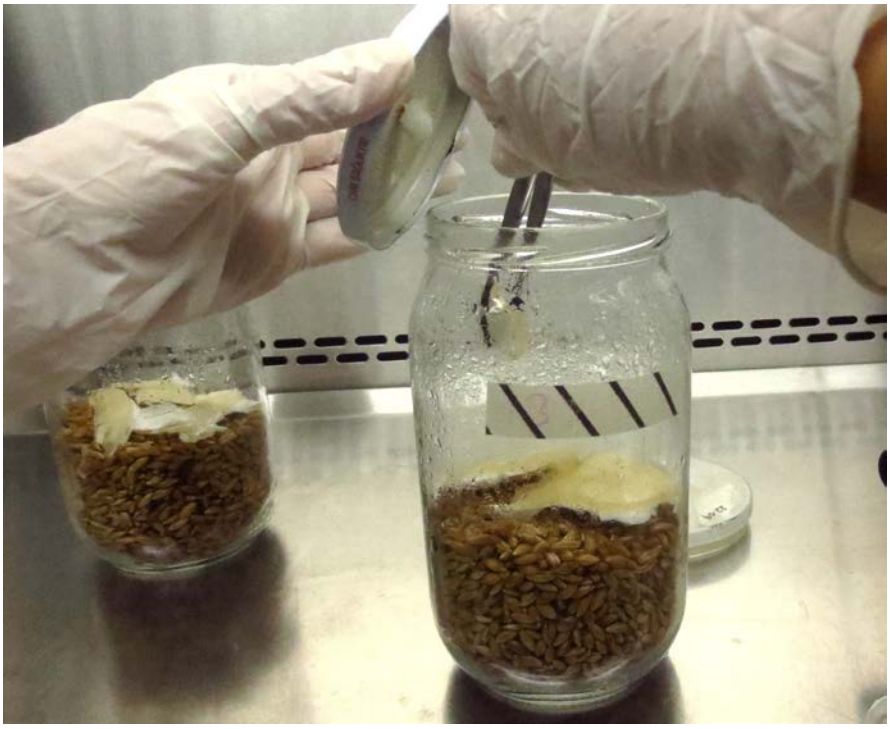


Figure 4.7: Grain inoculation.

Mycelium spread on barley grains after 4-5 days shown in **Figure 4.8**.

Figure 4.8: Mycelium spread on barley grains after 4-5 days.

When the mycelium thrived approximately ¾ of the jars like a white net, they were manually shaken very harshly to make the mycelium grow more vigoriously on grains (Kumar, 1995) (**Figure 4.9 a-b**).

Figure 4.9 : (a) Mycelium thrived approximately ¾ of the jars.

Figure 4.9 : (b) Barley grains after shaken.

After 15-21 days the grains for the purpose of the spawn preperation, the jars stored in +4^{o}C (**Figure 4.10**).

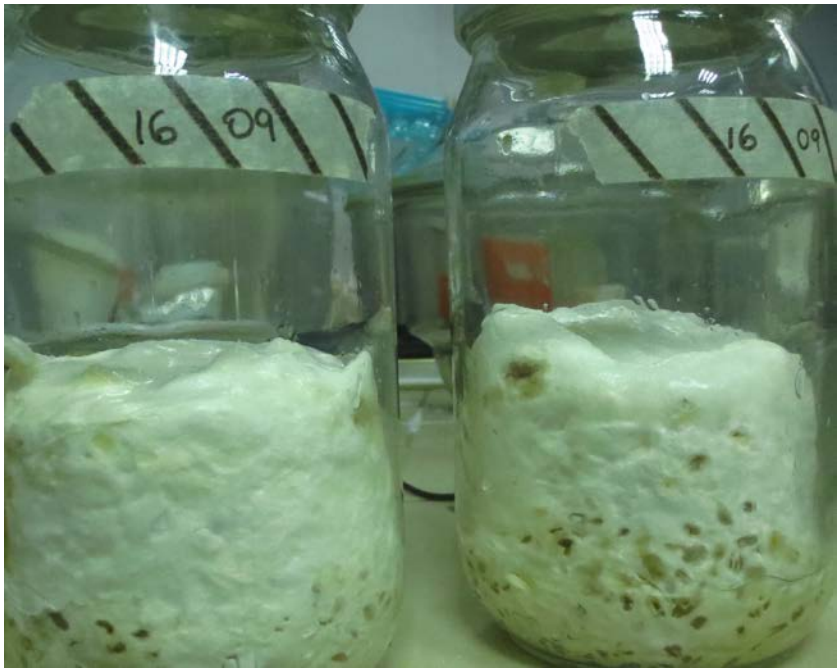


Figure 4.10: Mother spawn.

4.2.1.3 Mushroom spawn and straw inoculation

In the third and final phase of mushroom cultivation, mother spawn is used for straw inoculation. Inoculation on a different substrate than grain stimulate fruiting. The choice of a fruiting substrate will vary, depending on the mushroom species. In this experiment straw was used as bulk substrate.

Many of agricultural, food or even forestry and paper processing industry wastes can be used as substrates. These wastes represent a complex medium, sufficient amounts of carbon and all other nutrients as well.

These waste materials need to pretreatment before use in cultivation process:

- To increase the surface area waste materials grind, mill or cut which are use as substrate.
- Soak to soften and absorb water to adjust the water content,
- Pasteurize or autoclave to eliminate harmful organisms or kill unwanted organisms and partially degrade the structure (Moilanen et al., 2014).

Optimizing the particle size of substrate is important. It needs to be small enough to provide homogenity as well as increase the surface area of the particles where the mycelium is spreading and growing on. At the same time medium needs to remain porous to allow uniform oxygen distribution as well as dissipation of the carbon dioxide and heat which are generated by fungi (Moilanen et al., 2014).

First at all wheat straws were chopped into 2-5 cm lenghts one by one with scissors (**Figure 4.11**) to increase the surface area. Then the straw pieces were filled in a potato sack which has small holes allow water diffusion and soaked in warm water for one and half hour for water saturation. Then the sack was left hanging for removal of excess water until there is no dripping water out . In second step, moistured straw pieces were filled in oven bags which made of polyethilene. 300 g wet staw were weighted in double oven bags and closed tightly to became ready for pasteurization (**Fig. 4.12**).

Figure 4.11: Straws were cut into 2-5 cm lenght.

Bulk substrates like straw are generally pasteurized. Where as *sterilization* refers to the non selective killing of every living organism, *pasteurization* selectively kills the major competitors by preserving populations of noncompetitive thermotolerants. Pasteurization limits window of oppurtunity for contamination, and in turn favors the dominance of mushroom mycelium. Pasteurization typically occurs between 60-82^{o}C (140-180^{o}F) at atmospheric pressure (1 psi) (Stamets, 2000).

Figure 4.12: Pasteurization of straw containing oven bags.

Firstly the straw filled bags were placed in the pressure cooker and two big pieces of rock were put onto the bags to submerged in water completly, a pasteurization method called as "The Hot Water Bath Method: *Submerged Pasteurization*" (Stamets, 2000).

After that cold water was added up over the top. The pressure cooker was put on the stove and the temperature was constantly checked by a home type digital termometer (**Fig. 4.12**). When the temperature was reached on 71^{o}C, pasteurization started for one hour and it was maintained between 71-82^{o}C by turning on and off the stove. At the end of one hour the bags were taken out of pressure cooker and left to cool.

For each bags which contained already pasteurized 300 g wheat straw, 25% (w/w) (75 g) barley grains were used to trigger inoculation. But it was recommended 10-20 % by Stamets (2005) (**Figure 4.13 a-b**). After adding grains onto straw, they were mixed well by shaken for homogeneous dispersion.

Figure 4.13 : (a) Weighing of inoculated grains.

Figure 4.13 : (b) Straw inoculation with grains.

Spawn containing oven bags were modified as seen in the **Figure 4.14**. Bags was stored in a dark place for 20 days. The time refers that all straw pieces were surrounded with mycelium and together with wheat straw form a compact structure. The fungal cultivation steps were also summarized in **Fig. 4.15**.

Figure 4.14: Mushroom spawn of *Pleurotus ostreatus.*

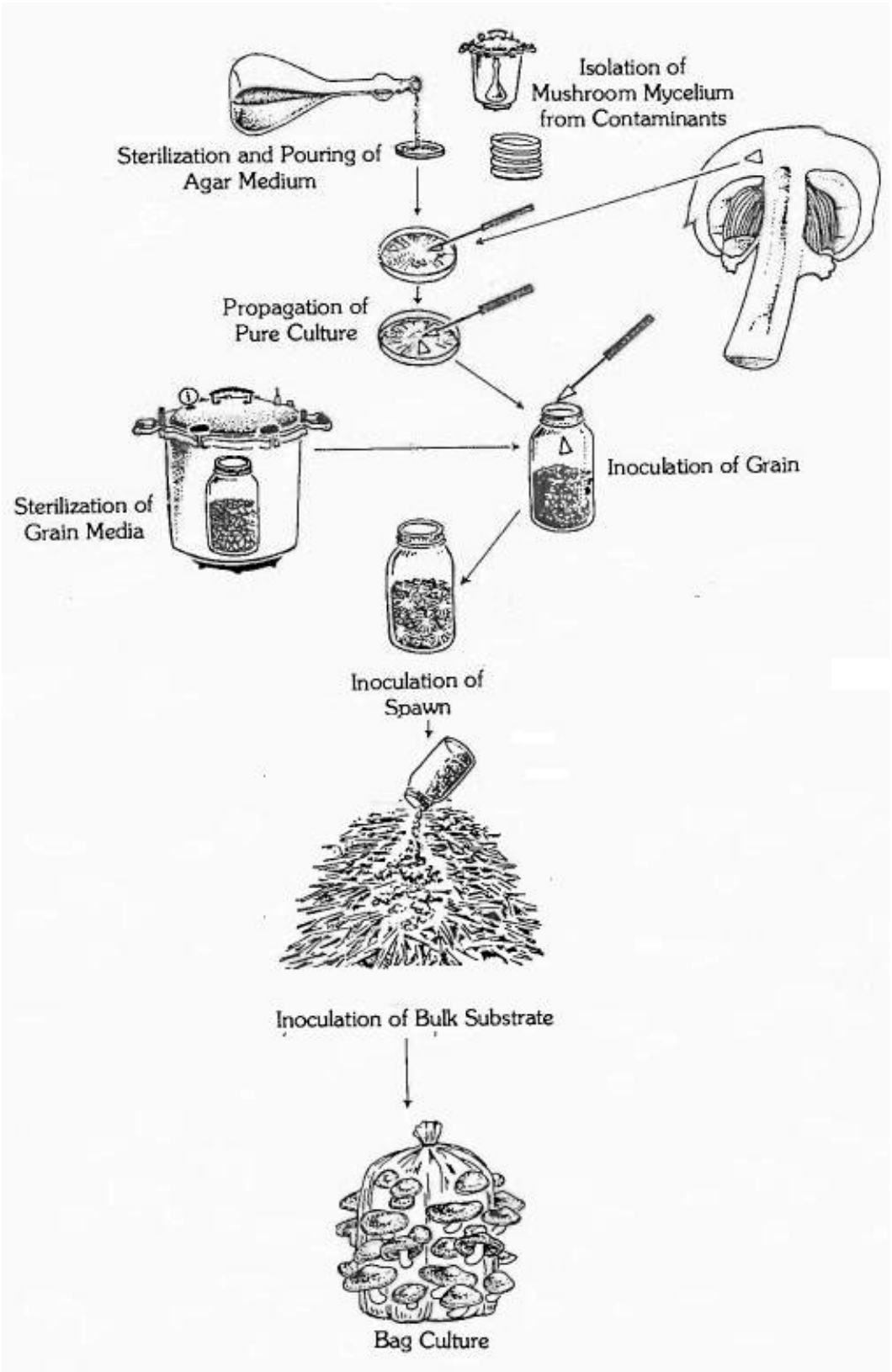


Figure 4.15: Overview tecniques for growing mushrooms. *Source:* Adapted and customized from Stamets, 2000.

4.2.2 Soil as a working environment

Soil is a extremely heterogenous matrix. Particle size of soil important for size and shape of air pores inside the soil, as well as the penetration of air and water. Because of compact structure of soil firstly it was sieved and stored in a covered plastic container at room temperature until using for experiments.

In all experiments sterilized soil was used. Soil pH was measured and moisture content (%) was calculated and results shown in **Table 4.1**.

4.2.2.1 Soil pH measurement

pH value of soil has paramount importance. It affects soils fertility with controlling how well nutrients are dissolved. pH may affect metal-fungal response.

Decreasing pH may increase the concentration of free metal ions. H^+ may compete with metal ions for cellular binding sites. This competition reduce potential interactions between metal ions and cells.

As for external pH may also affect the speciation of metal-binding ligands (Newby and Gadd, 1986; Gadd, 1986a).

For soil pH measurement, EPA Method 9045D was followed:

Procedure

- 20 g soil was weighted into 50 mL baker.
- Then 20 mL water added.
- Soil and water stirred for 5 minutes manually.
- Let the soil suspension stand for 1 hour to allow most of the suspended clay to settle out from the suspension.
- After that aquaous phase was transferred into a santrifuge tube using a pasteur pipet.
- Aquaous phase was santifuged in 9000 rpm for ten minutes.
- Then the supernatant transferred into a baker again.
- pH measured by using a pH meter which already calibrated (URL-7) and determineted result was given at **Table 4.1**.

4.2.2.2 Soil moisture content

The soil moisture content which is also referred as water content, describes the ratio of the mass of water to the mass of solids a given volume of soil. According to literature reveals such factors as moisture content, texture and structure, which affect soil aeration, have a profound influence on fungal activity (Griffin, 1963).

Determination of the moisture content of a soil is based on removing soil moisture by oven-drying a soil sample until the weight remains constant. The moisture content (%) is calculated from the sample weight before and after drying, also result was given at **Table 4.1** by following the procedure below (URL-8 and URL-9):

Procedure

- Weight an aluminum tin, and record this weight.
- Put 10 g of soil sample in the tin and record this weight as "wet soil + tare".
- Place the sample in the oven 105^{o}C and dry for 24 hours.
- Weigh the sample, and record this weight as weight of "dry soil + tare".
- Return the sample to the oven and dry for several hours, and determine the weight of "dry soil +tare".
- Repeat step 5 until there is no difference between any two consecutive measurements of the weight of "dry soil+tare".
- The moisture content (%) of the soil was determined by a formula given below:

$$MC\% = \frac{W_2 - W_3}{W_3 - W_1} x100 \qquad \textbf{(4.1)}$$

Where:

MC % = Moist Content %

W_1 = Weight of tin (g)

W_2 = Weight of moist soil + tin (g)

W_3 = Weight of dried soil + tin (g)

Table 4.1: pH and moisture content (%) of soil.

Soil Parametres	
pH	Moisture Content (%)
5.9	11.75

4.2.3 Experimental design

Two sets of experimental group were designed called as SET I and SET II. Incubation period was determined as one month for SET I (**Table 4.2**) and two month for SET II (**Table 4.3**). Each set composed of artificially contaminated soil with three different heavy metal concentration, their replicates and control groups.

200 g of soil was weighted into glass jars. Soils were contaminated with concerned concentration of Pb, Cd and Hg. After that staw pieces were added on top and were sterilized to become ready for fungal inoculum**.**

Table 4.2: SET I experimental design**.**

	Content 1 (C1)	Content 2 (C2)	Content 3 (C3)
SET I Control (Incubation time: 1 Month)	Soil (200 g) + 10 mL water + 0.5 mg Cd + 0.5 mg Hg + 0.5 mg Pb	Soil (200 g) + 10 mL water + 1 mg Cd + 1 mg Hg + 1 mg Pb	Soil (200 g) + 10 mL water + 2 mg Cd + 2 mg Hg + 2 mg Pb

Table 4.2 (continued): SET I experimental design.

SET I Experimental Group (Incubation time: 1 Month)	Soil (200 g) + 10 mL water + 0.5 mg Cd + 0.5 mg Hg + 0.5 mg Pb + Straw (25 g) + Spawn (15 g)	Soil (200 g) + 10 mL water + 1 mg Cd + 1 mg Hg + 1 mg Pb + Straw (25 g) + Spawn (15 g)	Soil (200 g) + 10 mL water + 2 mg Cd + 2 mg Hg + 2 mg Pb + Straw (25 g) + Spawn (15 g)
SET I Experimental Group (Replicate) (Incubation time: 1 Month)	Soil (200 g) + 10 mL water + 0.5 mg Cd + 0.5 mg Hg + 0.5 mg Pb + Straw (25 g) + Spawn (15 g)	Soil (200 g) + 10 mL water + 1 mg Cd + 1 mg Hg + 1 mg Pb + Straw (25 g) + Spawn (15 g)	Soil (200 g) + 10 mL water + 2 mg Cd + 2 mg Hg + 2 mg Pb + Straw (25 g) + Spawn (15 g)

Table 4.3: SET II experimental design having varied incubation time.

	Content 1 (C1)	Content 2 (C2)	Content 3 (C3)
SET II Control (Incubation time: 2 Month)	Soil (200 g) + 10 mL water + 0.5 mg Cd + 0.5 mg Hg + 0.5 mg Pb	Soil (200 g) + 10 mL water + 1 mg Cd + 1 mg Hg + 1 mg Pb	Soil (200 g) + 10 mL water + 2 mg Cd + 2 mg Hg + 2 mg Pb

Table 4.3 (continued): SET II experimental design having varied incubation time.

SET II Experimental Group (Incubation time: 2 Month)	Soil (200 g) + 10 mL water + 0.5 mg Cd + 0.5 mg Hg + 0.5 mg Pb + Straw (25 g) + Spawn (15 g)	Soil (200 g) + 10 mL water + 1 mg Cd + 1 mg Hg + 1 mg Pb + Straw (25 g) + Spawn (15 g)	Soil (200 g) + 10 mL water + 2 mg Cd + 2 mg Hg + 2 mg Pb + Straw (25 g) + Spawn (15 g)
SET II Experimental Group (Replicate) (Incubation time: 2 Month)	Soil (200 g) + 10 mL water + 0.5 mg Cd + 0.5 mg Hg + 0.5 mg Pb + Straw (25 g) + Spawn (15 g)	Soil (200 g) + 10 mL water + 1 mg Cd + 1 mg Hg + 1 mg Pb + Straw (25 g) + Spawn (15 g)	Soil (200 g) + 10 mL water + 2 mg Cd + 2 mg Hg + 2 mg Pb + Straw (25 g) + Spawn (15 g)

By reason of $Cd(NO_3)_2$ and $Pb(NO_3)_2$ stock solutions were dissolved in 0.5 M HNO_3 and $Hg(NO_3)_2$ standard solution was dissolved in 2 M HNO_3 it was predicted that increasing concentration of heavy metals caused pH decrease (**Figure 4.16**). First of all, solutions were mixed with water and pH measured and record as initial pH. After that pH value adjusted between 5.3-5.7 by adding 1 N NaOH (**Table 4.4**). For each concentration same procedure was followed.

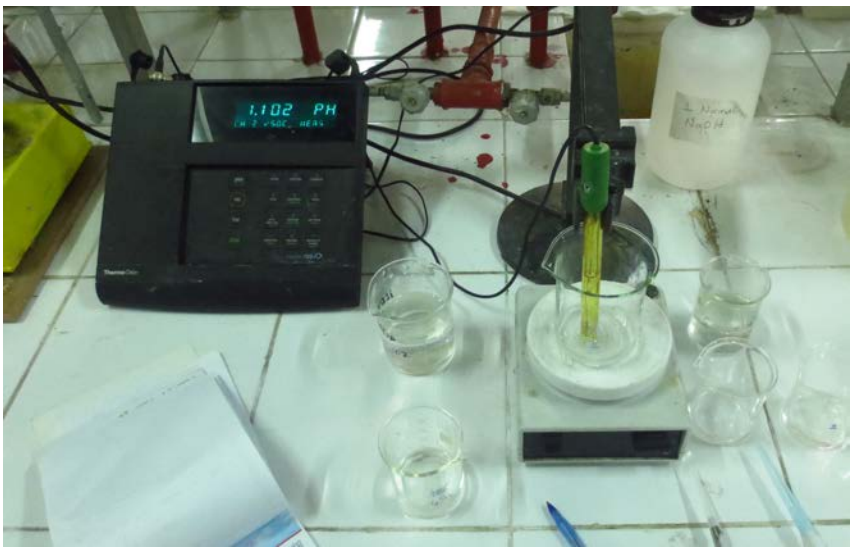


Figure 4.16: pH adjustment.

10 mL of distilled water was added for once. There were no water added after that.

Table 4.4: pH adjustment.

Concentrations	Initial pH	After amount of 1N NaOH consumed final pH	Amount of 1N NaOH consumed (mL)
C1	~1.5-1.7	5.6-5.7	1.05
C2	~0.9-1.0	5.3-5.5	2.05
C3	~0.8	5.3-5.6	4.15

pH adjusted heavy metal-water mixings were added to soil samples and mix by a wood lath in 2x20 cm size for ten minutes until to be sure it looks similar on the top and the bottom and the sides of the glass jars.

Wheat straws were cut into pieces 2-5 cm long to get it ready for use. 25 g of water saturated wheat straw were added on 200 g soil for each glass jar before autoclaved. 200 g of no heavy metal and no straw added experimental soil samples also autoclaved.

The glass jars which contains contaminated soil with stated concentrations of heavy metals and wheat straw, were autoclaved in 121 ˚C, 15 minutes. After sterilization process each glass jar was inoculated with 15 g vigoriously grown *Pleurotus*

ostreatus spawn (**Figure 4.17**). The aim of wheat straw addition was easifying spread out of mycelium toward to heavy metal contaminated soil particles.

Figure 4.17: Inoculation of sterilized, straw enriched, heavy metal contained soil by mushroom spawn.

5. RESULTS

SET I jars were opened at the end of 30 days and SET II jars were opened at the end of 60 days. For all jars, straw pieces and mycelium were removed carrefully by a plastic covered pens to prevent metal-metal interaction and each mycelium sample put in a freezer zipper bag separately (**Figure 5.1 a-b**). Then soil samples were mixed well by a wood lath before taken samples. Soil samples were put in freezer zipper bag separately too and samples labelled. Taken samples stored at +4^{o}C until sent for analysis.

For each set no tratment experimental soils (blanks) were analyzed too. The heavy metal analyses of soil and mycelium samples were performed by Bureau Veritas Mineral Laboratories, in Canada. Lead, cadmium and mercury analyses were realized by ICP-MS method.

Figure 5.1: (a) Mycelium and straw pieces were separated from soil carefully.

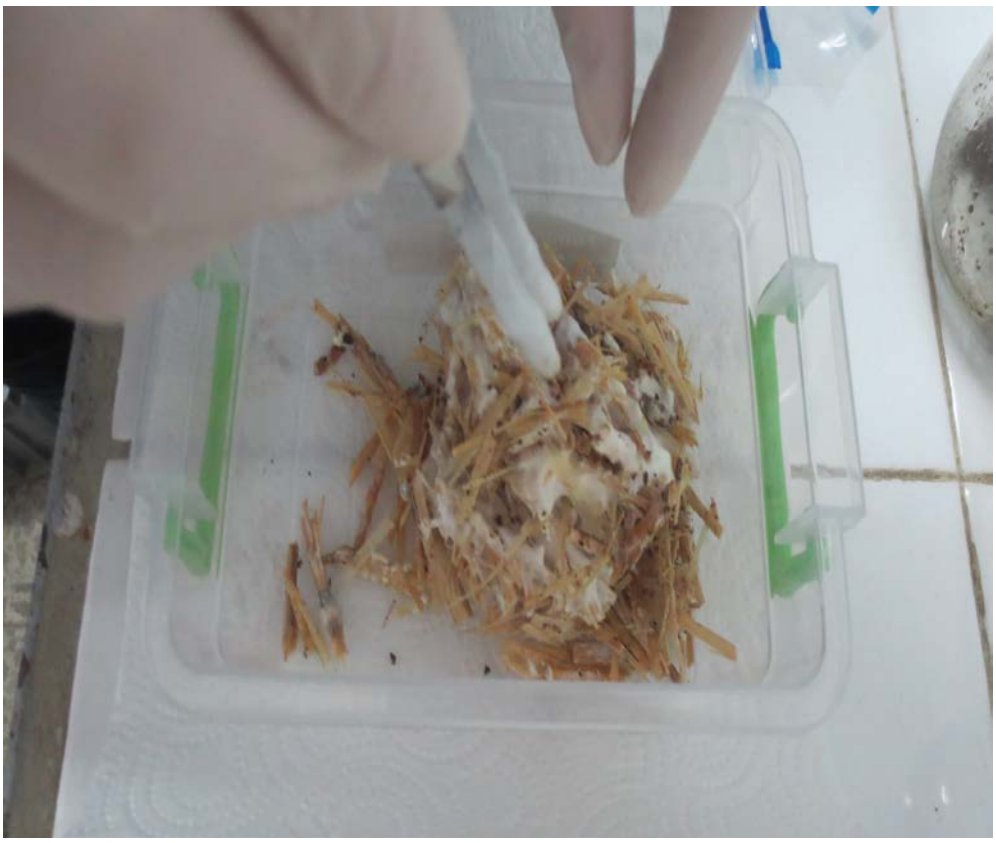


Figure 5.1 : (b) Mycelium samples were placed in zipper bags.

SET I experimental groups were shown in **Figure 5.2 (a-d).**

Figure 5.2 : (a) SET I, C1.

Figure 5.2 : (b) SET I, C2.

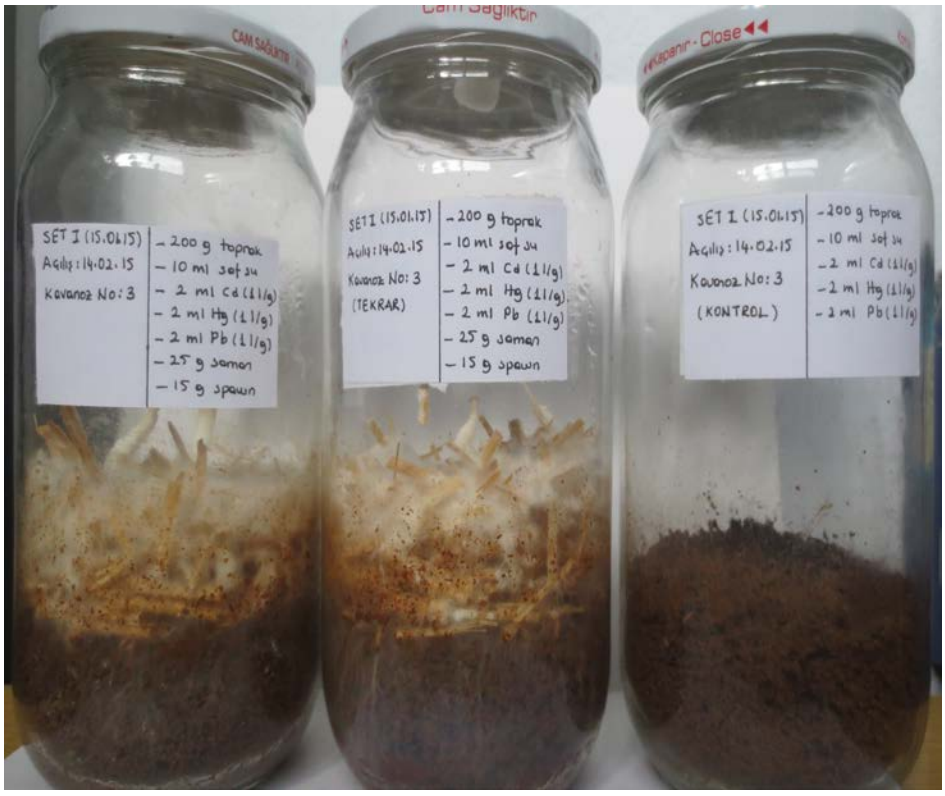


Figure 5.2 : (c) SET I, C3.

Figure 5.2 : (d) Mycelium network in contaminated soil.

SET II experimental groups were shown in **Figure 5.3 a-e**.

Figure 5.3 : (a) SET II, C1.

Figure 5.3 : (b) SET II, C2.

Figure 5.3 : (c) SET II, C3.

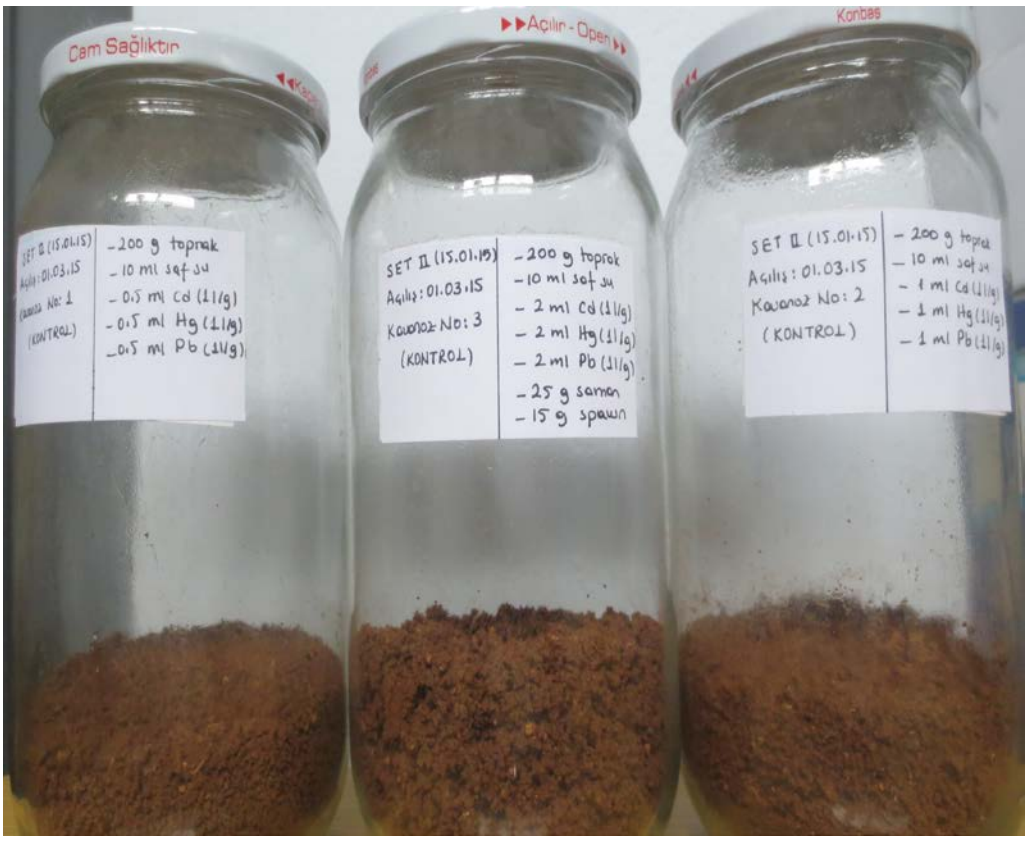


Figure 5.3 : (d) SET II, C1-2-3 control groups.

Figure 5.3 : (e) No treatment experimental soil for each set.

Bioaccumulation levels were compared between SET I and SET II, both in soil and in mycelium samples in factor of incubation time and increasing concentrations of heavy metals.

5.1 Soil Analyses of SET I and SET II

No treatment experimental soil analysis for SET I and SET II was shown in **Table 5.1**.

Table 5.1: Heavy metal contents of no treatment experimental soil.

Experimental Soil	Pb (ppm)	Cd (ppm)	Hg (ppm)
SET I Experimental Soil	36.2	0.3	0.23
SET II Experimental Soil	39.0	0.3	0.1

Experimental soil analyses show that it contains high amount of lead in comparison with cadmium and mercury. The artificially lead addition maybe affected its accumulation because of the its relatively high concentration in comparison with cadmium and mercury.

Heavy metal analyses of SET I and SET II soil samples and average heavy metal contents for C1, C2 and C3 were shown in **Table 5.2** and **Table 5.3**, respectively. These values pointed at the total concentrations of heavy metals which left in soil and bioaccumulated concentrations of each heavy metals into mycelium which were not allow to seperate from soil particules. Average heavy metal contents of SET I and SET II soil samples were also shown in **Figure 5.4** and **Figure 5.5**, respectively.

Table 5.2: Heavy metal analysis of SET I soils.

SET NO	Content		Heavy metal concentrations which were left in soil (ppm)		
			Pb	Cd	Hg
SET I (Incubation time: 30 days)	C1	1	37.6	2.9	3.0
		Replicate	38.2	3.3	3.9
		Average	37.9±0.4	3.1±0.28	3.45±0.6
		Control	39.6	3.3	4.0
	C2	2	40.6	5.5	6.3
		Replicate	42.5	6.2	6.5
		Average	41.55±1.3	5.85±0.49	6.4±0.14
		Control	42.8	6.4	6.9
	C3	3	49.1	11.9	11.3
		Replicate	47.8	11.9	11.8
		Average	48.45±0.9	11.9±0	11.55±0.35
		Control	51.6	12.0	13.8

Table 5.3: Heavy metal analysis of SET II soils.

SET NO	Content		Heavy metal concentrations which were left in soil (ppm)		
			Pb	Cd	Hg
SET II (Incubation time: 60 days)	C1	1	39.6	3.5	3.4
		Replicate	41.1	3.3	3.8
		Average	40.35±1.06	3.4±0.14	3.6±0.28
		Control	39.4	3.3	3.8
	C 2	2	43.7	6.1	6.8
		Replicate	43.5	6.5	8.4
		Average	43.6±0.14	6.3±0.28	7.6±1.13
		Control	44.1	6.4	7.2
	C3	3	53.6	11.9	14.3
		Replicate	50.5	11.1	11.0
		Average	52.05±2.19	11.5±0.56	12.65±2.3
		Control	49.7	11.4	12.4

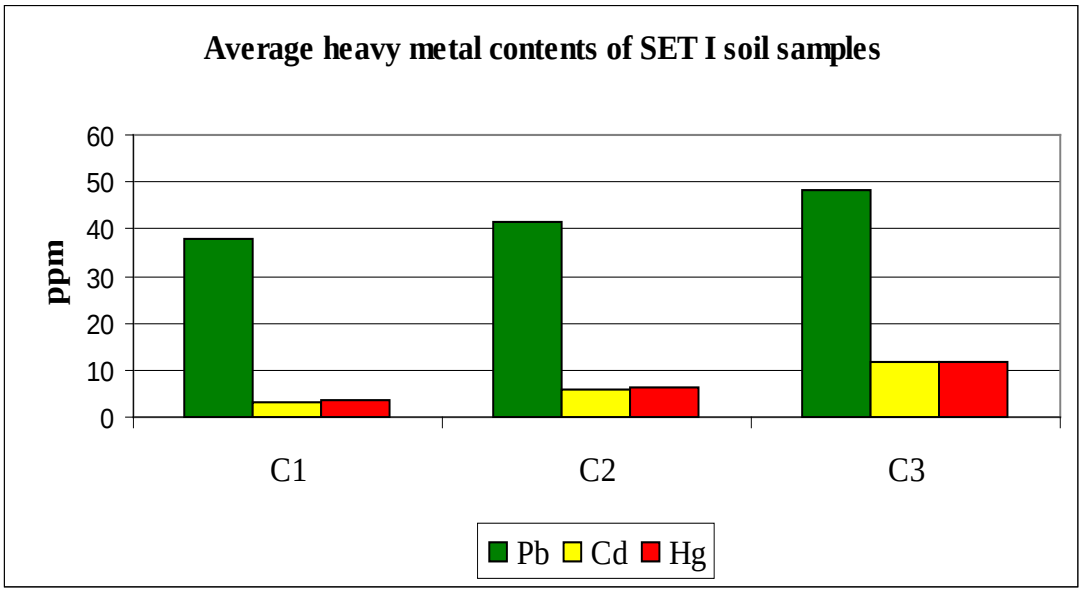


Figure 5.4: Average heavy metal contents of SET I soil samples.

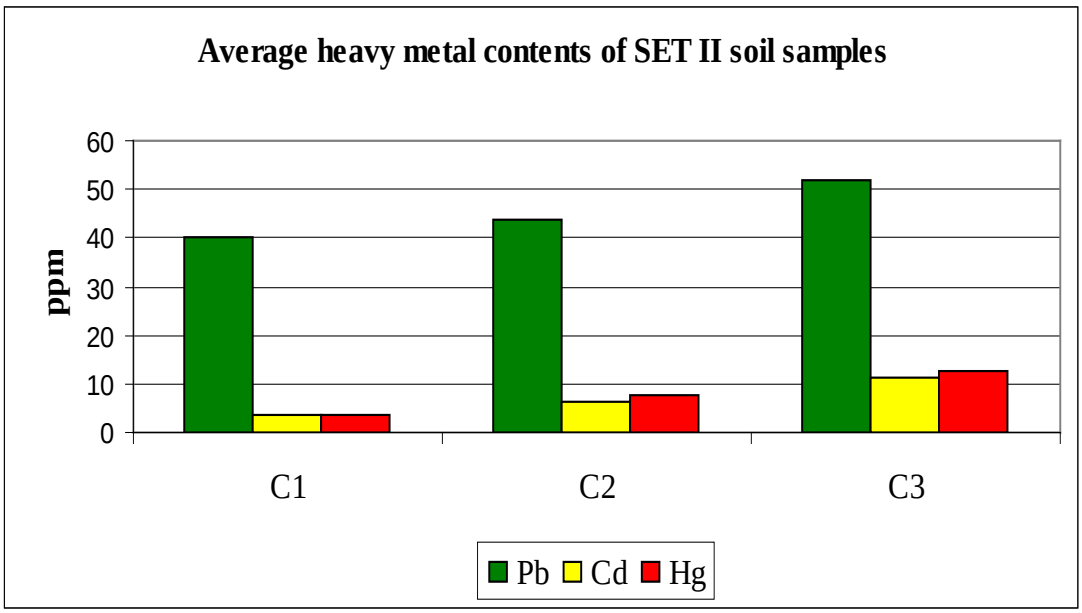


Figure 5.5: Average heavy metal contents of SET II soil samples.

The heavy metal concentrations were found in soil did not show the bioaccumulation level stand alone. Thin mycelium threads which were penetrated into soil and were not allowed to separate, caused misimpression. The heavy metals which were accumulated into mycelium, seem like they were still in soil. One month and two month incubation time were not sufficient to form mature fruting body and to complete bioaccumulation of heavy metals from soil to aboveground structure (fruiting body) of fungi. This situation caused false pretence the heavy metals concentration in experiment groups and/or their replicates (**Figure 5.6**).

(a)

(b)

Figure 5.6: (a-b) Mycelium which penetrated into soil.

Heavy metal analyses of SET I and SET II control groups showed in **Table 5.4**.

Table 5.4: Heavy metal analysis of SET I and SET II control groups.

SET NO	Content	Pb (ppm)	Cd (ppm)	Hg (ppm)
SET I SOIL SAMPLES	Control C1	39.6	3.3	4.0
	Control C2	42.8	6.4	6.9
	Control C3	51.6	12.0	13.8
SET II SOIL SAMPLES	Control C1	39.4	3.3	3.8
	Control C2	44.1	6.4	7.2
	Control C3	49.7	11.4	12.4

5.2 Mycelium Analyses of SET I and SET II

Heavy metal analysis of SET I and SET II mycelium samples showed in **Table 5.5** and **Table 5.6**, respectively.

Table 5.5: Mycelium heavy metal analyses of SET I.

SET I MYCELIUM SAMPLES	Pb (ppm)	Cd (ppm)	Hg (ppm)
C1	1.8	0.26	0.7
C1 (Rep.)	0.7	0.2	1.0
Average of C1	1.25±0.78	0.23±0.04	0.85±0.2
C2	0.7	0.3	2.4
C2 (Rep.)	0.8	0.5	3.3
Average of C2	0.75±0.07	0.4±0.14	2.85±0.6
C3	0.5	0.7	6.7
C3 (Rep.)	0.6	0.9	8.2
Average of C3	0.55±0.07	0.8±0.14	7.45±1.06

Table 5.6: Mycelium heavy metal analyses of SET II.

SET II MYCELIUM SAMPLES	Pb (ppm)	Cd (ppm)	Hg (ppm)
C1	1.4	0.4	1.6
C1 (Rep.)	1.6	0.4	2.6
Average of C1	1.5±0.14	0.4±0	2.1±0.7
C2	1.1	0.4	3.7
C2 (Rep.)	2.0	0.7	5.5
Average of C2	1.55±0.6	0.55±0.2	4.6±1.27
C3	1.3	1.1	9.5
C3 (Rep.)	1.8	1.5	11.7
Average of C3	1.55±0.35	1.3±0.28	10.6±1.55

Average heavy metal concentrations of mycelium samples which were belonging to SET I and SET II, shown in **Figure 5.7** and **Figure 5.8**, respectively. Heavy metal analyses both in soil and mycelium samples showed that bioaccumulation were increased with incubation time. It was found that bioaccumulated concentration of lead, cadmium and mercury were higher in SET II mycelium samples than SET I. It pointed at *Pleurotus ostreatus* colonization into soil particules and pinhead formation.

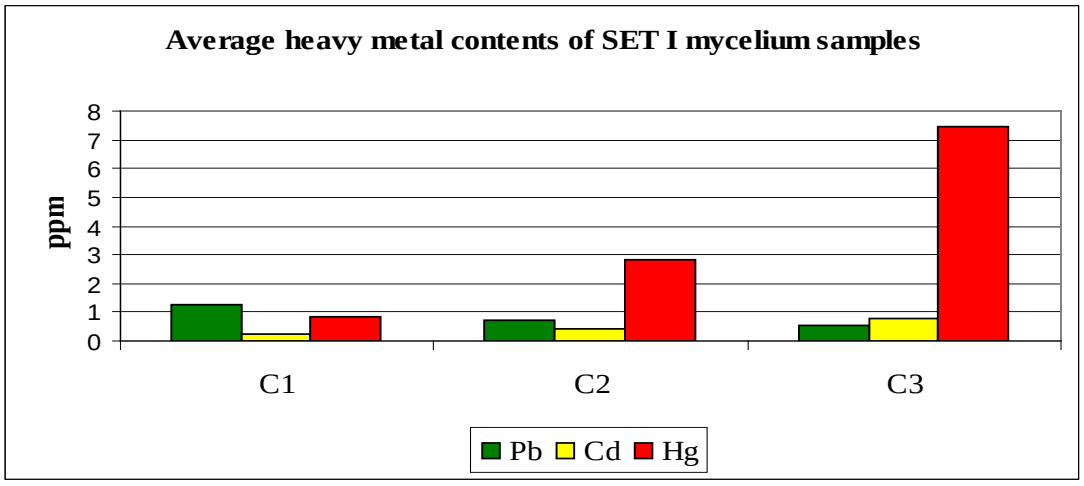


Figure 5.7: Average concentrations of heavy metals which were accumulated to SET I mycelium samples.

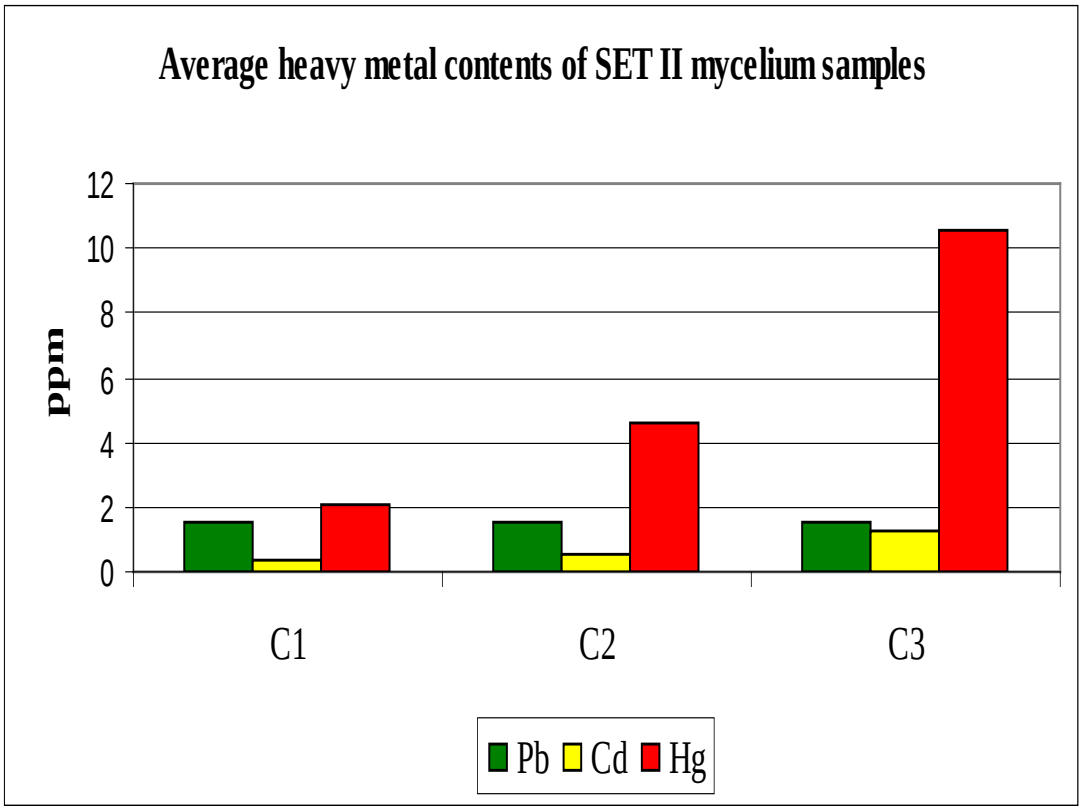


Figure 5.8: Average concentration of heavy metals which were accumulated to SET II mycelium samples.

The mycelium analyses showed that increasing concentration of lead caused a decrease in its bioaccumulation in SET I and bioaccumulated concentration of Pb was not showed significant difference in all concentrations in SET II.

It was observed that mercury was the highest acumulated heavy metal either SET I and SET II mycelium samples and bioaccumulation of cadmium increased by its increasing concentrations for one and two month incubation periods.

5.3 Pinhead Formation and Effect of Incubation Time

For one and two month incubation time of *Pleurotus ostreatus*, pinhead (young mushrooms that look like a fat white pencil tip) formation was observed in experiment groups which had three different heavy metal contents (**Figure 5.9 a-c**). On the other hand incubation periods which were determined for this experiment were not sufficient to observe mature fruiting bodies.

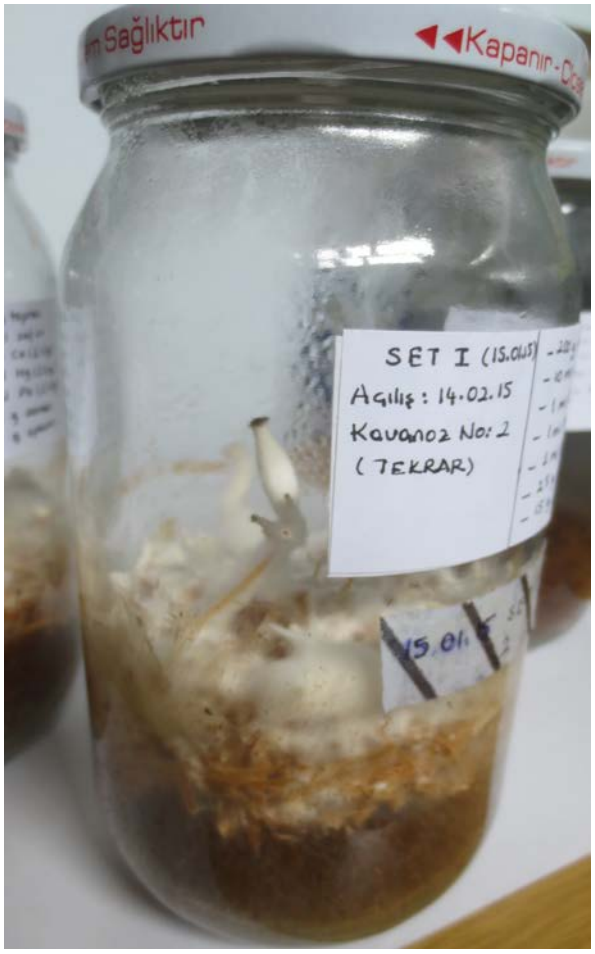


Figure 5.9 : (a) Pinhead formation in SET I, C2.

Figure 5.9: (b)Pinhead formation in SET II, C2.

Figure 5.9: (c) Pinhead formation in SET II, C3.

5.4 Determination of Bioaccumulation Factor and Bioaccumulation Percentages

The ratio of the metal concentration in the bioaccumulator (e.g fruting body and mycelium) to the metal concentration in its environment (e.g soil, water, etc.) is considered to be the characterization of bioaccumulation efficiency and named as *bioaccumulation factor*. If bioaccumulation value greater than 1, it points at high accumulation potential of concern species (Damodaran et al., 2014).

$$Bioaccumulation\ \ factor = \frac{\text{Conc. of metal in dried biomass (mg/kg)}}{\text{Conc. of metal in the soil (mg/kg)}} \quad \textbf{(5.1)}$$ (Damodaran et al., 2014)

Bioaccumulation factor of each heavy metal for SET I and SET II represent in **Table 5.7**.

Table 5.7: Bioaccumulation factor of each heavy metal for SET I and SET II.

SET No & Content	Heavy Metals		
	Pb	Cd	Hg
SET I C1	0.03	0.069	0.21
SET I C2	0.018	0.0625	0.41
SET I C3	0.011	0.066	0.54
SET II C1	0.038	0.12	0.55
SET II C2	0.035	0.086	0.64
SET II C3	0.031	0.11	0.85

Bioaccumulation factor of Pb, Cd and Hg represented in **Figure 5.10**, **Figure 5.11** and **Figure 5.12**, respectively.

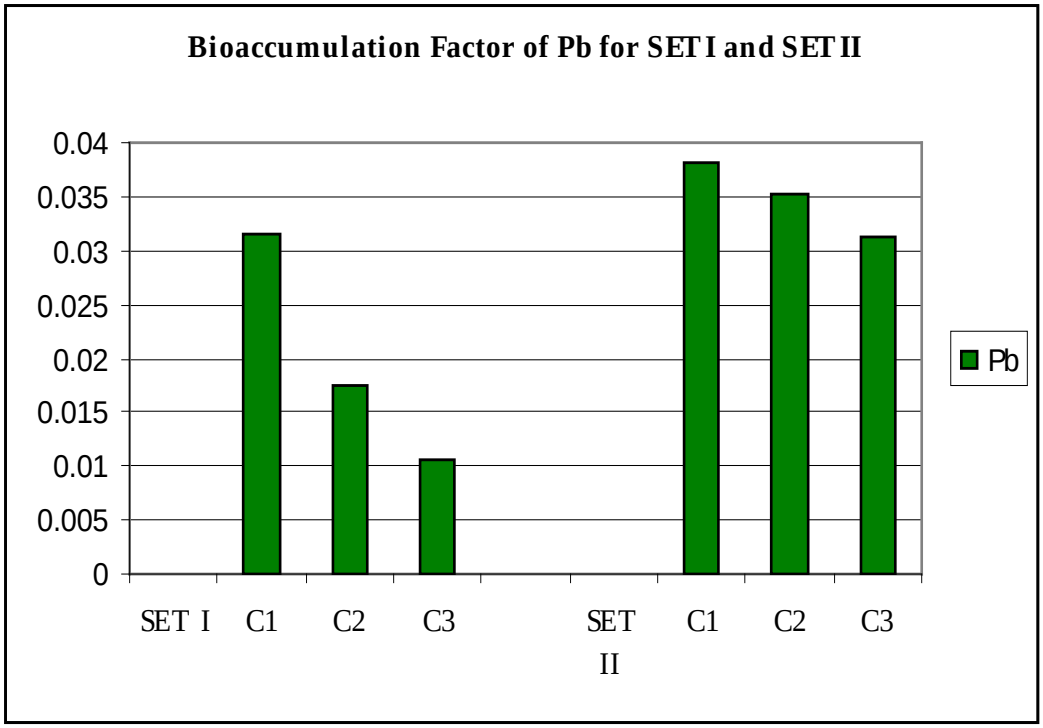


Figure 5.10: Bioaccumulation factor of Pb.

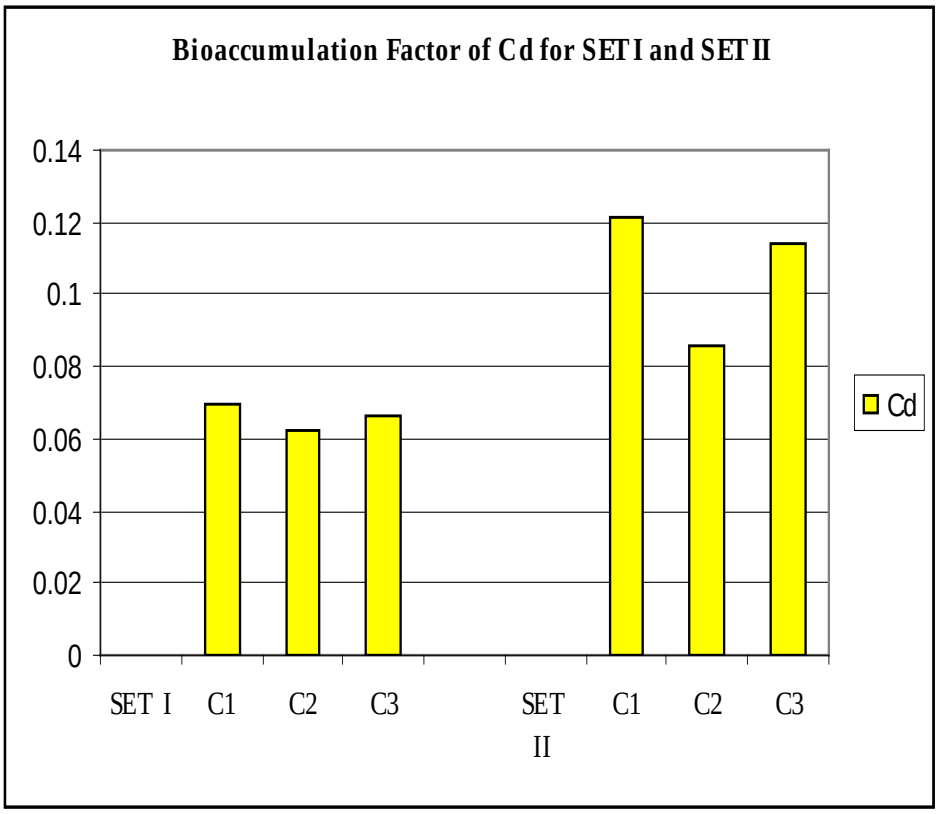


Figure 5.11: Bioaccumulation factor of Cd.

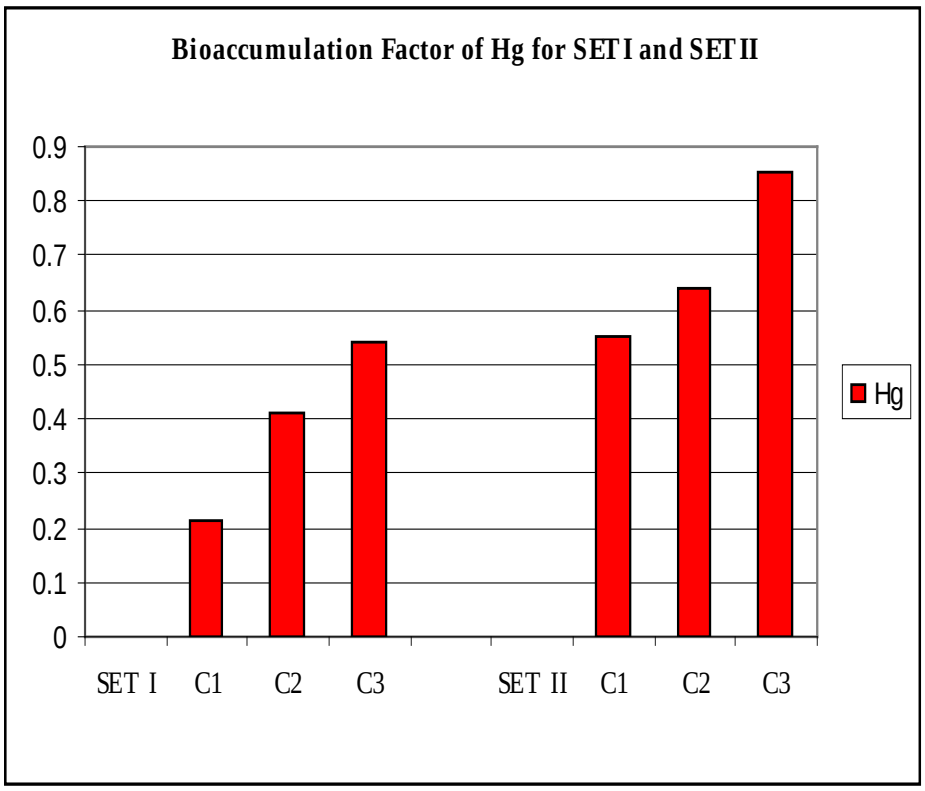


Figure 5.12: Bioaccumulation factor of Hg.

According to heavy metal analysis both in soil and mycelium, "Bioaccumulation (%)" was calculated based on the ratio between bioaccumulated concentration of heavy metals which found in mycelium to heavy metal concentrations of control groups by following this formula:

$$Bioaccumulation\ (\%) = \frac{\text{Conc. of metal in dried biomass (mg/kg)}}{\text{Conc. of metal in the soil (mg/kg)}} x100 \qquad \textbf{(5.2)}$$

SET I and SET II bioaccumulation (%) were showed in **Table 5.8** and **Table 5.9**.

Table 5.8: Bioaccumulation ratio (%) of SET I.

	Content of Control Groups of SET I (ppm)			Average heavy metal contents in mycelium samples of SET I (ppm)			Accumulation (%)		
Content	Pb	Cd	Hg	Pb	Cd	Hg	Pb	Cd	Hg
C1	39.6	3.3	4	1.25	0.23	0.85	3.16	6.97	21.25
C2	42.8	6.4	6.9	0.75	0.4	2.85	1.75	6.25	41.3
C3	51.6	12	13.8	0.55	0.8	7.45	1.06	6.66	53.98

Table 5.9: Bioaccumulation ratio (%) of SET II.

	Content of Control Groups of SET II (ppm)			Average heavy metal contents in mycelium samples of SET II (ppm)			Accumulation (%)		
Content	Pb	Cd	Hg	Pb	Cd	Hg	Pb	Cd	Hg
C1	39.4	3.3	3.8	1.5	0.4	2.1	3.80	12.12	55.26
C2	44.1	6.4	7.2	1.55	0.55	4.6	3.51	8.59	63.88
C3	49.7	11.4	12.4	1.55	1.3	10.6	3.12	11.40	85.48

SET I and SET II bioaccumulation ratio (%) were showed in **Figure 5.13** and **Figure 5.14** respectively.

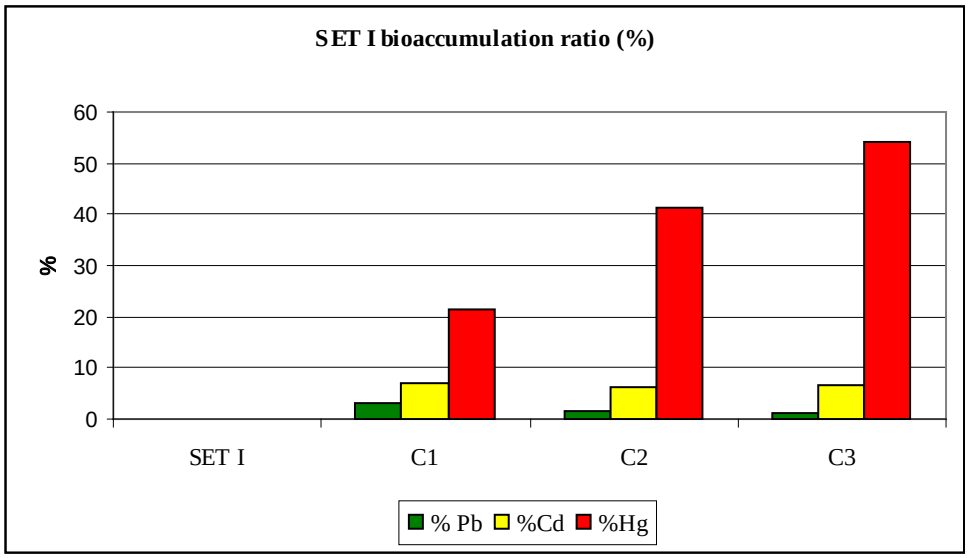


Figure 5.13: Bioaccumulation ratio (%) of SET I.

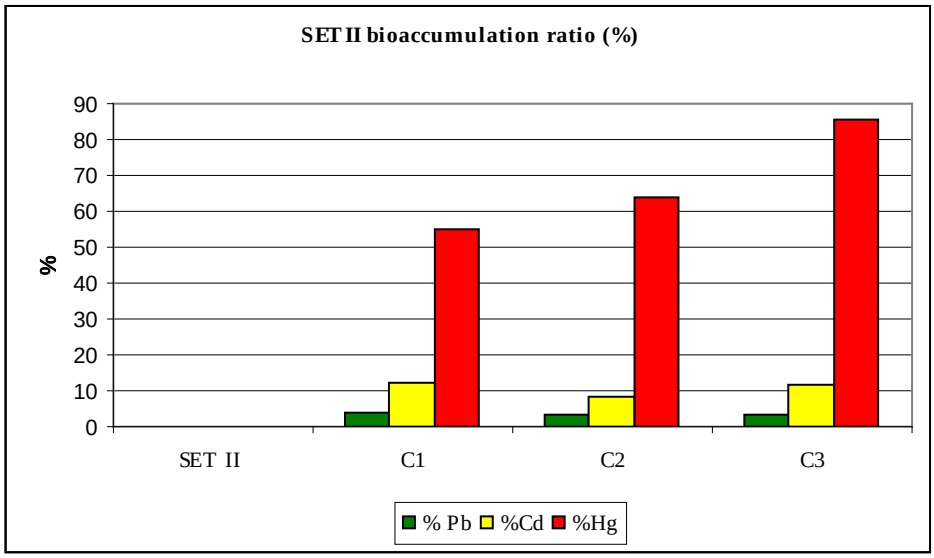


Figure 5.14: Bioaccumulation ratio (%) of SET II.

According to bioaccumulation percentage calculations for lead, it showed a decrease for its increasing concentrations. For one month incubation of *Pleurotus ostreatus*, Pb bioaccumulation showed a significant decreace by its increasing concentration and it was not found a significant difference in C1, C2 and C3 for two month incubation period. On the other hand according to bioaccumulation percentage calculations for cadmium, C2 which had 5 ppm Cd showed the lowest bioaccumulation (%) ratio in comparison to C1 and C3, both SET I and SET II.

Mercury bioaccumulation was increased by its increasing concentration and incubation period.

Bioaccumulation percentages of heavy metals under study were lead, cadmium and mercury also comprised one by one for one and two month incubation periods. Comparison of Pb, Cd and Hg bioaccumulation percentages for SET I and SET II shown in **Figure 5.15**, **Figure 5.16** and **Figure 5.17**, respectively.

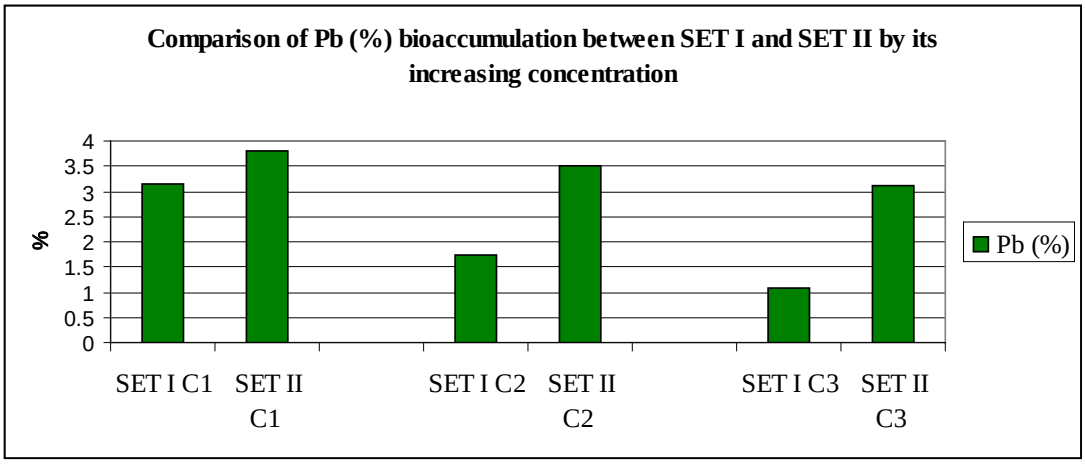


Figure 5.15: Comparison of Pb bioaccumulation ratio (%) between SET I and SET II.

While lead bioaccumulation percentage increasing by incubation period, showed a decrease by its increasing concentration.

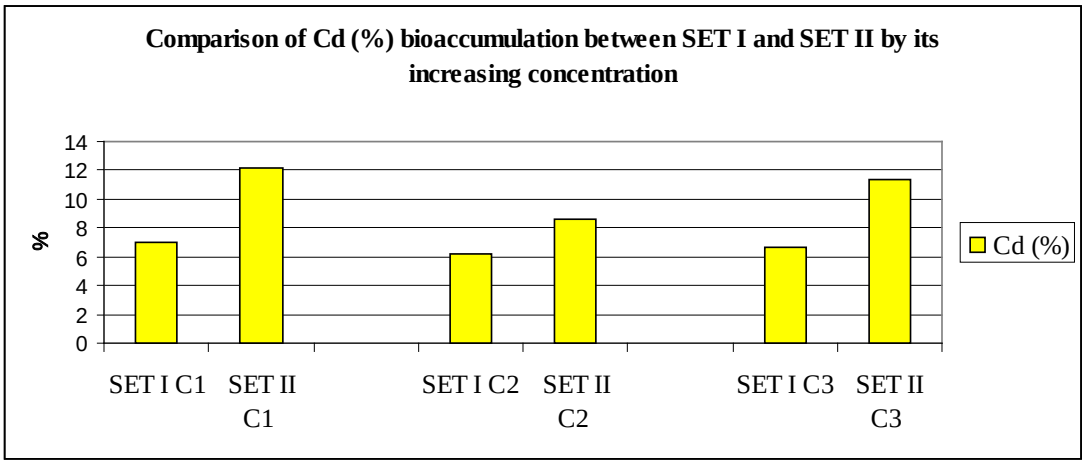


Figure 5.16: Comparison of Cd bioaccumulation ratio (%) between SET I and SET II.

Cadmium bioaccumulation (%) ratio exhibited interesting manner by its increasing concentration in soil. While Cd bioaccumulation increased by incubation time, the

highest bioaccumulation (%) ratio was observed at the lowest concentration of cadmium either SET I and SET II.

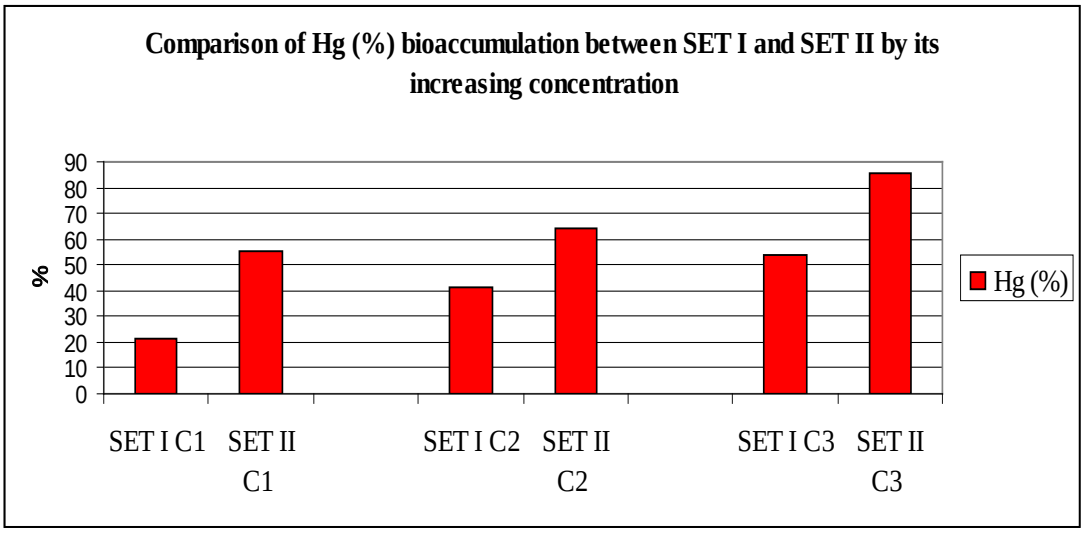


Figure 5.17: Comparison of Hg bioaccumulation ratio (%) between SET I and SET II.

Bioacumulated concentration of mercury by *Pleurotus ostreatus* showed a significant difference both its increasing concentrations and incubation period. Heavy metal analyses of mycelium samples show that the most bioaccumulated heavy metal by *Pleurotus ostreatus* was mercury.

The mycelium samples belonged to 10 ppm mercury contained soils showed 54 % Hg bioaccumulation and 85 %, in SET I and SET II, respectively.

5.5 Statistical Analyses

5.5.1 Pearson correlation analyses

In statistics, the *Pearson correlation coefficient* is a measure of the linear correlation between two variables *X* and *Y*, giving a value between +1 and −1 inclusive, where 1 is total positive correlation, 0 is no correlation, and −1 is total negative correlation.

It is widely used in the sciences as a measure of the degree of linear dependence between two variables (URL-12). Pearson correlation analyses were performed with the software Statistica version 6.0 (StatSoft, Inc.).

5.5.1.1 Pearson analysis for soil samples

The correlation between lead, cadmium and mercury in soil represent in **Table 5.10**.

Table 5.10: Pearson correlation of the heavy metals in soil.

Heavy Metals	Pb	Cd	Hg
Pb	1.00		
Cd	0.97	1.00	
Hg	0.96	1.00	1.00

Correlations (aaa) Marked correlations are significant at p < .05000 N=8 (Casewise deletion of missing data)

5.5.1.2 Pearson corelation analyses for mycelium samples

The correlation between mycelium samples which belong to SET I and SET II by increasing heavy metal concentrations in soil samples, represent in **Table 5.11**.

Table 5.11: Pearson correlation of mycelium samples of SET I and SET II.

Set No & Content	ST1 C1	ST1 C2	ST1 C3	ST2 C1	ST2 C2	ST2 C3
ST1 C1	1.00					
ST1 C2	0.25	1.00				
ST1 C3	0.11	0.99	1.00			
ST2 C1	0.75	0.83	0.74	1.00		
ST2 C2	0.37	0.99	0.96	0.89	1.00	
ST2 C3	0.16	1.00	1.00	0.77	0.98	1.00

Correlations (aaa) Marked correlations are significant at p < .05000 N=3 (Casewise deletion of missing data)

5.5.2 Principal component analyses

Principal component analysis is a statistical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables (URL-13). Principal component analyses performed with the software Statistica version 6.0 (StatSoft, Inc.).

5.5.2.1 Principal component analysis for soil samples

Principle component analysis of soil samples showed that while cadmium and mercury show 100 % similarity, lead showed a different behavior in soil (**Figure 5.18**).

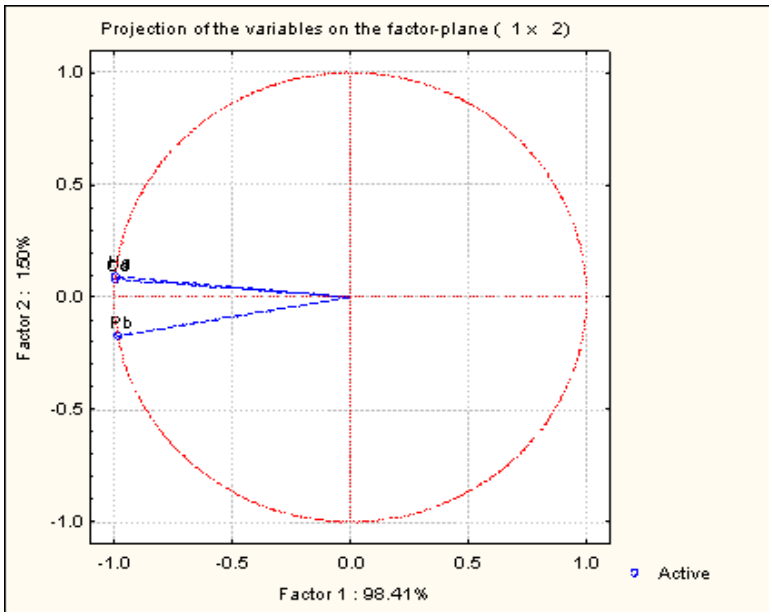


Figure 5.18: Principle component analysis for heavy metals behavior in soil.

5.5.2.2 Principal component analysis for mycelium samples

Principle component analysis of mycelium samples showed that while cadmium and mercury show close relation, lead showed a significant different manner (**Figure 5.19**).

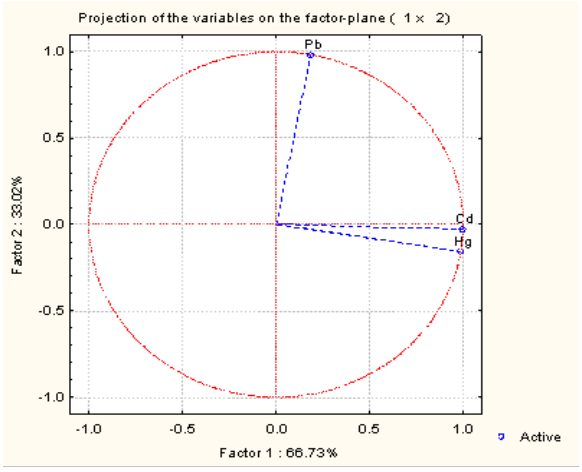


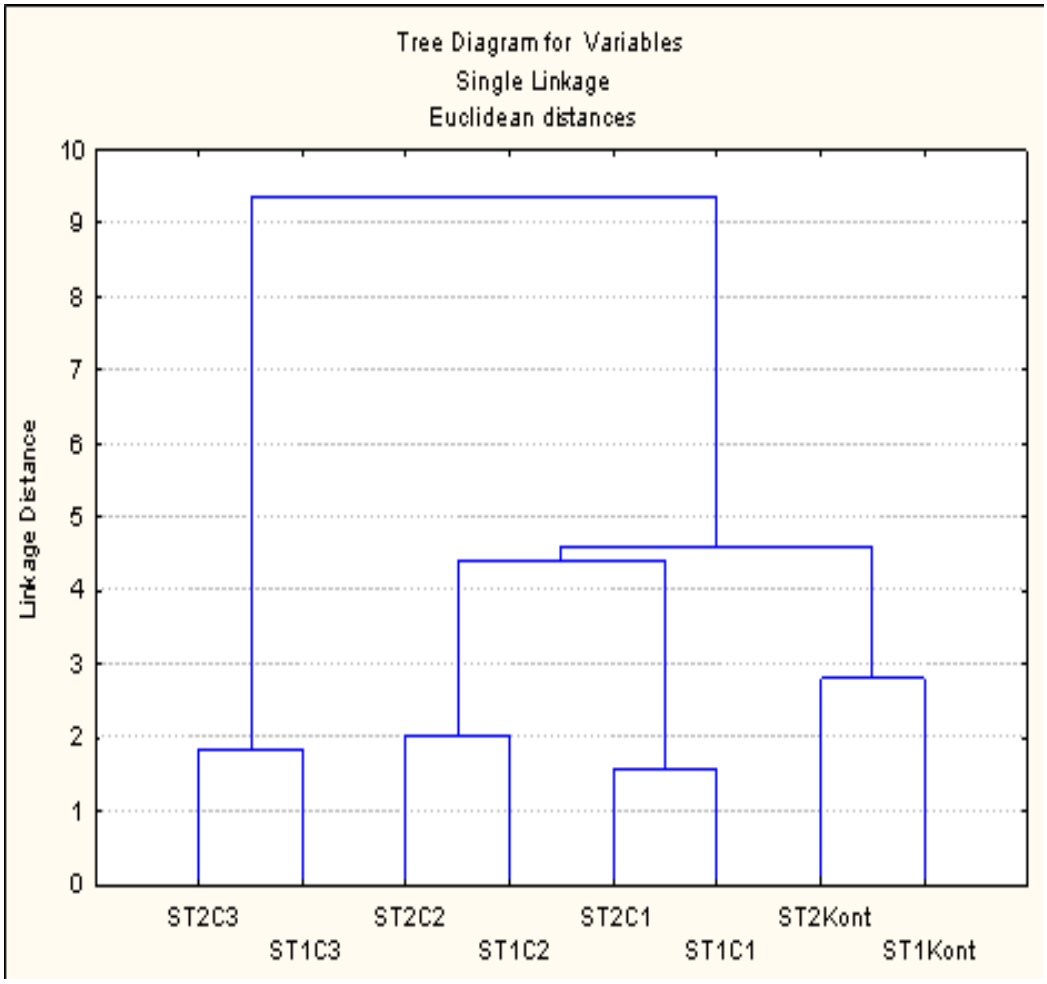
Figure 5.19: Principle component analysis for heavy metals behavior in mycelium.

5.5.3 Cluster analyses

Cluster analysis is the task of grouping a set of objects in such a way that objects in the same group (called a cluster) are more similar (in some sense or another) to each other than to those in other groups (clusters) (URL-14). Cluster analyses were performed with the software Statistica version 6.0 (StatSoft, Inc.).

5.5.3.1 Cluster analysis of soil samples

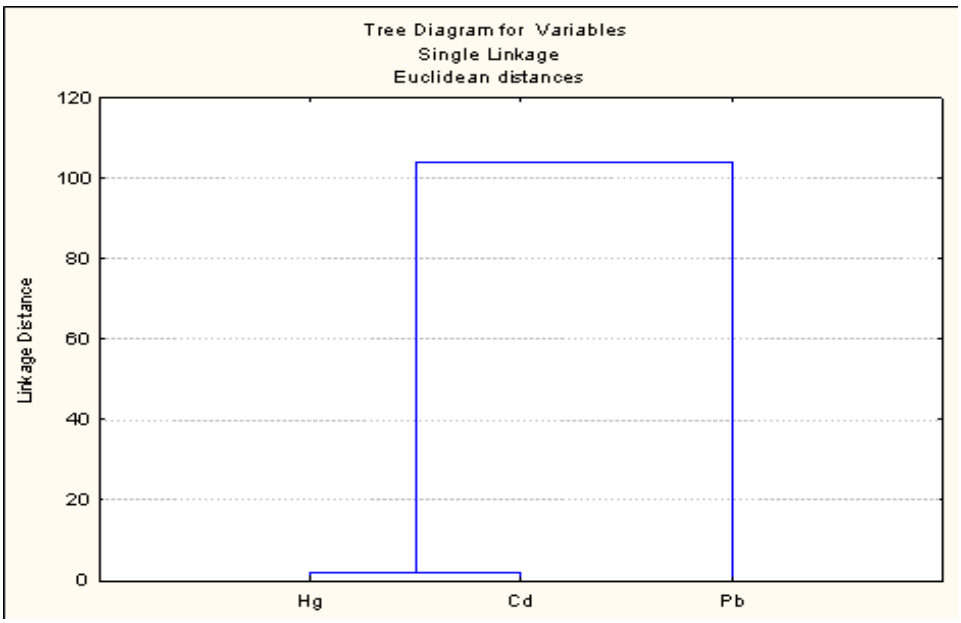
Cluster analysis of soil samples showed that soil samples which have similar concentrations form clusters between each others. The linkage distance refers relationship between increasing concentrations of heavy metals by incubation time (**Figure 5.20 a**).



(a)

Figure 5.20: (a) Cluster analysis of soil samples.

While mercury and cadmium show close relation, lead show significantly different behavior with cadmium and mercury in soil (**Figure 5.20 b**).

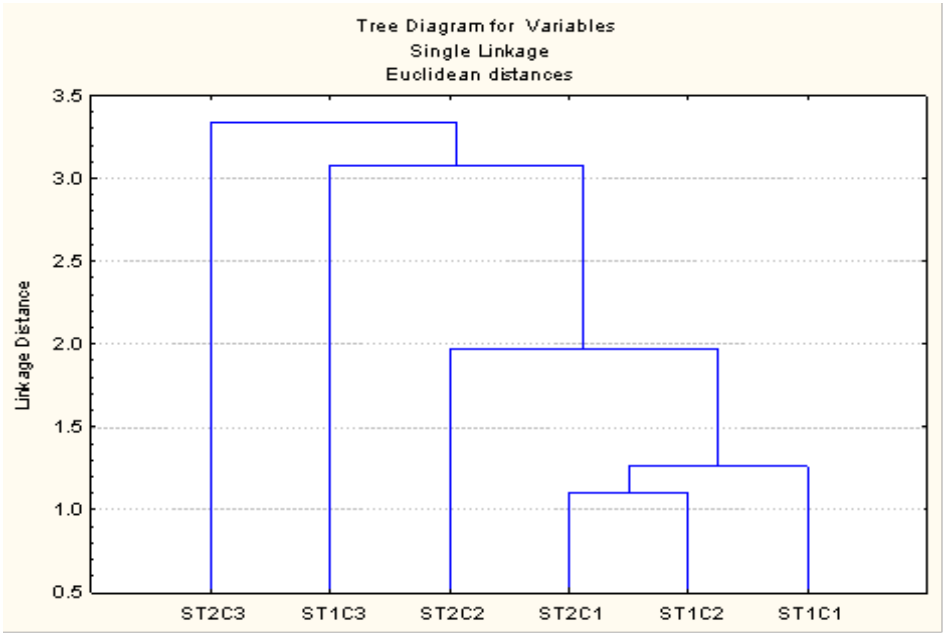


(b)

Figure 5.20: (b) Cluster analysis of heavy metals behavior in soil.

5.5.3.2 Cluster analysis of mycelium samples

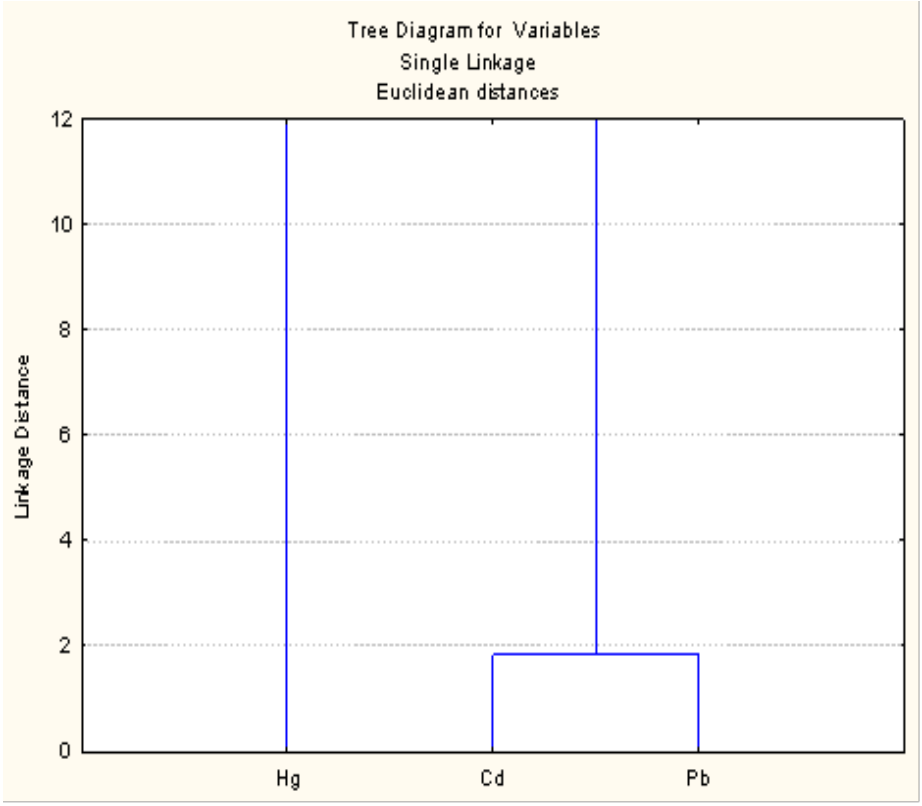
Cluster analysis of mycelium samples which belong to SET I and SET II form clusters in echelons of increasing concentrations of heavy metal contents along with incubation time (**Figure 5.21 a**).



(a)

Figure 5.21:.(a) Cluster analysis of mycelium samples.

Lead and cadmium show close relation, mercury behavior significantly distant relation with cadmium and lead in mycelium (**Figure 5.21 b**).



(b)

Figure 5.21: (b) Cluster analysis of heavy metals behavior in mycelium.

6. CONCLUSION

Since we all inhabit the Earth, it encompasses each and every one of us, warm or cold-blooded, mammal, vertebrate or invertebrate, bird, reptile, amphibian, fish, and human alike. Humans, therefore, being not the only species on this planet, share this world with millions of other living creatures as we all evolved here together (Earthlings, 2005) (URL-15). The invasion of industrialization, usage of organic and inorganic chemicals in agriculture and improper waste disposal activities caused anthropogenic contamination of soil and ground water with heavy metals and becoming a major problem worldwide for earthlings.

On the other hand the metal-uptake, metal tolerance/resistance and defence ability of organisms like plants, fungi, algea and bacteria has still been studying. The metal removal mechanism which is carried out by microorganisms activity is a complex process, affected by chemistry of metal ions, cell wall compositions of microorganisms, cell physiology, pH, temperature, time, ionic strength and metal concentration (Morsy et al., 2010).

The main transport membranes of fungi are plasma and vacuolar membranes (Sanders,1990). The great importance of K^+ and Ca^{2+} in fungal growth, metabolism and differentiation, most work on metal ion transport in fungi has concerned K^+ and Ca^{2+} (Gadd, 1992).

Fungal cellular transport systems are generally classed as either carrier or channel systems. Carriers consist of all metabolically-coupled and H^+ gradient driven transport systems. Ion channels are a class of proteins in plasma membrane which allow the flow of ions down electrical and/or chemical gradients (Gustin et al., 1986). Channels have higher turn over rates than carriers, 10^{7-8} s^{-1} compared to 10^{2-4} s^{-1}, respectively (Sanders, 1990).

The main primary transport systems which have been characterized in fungi derive energy from ATP hydrolysis and pump H^+ electrogenically from the cytosol, creating a transmembrane electrochemical H^+ gradient, negative and alkaline inside. This electrochemical gradient can drive the transport of ionizable substances across

membranes (Gadd, 1992). The secondary gradient coupled transport systems energized by coupling with passive refflux of H^+ proton and solute fluxes maybe in the same (symport) or opposing (antiport) directions (Sanders, 1990).

Metal-binding proteins are important in the modulation of intracellular concentrations of both potentially-toxic and essential metal ions which are called as *metallothioneins* (Hamer, 1986; Gadd 1992). These proteins are subdivision into three classes has been recommended as Class I, Class II , Class III (Rauser, 1990).

Class I includes most vertebrate forms as well as some fungal metallothioneins, e.g. those of *Neurospora crassa* and *Agaricus bisporus* (Lerch and Beltramini, 1983; Munger and Lerch, 1985). Class II metallothioneins are found in *Saccharomyces cerevisiae* (Steffens, 1990). Algae, plants and certain fungi, e.g. *Schizosaccharomyces pombe* and *Candida glabrata*, produce class III metallothionein (Rauser, 1990). Metallothioneins are small, cysteine-rich polypeptides that can bind essential metals, e.g. Cu and Zn, as well as inessential metals such as Cd (Gadd, 1992).

In addition to cysteine-rich polypeptides of the metallothionein family, fungi can synthesize another group of metal-binding molecules. These are short, cysteine-containing γ-glutamyl peptides, encompassed by the common name *phytochelatins* (Winge et al., 1989; Rauser, 1990). These γ-glutamyl peptides are the main metal detoxification mechanism in algae and plants (Grill et al., 1988; Gekeler et al., 1988) as well as in arrange of filamentous fungi and yeasts.

The similarity in structure between glutathione and γ-glutamyl peptides indicates that biosynthesis of these peptides shares common features with that of glutathione (Steffens, 1990) and they may be viewed as linear polymers of the γ-glutamyl-cysteine (γ-Glu-Cys) portion of glutathione (Grill et al., 1989) (**Figure 6.1**).

Mutoh and Hayashi, 1988 reported that γ-glutamyl peptides were essential for Cd-detoxification and clearly demonstrated biochemical linkage with glutathione metabolism. It is now known that γ-glutamyl peptides are synthesized by the enzyme γ-glutamyl-cysteine dipeptidyl transpeptidase (phytochelatin synthase).

Moreover Cd^{2+} is the best activator of the enzyme followed by Ag^+, Bi^{3+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+} and Au^+ and it appears that there action product (γ-glutamyl peptide) chelates the activating metal ions thus terminating the reaction (Grill et al., 1989; Loeffler et al., 1989).

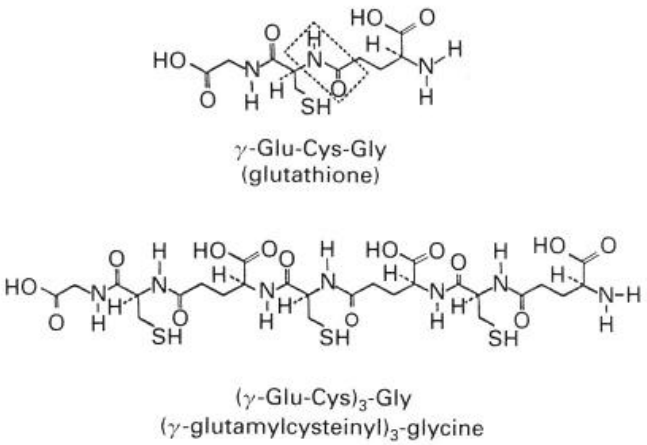


Figure 6.1: Structures of glutathione and the metal-binding polypeptide, (γ-glutamyl-cysteinyl)$_3$-glycine. *Source*: Adapted from Steffens, 1990.

Schematic representation of cellular metal tolerance mechanisms in ectomycorrhizal fungi was shown in **Figure 6.2**.

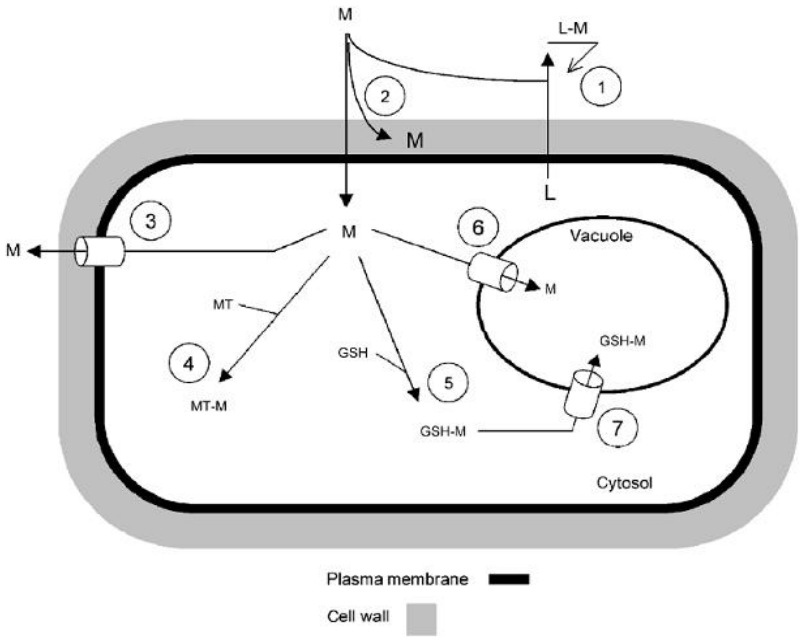


Figure 6.2: Schematic representation of cellular mechanisms potentially involved in metal tolerance in ectomycorrhizal fungi. M, metal-ion; 1, extracellular chelation by excreted ligands (L); 2, cell-wall binding; 3, enhanced efflux; 4, intracellular chelation by metallothionein (MT); 5, intracellular chelation by gluthathione (GSH); 6, subcellular compartmentation (vacuole or other internal compartments); 7, vacuolar compartmentation of GSH-M complex. *Source:* Adapted from Bellion et al., 2006.

Bioaccumulation of metallic elements, metalloids and non-metals in fruit bodies is highly species specific. Some macrofungi are able to accumulate elements much more effectively than other species; the ability to accumulate a particular element is a

characteristic feature of a particular fungal species (Falandysz and Borovička, 2013). The ratio of an element in fruit body to its concentration in soil called as *bioaccumulation factor*.

The hyphae (mycelium), rhizomorphs, which are root-like structures of filementous fungi can serve as biological filters since their aerial structures consist of large biomass to be diffuse a huge area, makes them potential sorbents for water and mineral compounds uptake from soils (Volesky and Holan, 1995; Damodaran et al., 2013).

Under suitable conditions mycelia spread over an available area like soil, wood residues etc. and by absorbing metal ions from environment, accumulate them in their cytosol. The mycelia mimic the roots of the plants in removing heavy metals from contaminated soil, known as mycofiltration which pioneer to mycoremediation of contaminated soil. In this context, the potential of macro fungi belonging to Basidiomycetes act as an excelent mycoremediation tool. Comparing to plants, mushrooms have shorter life cycle (30 days) and show better adaptability to environment. Therefore mycoremediation can be regarded as an evolved remediation technique (Volesky and Holan, 1995; Damodaran et al., 2013).

Based on the studies between mushrooms and heavy metal interaction in soil, it has been reported that mushrooms can concentrate high concentrations of heavy metals such as lead, cadmium and mercury in comparison to plants (Gast et al., 1988).

Fungi have low potential to bioaccumulate lead and Pb relatively abundant in forest topsoil compared to Cd or Hg. Cadmium and mercury are well bioaccumulated heavy metals and while cadmium can be found at elevated concentrations in wild-grown mushrooms, mercury is present in small concentrations in forest soils (Falandysz and Borovička, 2013). Wood-decaying mushroom species are low in Hg compared to the numerous soil mushrooms, and good examples are *Pleurotus* spp. with *P. ostreatus* (Nnorom et al., 2012).

Mushrooms are valued due to their aroma, taste and their medical properities, as well as their protein, vitamins, minerals and low fat contents. Apart from the abundance of minerals desired, mushrooms may contain undesired potentially toxic elements including Cd and Hg sometimes at elevated concentrations (Falandysz and Borovička, 2013).

- Nonessential heavy metals are known to act as enzymatic inhibitors by denaturating proteins due to their chemical similarity to essential microelements. Therefore only some species that can successfully tolerate these physiological stresses, can survive in heavy metal contaminated environment (Damodaran et al, 2013).

- In this study a wood-degrading white-rot fungus *Pleurotus ostreatus* was used to test its heavy metal bioremediation capacity for heavy metals under study were lead, cadmium and mercury from straw enriched soil for its one month and two month incubation periods.

- Heavy metal analyses of soil and mycelium samples showed that the incubation periods have significant effect on bioaccumulation of metals from the soil and bioaccumulation percentages were increased for each metal by incubation periods, in order of SET I C1<SET II C1, SET I C2<SET II C2, SET I C3<SET II C3.

- Bioaccumulated concentration of lead, cadmium and mercury were found higher in SET II mycelium samples than SET I. It pointed at better colonization of *Pleurotus ostreatus* in soil particules.

- When lead bioaccumulation increasing by incubation period, it decearesed by its increasing concentration in SET I and SET II, in order of SET I C3<SET I C2<SET I C1 and SET II C3<SET II C2<SET II C1. Cadmium and mercury accumulation increased by their increasing concentrations both SET I and SET II, in order of SET I C1< SET I C2<SET I C3 and SET II C1<SET II C2<SET II C3.

- Various researchers have found that the heavy metal accumulation ability varies from species to species (Falandysz and Borovička, 2013). The bioaccumulation potential of *P. ostreatus* was found to be higher than some mushroom species reported in the literature. Lead, cadmium and mercury content of some mushrooms represent in **Table 6.1**.

- For one and two month incubation periods did not allow to form mature fruting body. Pinhead formation was observed exclusively.

- It was found that the most bioaccumulated heavy metal by *Pleurotus ostreatus* was mercury. The mycelium samples belonged to 10 ppm (C3) mercury contained soil showed 54 % Hg bioaccumulation and 85 % ratio, in SET I and SET II, respectively.

Table 6.1: Heavy metal content in fruiting body of various tolerant mushrooms.

Mushroom species	Metal content in fruiting body, ppm of dry wt.	References
Lepiota rhacodes	Pb (66), Cd (3.7)	Srivastava and Thakur (2006).
Paxillus rubicondulus	Pb (0.69), Cd (0.78)	Srivastava and Thakur (2006).
Pleurotus sp.	Pb (3.4), Cd (1.18)	Mitra (1994).
Omphalotus olearius	Cd (1.3), Pb (5.2)	Yılmaz et al. (2002).
Poria sp.	Pb (1.0), Cd (0.1)	Ita et al. (2006).
Nectria cinnabarina	Pb (1.9), Cd (0.2)	Ita et al. (2006).
Ganoderma lucidum	Pb (0.7), Cd (0.31)	Ita et al. (2006).
Polyporus frondosus	Pb (0.4), Cd (0.2)	Ita et al. (2006).
Phellinus badius	Cd (110), Hg (61)	Bardan et al. (2012).
Phellinus sanguineus	Cd (80), Hg (35)	Bardan et al. (2012).
Pleurotus platypus	Cd (34.9), Pb (27.1)	Vimala and Das (2009).
Agrocybe aegerita	Cd (0.01), Pb (0.018)	Konuk et al. (2007).
Galerina sp.	Cd (85), Pb (900)	Damodaran et al. (2013).
Pleurotus ostreatus	Pb C1 (1.25*-1.5**) Pb C2 (0.75*-1.55**) Pb C3 (0.55*-1.55**) Cd C1 (0.23*-0.4**) Cd C2 (0.4*-0.55**) Cd C3 (0.8*-1.3**) Hg C1 (0.85*-2.1**) Hg C2 (2.85*-4.6**) Hg C3 (7.45*-10.6**)	

*** 30 days incubation period (SET I)**

**** 60 days incubation period (SET II)**

Source: Adapted and custumized from Damodaran et al., 2013.

- The strong correlation between cadmium and mercury indicates their strong bioaccumulation capacity in *Pleurotus ostreatus* comparison to lead. In the course of the accumulation of cadmium and mercury in fungi seems to be related and no competition between the uptake of these matals are as though accumulated by different physiological mechanisms (Brunnet and Zadrazil, 1981; Kojo and Lodenius, 1989). Cadmium and mercury were accumulated more efficiently by *P. ostreatus* for concentrations of C1, C2, C3 in both SET I and SET II.

- Because of the white-rot fungi can be grown on a number of inexpensive agricultural or forest wastes such as wheat straw, corn cobs and sawdust, using them as bioremediation tools are expected to be relatively economical way for remediation. On the way of searching for economical and ecologic methods for

environmental remediation, the use of mushrooms is a very good approach. More intensive research needs to be find out ecology of mushrooms and their potentials for bioremediation.

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APPENDICES

APPENDIX A: Laboratory Equipment.
APPENDIX B: Chemicals.
APPENDIX C: Solutions.
APPENDIX D: Soil ICP-MS Heavy Metal Analyses Results.
APPENDIX E: Mycelium ICP-MS Heavy Metal Analyses Results.

APPENDIX A

Laboratory equipment

Analytical balance: METTLER TOLEDO AB204-S

Autoclave: NÜVE 0T 032

Centrifuges: Universal 320 Hettich

Digital Thermo-Hygrometer: TFA

Drying oven (105 ^{0}C): MEMMERT

Home type termometer

Labwater : RIOS-D1 UV Millipore

Laboratory balance: METTLER TOLEDO PB1502-L

Laminar flow: Labculture® Class II, Type A2

Magnetic stirrer, heater: WiseStir MSH-20A

pH meter: Orion 720A+

Pure water systems: SYNERGY UV Milipore

Refrigerator: INDESIT Total No Frost

APPENDIX B

Chemicals

Agar-Agar: Merck
Malt Extract Agar**:** Merck

APPENDIX C

Solutions

Cadmium Standard Solution: Merck

Lead Standard Solution: Merck

Mercury Standard Solution: Merck

APPENDIX D

Soil ICP-MS Heavy Metal Analyses

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Client: **Istanbul Technical University**
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Department, Ayazaga Campus
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Submitted By: Mustafa Sami Yazgan
Receiving Lab: Turkey-Ankara
Received: March 19, 2015
Report Date: April 06, 2015
Page: 1 of 2

CERTIFICATE OF ANALYSIS ANK15000203.1

CLIENT JOB INFORMATION

Project: Mycoremediation of Heavy Metals from Soil Using Pleurotus
Shipment ID:
P.O. Number
Number of Samples: 20

SAMPLE DISPOSAL

DISP-PLP Dispose of Pulp After 90 days

Bureau Veritas does not accept responsibility for samples left at the laboratory after 90 days without prior written instructions for sample storage or return.

SAMPLE PREPARATION AND ANALYTICAL PROCEDURES

Procedure Code	Number of Samples	Code Description	Test Wgt (g)	Report Status	Lab
Dry at 60C	20	Dry at 60C			ANK
SS80	20	Dry at 60C sieve 100g to -80 mesh			ANK
SHP01	20	Per sample shipping charges for branch shipments			ANK
MA200	20	4 Acid digestion ICP-MS analysis	0.25	Completed	VAN
AQ200-HG	20	Hg by AR digestion ICP-MS analysis	0.5	Completed	VAN
DISP2	20	Heat treatment of Soils and Sediments			VAN

ADDITIONAL COMMENTS

Mycoremediation of Heavy Metals from Soil Using Pleurotus ostreatus

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All results are considered the confidential property of the client. Bureau Veritas assumes the liabilities for actual cost of analysis only. Results apply to samples as submitted.
** asterisk indicates that an analytical result could not be provided due to unusually high levels of interference from other elements.

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Project: Mycoremediation of Heavy Metals from Soil Using Pleurotus ostreatu
Report Date: April 06, 2015

CERTIFICATE OF ANALYSIS ANK15000203.1

Method		MA200	MA200	AQ200
Analyte		Pb	Cd	Hg
Unit		ppm	ppm	ppm
MDL		0.1	0.1	0.01
DS203	Soil	53.6	11.9	14.29
DS23D	Soil	50.5	11.1	11.05
DS23C	Soil	49.7	11.4	12.39
DS101	Soil	37.6	2.9	3.08
DS11C	Soil	39.6	3.3	4.05
DS11D	Soil	38.2	3.3	3.85
DS202	Soil	43.7	6.1	6.77
DS22C	Soil	44.1	6.4	7.22
DS22D	Soil	43.5	6.5	8.40
DS201	Soil	39.6	3.5	3.44
DS21C	Soil	39.4	3.3	3.80
DS21D	Soil	41.1	3.3	3.74
DS102	Soil	40.6	5.5	6.28
DS12C	Soil	42.8	6.4	6.90
DS12D	Soil	42.5	6.2	6.52
DS103	Soil	49.1	11.9	11.34
DS13C	Soil	51.6	12.0	13.76
DS13D	Soil	47.8	11.9	11.78
DS200	Soil	39.0	0.3	0.10
DS100	Soil	36.2	0.3	0.23

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Project: Mycoremediation of Heavy Metals from Soil Using Pleurotus ostreatu
Report Date: April 06, 2015

QUALITY CONTROL REPORT ANK15000203.1

Method		MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200
Analyte		Mo	Cu	Pb	Zn	Ag	Ni	Co	Mn	Fe	As	U	Th	Sr	Cd	Sb	Bi	V	Ca	P	La
Unit		ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	%	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	%	%	ppm
MDL		0.1	0.1	0.1	1	0.1	0.1	0.2	1	0.01	1	0.1	0.1	1	0.1	0.1	0.1	1	0.01	0.001	0.1
Pulp Duplicates																					
DS100	Soil	1.2	76.1	36.2	104	0.1	84.2	25.6	1950	5.17	15	2.1	8.9	41	0.3	1.5	0.4	123	0.49	0.066	33.4
REP DS100	QC	1.3	75.2	36.7	102	<0.1	82.4	24.5	1868	5.03	14	2.0	9.3	39	0.2	1.2	0.4	125	0.48	0.067	36.0
Reference Materials																					
STD DS10	Standard																				
STD OREAS25A-4A	Standard	2.3	35.7	25.6	47	<0.1	45.6	7.5	453	5.96	9	3.0	15.6	44	<0.1	0.7	0.3	147	0.27	0.049	21.0
STD OREAS45EA	Standard																				
STD OREAS45E	Standard	2.2	741.1	19.7	46	0.3	450.7	57.8	519	23.30	16	2.6	12.2	15	<0.1	1.0	0.3	292	0.06	0.034	8.9
STD DS10 Expected																					
STD OREAS25A-4A		2.55	33.9	25.2	44.4		45.8	8.2	470	6.6		2.94	15.8	48.5		0.67	0.35	157	0.309	0.048	21.8
STD OREAS45E Expected		2.4	780	18.2	46.7	0.311	454	57	570	24.12	16.3	2.41	12.9	15.9	0.06	1	0.28	322	0.065	0.034	11
BLK	Blank																				
BLK	Blank	<0.1	<0.1	0.3	<1	<0.1	<0.1	<0.2	2	<0.01	<1	<0.1	<0.1	<1	<0.1	<0.1	<0.1	<1	<0.01	<0.001	<0.1

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Project: Mycoremediation of Heavy Metals from Soil Using Pleurotus ostreatu
Report Date: April 06, 2015

Page: 1 of 1 Part: 2 of 3

QUALITY CONTROL REPORT ANK15000203.1

	Method	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200	MA200
	Analyte	Cr	Mg	Ba	Ti	Al	Na	K	W	Zr	Ce	Sn	Y	Nb	Ta	Be	Sc	Li	S	Rb	Hf
	Unit	ppm	%	ppm	%	%	%	%	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	%	ppm	ppm
	MDL	1	0.01	1	0.001	0.01	0.001	0.01	0.1	0.1	1	0.1	0.1	0.1	0.1	1	1	0.1	0.1	0.1	0.1
Pulp Duplicates																					
DS100	Soil	100	0.89	392	0.350	8.65	0.472	1.77	1.3	62.7	56	2.8	45.9	9.7	0.6	3	19	43.4	<0.1	80.7	1.9
REP DS100	QC	96	0.87	392	0.353	8.73	0.457	1.77	1.3	61.2	58	2.6	46.4	9.5	0.7	3	19	44.9	<0.1	79.5	1.8
Reference Materials																					
STD DS10	Standard																				
STD OREAS25A-4A	Standard	116	0.29	136	0.950	8.71	0.126	0.45	1.9	141.4	45	3.6	10.2	19.3	1.4	<1	12	38.4	<0.1	54.0	4.1
STD OREAS45EA	Standard																				
STD OREAS45E	Standard	925	0.14	235	0.508	6.25	0.055	0.32	1.0	91.9	20	1.2	7.1	6.0	0.5	<1	86	6.3	<0.1	19.1	2.8
STD DS10 Expected																					
STD OREAS25A-4A		115	0.327	147	0.977	8.87	0.134	0.482	2.1		48.9	4.06	12.3	22.4	1.6	1.02	13.7	36.7	0.051	61	4.53
STD OREAS45E Expected		979	0.156	252	0.559	6.78	0.059	0.324	1.07	97	23.5	1.32	8.28	6.8	0.54		93	6.58	0.046	21.2	3.11
BLK	Blank																				
BLK	Blank	<1	<0.01	<1	<0.001	<0.01	0.001	<0.01	<0.1	<0.1	<1	<0.1	<0.1	<0.1	<0.1	<1	<1	0.1	<0.1	<0.1	<0.1

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Project: Mycoremediation of Heavy Metals from Soil Using Pleurotus ostreatu
Report Date: April 06, 2015

Page: 1 of 1 Part: 3 of 3

QUALITY CONTROL REPORT ANK15000203.1

	Method	MA200	MA200	MA200	MA200	MA200	AQ200
	Analyte	In	Re	Se	Te	Tl	Hg
	Unit	ppm	ppm	ppm	ppm	ppm	ppm
	MDL	0.05	0.005	1	0.5	0.5	0.01
Pulp Duplicates							
DS100	Soil	0.11	<0.005	<1	<0.5	0.5	0.23
REP DS100	QC	0.09	<0.005	<1	<0.5	0.6	
Reference Materials							
STD DS10	Standard						0.36
STD OREAS25A-4A	Standard	0.11	<0.005	2	<0.5	<0.5	
STD OREAS45EA	Standard						0.10
STD OREAS45E	Standard	0.08	<0.005	2	<0.5	<0.5	
STD DS10 Expected							0.3
STD OREAS25A-4A						0.35	
STD OREAS45E Expected		0.099		2.97	0.1	0.09	
BLK	Blank						0.02
BLK	Blank	<0.05	<0.005	<1	<0.5	<0.5	

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APPENDIX E

Mycelium ICP-MS Heavy Metal Analyses

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Submitted By: Mustafa Sami Yazgan
Receiving Lab: Turkey-Ankara
Received: March 19, 2015
Report Date: April 06, 2015
Page: 1 of 2

CERTIFICATE OF ANALYSIS ANK15000202.1

CLIENT JOB INFORMATION

Project: Mycoremediation of Heavy Metals from Soil Using Pleurotus
Shipment ID:
P.O. Number
Number of Samples: 12

SAMPLE DISPOSAL

DISP-PLP Dispose of Pulp After 90 days

SAMPLE PREPARATION AND ANALYTICAL PROCEDURES

Procedure Code	Number of Samples	Code Description	Test Wgt (g)	Report Status	Lab
SHP01	12	Per sample shipping charges for branch shipments			ANK
VGMAS	12	Plant Maceration to 1mm			VAN
VG101	12	Aqua Regia digestion ICP-MS analysis	1	Completed	VAN
DRPLP	12	Warehouse handling / disposition of pulps			ANK

ADDITIONAL COMMENTS

Mycoremediation of Heavy Metals from Soil Using Pleurotus ostreatus

Bureau Veritas does not accept responsibility for samples left at the laboratory after 90 days without prior written instructions for sample storage or return.

Invoice To: Istanbul Technical University
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Istanbul ISTANBUL 34469
TURKEY

CC:

BRITISH COLUMBIA CERTIFIED ASSAYER
Marcus Lau
MARCUS LAU

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All results are considered the confidential property of the client. Bureau Veritas assumes the liabilities for actual cost of analysis only. Results apply to samples as submitted.
** asterisk indicates that an analytical result could not be provided due to unusually high levels of interference from other elements.

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Project: Mycoremediation of Heavy Metals from Soil Using Pleurotus ostreatu
Report Date: April 06, 2015

CERTIFICATE OF ANALYSIS ANK15000202.1

Method		VG101	VG101	VG101
Analyte		Pb	Cd	Hg
Unit		ppm	ppm	ppb
MDL		0.01	0.01	1
DS2M03	Vegetation	1.31	1.10	9495
DS2M3D	Vegetation	1.79	1.46	11724
DS1M01	Vegetation	1.76	0.26	682
DS1M1D	Vegetation	0.68	0.24	1022
DS2M02	Vegetation	1.10	0.41	3655
DS2M2D	Vegetation	2.07	0.74	5493
DS2M01	Vegetation	1.42	0.39	1559
DS2M1D	Vegetation	1.60	0.43	2577
DS1M02	Vegetation	0.67	0.34	2359
DS1M2D	Vegetation	0.75	0.50	3282
DS1M03	Vegetation	0.47	0.69	6732
DS1M3D	Vegetation	0.63	0.88	8200

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Project: Mycoremediation of Heavy Metals from Soil Using Pleurotus ostreatu
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Page: 1 of 1 Part: 1 of 2

QUALITY CONTROL REPORT ANK15000202.1

	Method	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101
	Analyte	Mo	Cu	Pb	Zn	Ag	Ni	Co	Mn	Fe	As	U	Au	Th	Sr	Cd	Sb	Bi	V	Ca	P
	Unit	ppm	ppm	ppm	ppm	ppb	ppm	ppm	ppm	%	ppm	ppm	ppb	ppm	ppm	ppm	ppm	ppm	ppm	%	%
	MDL	0.01	0.01	0.01	0.1	2	0.1	0.01	1	0.001	0.1	0.01	0.2	0.01	0.5	0.01	0.02	0.02	2	0.01	0.001
Pulp Duplicates																					
DS1M3D	Vegetation	0.25	3.04	0.53	18.4	12	1.0	0.24	45	0.056	0.2	<0.01	0.8	0.06	19.5	0.88	0.06	<0.02	<2	0.37	0.062
REP DS1M3D	QC	0.24	3.47	0.61	17.0	10	0.9	0.24	42	0.051	0.2	<0.01	0.8	0.05	19.4	0.89	0.06	<0.02	<2	0.37	0.060
Reference Materials																					
STD CDV-1	Standard	0.19	7.52	0.87	19.0	8	5.8	1.73	363	0.266	1.3	0.15	1.2	0.56	112.7	0.04	0.05	<0.02	8	1.92	0.036
STD V16	Standard	1.83	7.04	2.93	38.8	49	10.3	1.14	638	0.531	1.4	<0.01	10.3	<0.01	10.7	0.10	0.13	<0.02	155	0.29	0.043
STD V16 Expected		1.6	6.89	3	39.2	32	7.4	1.11	720	0.4125	1.6		0.9		11.2	0.086	0.07			0.3	0.0488
STD CDV-1 Expected		0.2	8.61	1	23.3	9	6.4	2	385	0.256	1.3	0.17	2.3	0.61	112	0.04	0.03	0.02	4.2	1.94	0.038
FLOUR	Blank	0.53	3.11	0.03	24.2	<2	0.2	<0.01	30	0.004	<0.1	<0.01	0.3	<0.01	1.3	0.02	0.03	0.04	<2	0.03	0.283
BLK	Blank	<0.01	0.04	<0.01	0.1	<2	<0.1	<0.01	<1	<0.001	<0.1	<0.01	<0.2	<0.01	<0.5	<0.01	<0.02	<0.02	<2	<0.01	<0.001
Prep Wash																					
RICE	Prep Blank	0.14	2.04	0.04	13.3	3	0.2	0.01	7	<0.001	0.5	<0.01	<0.2	<0.01	<0.5	<0.01	<0.02	0.02	<2	<0.01	0.065

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Project: Mycoremediation of Heavy Metals from Soil Using Pleurotus ostreatu
Report Date: April 06, 2015

Page: 1 of 1 Part: 2 of 2

QUALITY CONTROL REPORT ANK15000202.1

	Method	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101	VG101
	Analyte	La	Cr	Mg	Ba	Ti	B	Al	Na	K	W	Sc	Tl	S	Hg	Se	Te	Ga
	Unit	ppm	ppm	%	ppm	ppm	ppm	%	%	%	ppm	ppm	ppm	%	ppb	ppm	ppm	ppm
	MDL	0.01	0.1	0.001	0.1	1	1	0.01	0.001	0.01	0.1	0.1	0.02	0.01	1	0.1	0.02	0.1
Pulp Duplicates																		
DS1M3D	Vegetation	0.44	2.2	0.070	38.4	4	1	0.03	0.065	0.32	<0.1	0.4	<0.02	0.08	8200	<0.1	<0.02	<0.1
REP DS1M3D	QC	0.42	2.2	0.065	35.2	3	<1	0.03	0.061	0.31	<0.1	0.2	<0.02	0.09	7363	<0.1	<0.02	<0.1
Reference Materials																		
STD CDV-1	Standard	2.32	11.5	0.111	8.4	26	12	0.11	0.006	0.18	<0.1	1.0	<0.02	0.11	48	0.2	<0.02	0.4
STD V16	Standard	0.04	350.6	0.050	1.9	9	5	0.04	0.001	0.22	<0.1	0.2	<0.02	<0.01	47	<0.1	<0.02	0.1
STD V16 Expected		0.05	323.1	0.0525	1.9	12	5	0.0454	0.0015	0.22				0.0177	41			0.2
STD CDV-1 Expected		2.31	12.1	0.12	9	30	12	0.15	0.0052	0.18		0.8		0.1	41	0.3		0.5
FLOUR	Blank	<0.01	1.8	0.112	2.7	4	<1	<0.01	0.001	0.32	<0.1	0.3	<0.02	0.18	<1	0.6	<0.02	<0.1
BLK	Blank	<0.01	0.2	<0.001	<0.1	<1	<1	<0.01	<0.001	<0.01	<0.1	<0.1	<0.02	0.01	3	<0.1	<0.02	<0.1
Prep Wash																		
RICE	Prep Blank	<0.01	1.8	0.009	0.1	<1	<1	<0.01	<0.001	0.06	<0.1	0.3	<0.02	0.12	9	0.2	<0.02	<0.1

This report supersedes all previous preliminary and final reports with this file number dated prior to the date on this certificate. Signature indicates final approval; preliminary reports are unsigned and should be used for reference only.

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