Doctoral Dissertation

BIOREMEDIATION OF MERCURY CONTAMINATED SOIL USING *Aspergillus flavus* STRAIN KRP1

EVI KURNIATI

Division of Environmental Science and Engineering
Graduate School of Science and Engineering
Yamaguchi University, Japan
September 2014

博士論文
Doctoral Dissertation

BIOREMEDIATION OF MERCURY CONTAMINATED SOIL USING *Aspergillus flavus* STRAIN KRP1

(*Aspergillus flavus* STRAIN KRP1 を用いた水銀汚染土壌の バイオレメディエーション に関する研究)

エビ　クリニアティ

EVI KURNIATI

A dissertation submitted to the Division of Civil and Environmental Science and Engineering of Yamaguchi University in partial fulfillment of the requirements for the degree of Doctor of Engineering

Advisor: Prof. Tsuyoshi Imai
(Div. of Environmental Science and Engineering)

Committee Members:
1. **Prof. Tsuyoshi Imai** (Div. of Environmental Science and Engineering)
2. **Prof. Masahiko Sekine** (Div. of Civil and Environmental Engineering)
3. **Prof. Masakazu Niinae** (Div. of Environmental Science and Engineering)
4. **Assoc. Prof. Toorisaka Eiichi** (Div. of Environmental Science and Engineering)
5. **Assoc. Prof. Takaya Higuchi** (Div. of Environmental Science and Engineering)

山口大学大学院理工学研究科環境共生系専攻

Division of Environmental Science and Engineering
Graduate School of Science and Engineering
Yamaguchi University, Japan

September 2014

ABSTRACT

Heavy metal presence in agricultural soil might be caused by long use of fertilizer, pesticides, as well as polluted water for irrigation. Mercury contamination in agricultural soil is generally due to application of municipal wastewater and industrial effluent for crop irrigation. Those might drive to its absorption by plant which is caused dangerous if it consumed by human or livestock. Fungi are known to tolerate and detoxify metals by several mechanisms including valence transformation, extra and intracellular precipitation and active uptake in associated with the production of antibiotics, enzymes and organic acid which is drive to future application for metal remediation from soil.

This research aims to observe the capability of filamentous fungi isolated from forest soil for bioremediation of mercury contamination. Six fungal strains were selected based on their capability to grow in 25 mg/L Hg^{2+}-contaminated potato dextrose agar plates. Fungal strain KRP1 showed the highest ratio of growth diameter, 0.831, thus was chosen for further observation. Identification based on colony and cell morphology carried out by 18S rRNA analysis gave a 98% match to *Aspergillus flavus* strain KRP1. The fungal characteristics in mercury(II) contamination such as range of optimum pH, optimum temperature and tolerance level were 5-7 and 27.5-35 °C and 100 mg/L respectively. The concentration of mercury in the media affected fungal growth during lag phases.

The fungal strain was also evaluated in vitro for the potential use in bioremediation of soil contaminated with mercury through observation of the growth profile and the mercury concentration in culture medium. The growth profiles of *Aspergillus flavus* strain KRP1 showed considerable growth in culture medium containing mercury. This result was supported by the decrease of mercury concentration which indicates a utilization process for mercury and might have mechanism for utilization. The capability of the fungal strain to remove the mercury(II) contaminant was evaluated in 100 mL sterile 10 mg/L Hg^{2+}-contaminated potato dextrose broth media in 250 mL Erlenmeyer flasks inoculated with 10^8 spore/mL fungal spore suspension and incubation at 30°C for 7 days. The mercury(II) utilization was observed for flasks shaken in a 130 r/min orbital shaker (shaken) and non-shaken flasks (static) treatments. Flasks containing contaminated media with no fungal spores were also provided as control. All treatments were done in triplicate. The strain was able to remove 97.50% and 98.73% mercury from shaken and static systems respectively. *A. flavus* strain KRP1 seems to have potential use in bioremediation of aqueous substrates containing mercury(II) through a bio sorption mechanism.

Plants are originally known to have capability to uptake heavy metals from contaminated sites through phytoremediation. This process is potentially noxious if the plant is a consumed plant because it will lead to bio-magnification mainly in case of mercury contamination. The results showed that the presence of mercury contaminant affected the total number of microbe yet tend to decrease the mercury contaminant from soil. The presence of plant itself is possible to remove mercury from soil as well as support the microbial growth resulted that combination between plant and fungal augmentation perform better in mercury removal from soil. In case of bioremediation, the selection of plant species is important either for better remediating performance or avoiding bio-magnification of mercury on food chain.

Keywords: soil fungi, mercury(II) contaminated soil, bioremediation, *Aspergillus flavus* strain KRP1

ACKNOWLEDGEMENTS

The greatest gratitude is for the Great Allah SWT for all blessing me and my family so that I am possible to have opportunity to study here. I would like to thanks to all supporters for the success of this dissertation for the invaluable support.

Great respectful to my academic advisor, Prof. TSUYOSHI IMAI for the guidance, supported facility, expert overview, and encouragement during my study stay in Japan.

Also a gratitude to Prof. MASAHIKO SEKINE, Assoc. Prof. TAKAYA. HIGUCHI, Assoc. Prof. K. YAMAMOTO and Asst. Prof. ARIYO KANNO, for the expert and great advices as well as their kind attention in many aspect.

I would like to acknowledge to The Indonesia Education Ministry for financial support through the DIKTI scholarship.

My warmest gratitude to Toshimi Yamamoto, Sun Xiaofeng, Wei Jie, Le Van Tuan, Vo Thanh Huy, Rina Febrina, Riyanto Haribowo, Kanthima Pummala, Ahmad Rivai, Yatnanta Padma Devia, Waranya Suwannasing, Thanhloc Dang Thi, Girija Pavatkhar and other friend for sharing ideas, knowledge, literature, joyful, smile and painful during my life in Japan.

Finally, I would like to express my heartfelt gratitude to my big family, my great man EDI SISWANTO for the love, support, patience and understanding, my priceless stars (FARIS, SHAFLY and SOFI) for their patience and understanding, big family M. THOHA AR and ENDRO SANTOSO the immeasurable long distance support and love.

Evi Kurniati

September 2014

TABLE OF CONTENTS Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1
INTRODUCTION

1.1 Background

Heavy metals contamination on agriculture soil decreases the economic value of the soil. Once it contaminated, so it is risky for use for agriculture because of potential absorption of hazardous heavy metals on the consumable plant crop. Heavy metals contamination may come from a long term use of heavy metals containing fertilizer, pesticide, fungicide, and also the use of municipal wastewater use for irrigation.

Mercury in soils has a long retention times and having a negative impact towards living organism (Cooper and Gillespie, 2001; Erdogrul, 2007; Spry and Wiener, 1991). The soil contaminated with mercury was mainly due to coal combustion, mercury and gold mining activities as well as industrial activities (Karunasagar *et al*., 2003). The remediation of mercury polluted soil is particularly important because mercury does not degrade and thus persist almost indefinitely in the environment. Methods such as excavation and disposal, stabilization/solidification, electro-remediation, soil washing/leaching, and as well as thermal desorption was known to be less economic favor.

Mercury contaminations in agricultural soil were generally due to application of municipal wastewater and industrial effluent for crop irrigation that often occurs in developing country such as India for example (Thippeswamy *et al*., 2012). The other sources mercury in agriculture fields also come from fertilizers, fungicides and pesticides, although the use of mercury in these products has been greatly reduced (UNEP, 2013). Mercury in soil is firmly bound to organic matter or precipitated as sulphide, and is found in trace concentrations in soil solutions (Schuster, 1991).

Some remediation technologies for mercury-contaminated soil have been developed. In general, the critical point of mercury concentration in soil for the application of remediation technologies is at 260 mg/kg (Wang *et al*., 2012). Extraction methods are required to remove mercury greater than 260 mg/kg, while stabilization methods are available to treat mercury concentrations less than 260 mg/kg. Biological roles in remediation of mercury-contaminated soil were continuously studied. Remediation

technologies of metals in soil using biological treatment are good for cost effective, toxicity and mobility reduction (Evanko and Dzombak, 1997). The remediation processes occur by adsorption, oxidation and reduction reaction, and methylation (Means and Hinchee, 1994). Those technologies are bioaccumulation, phytoremediation, bioleaching, and biochemical processes. Bioremediation technologies used for mercury contaminated soil are still limited on the use of genetically engineered organism (Smith and Atwater, 1991). It has been demonstrated in bioreduction of mercury but still in bench scale (Smith *et al*., 1995). The use of plants through phytoremediation has wider application than the use of microorganism. The technology are continuously being developed, however, it still needs more effort to become worthwhile in mercury remediation.

Aspergillus are saprophytic fungi that having high capability to grow in highly aerobic environment and can be found in oxygen-rich environment (loose soil). *Aspergillus* are also have economical feature for multiple use in agriculture, industry and environment. *Aspergillus* demonstrates oligotrophic characteristic which is capable to grow in less-nutrient environment. Less-nutrients mean less organic matter referring to non-acidic environment. The environment that commonly found on soil contaminated with hazardous contaminant. This appears that Aspergillus is having potential use for recovery of contaminated soil.

1.2 Significance

The dangerous effect of mercury has been awareness worldwide since Minamata case on 1950s. Many research and studies have also been conducted. Some result regarding to the awareness are the reduce of mercury containing material use for living aspect such like the band of the use of phenyl mercuric acetate (PMA) as a common fungicide in agriculture on 1986. Yet, the mercury problem still exists until now due to its natural occurrence and long preserve in the environment. Thus, it still possibly endangers human life. Therefore, the research about mercury is still continuously done.

1.3 Objectives

This study was conducted to examine fungal strain isolated from forest and plantation soil to be used in bioremediation of soil contaminated with mercury. Such information as expression and characteristics of the fungal strain in the presence of mercury contaminants that will be useful as new knowledge regarding fungal expression

with the presence of harmful mercury on soil were observed. In order to reach the objective, the study was divided into three stages. The objective of each stage is stated as follows:

1. To select and identify the fungal strain for use in bioremediation.
2. To observe the characteristics of the fungal strain for bioremedation.
3. To observe the potential use of the fungal strain in bioremediation: liquid and soil.

1.4 Scope of thesis

This thesis consists of six chapters. **Chapter 1** explains the background, the significance and the objectives of this study. **Chapter 2** presents a literature review on mercury (chemical structure and properties, environmental fate, toxicity, and mercury utilization microbial), fungi (habitat, characteristics, and ecological adaptability of A. flavus, and the existence bioremediation technology of mercury contamination using fungi. **Chapter 3** describes about screening and identification of fungal strain and its capability for living in contaminated media. **Chapter 4** provides the characteristic of fungal strain on mercury contaminated media. **Chapter 5** concerns about the potential use of fungal strain for bioremediation of mercury. **Chapter 6** is the conclusion.

1.5 References

Evanko, C.R. and Dzombak, D.A., 1997. Remediation of metals-contaminated soil and groundwater. http://www.cluin.org/download/toolkit/metals.pdf. Pittsburg, PA.

Karunasagar, D., Arunachalam, J., Rashmi, K., Naveena-Lavanya-Latha, J., Maruthi-Mohan, P., 2003. Biosorption of inorganic and methyl mercury by a biosorbent from *Aspergillus niger*. World J. Microbiol. Biotechnol. 19, 291-295.

Means, J.L. and Hinchee, R.E., 1994, Emerging technology for bioremediation of metals. Lewis Publishers. Boca Raton. FL.

Schuster, E., 1991. The behaviour of mercury in the soil with special emphasis on complexation and adsorption process – a review of the literature. Water Air & Soil Poll. 56, 667-680.

Thippeswamy, B., Shivakumar, C.K., Krishnappa, M., 2012. Bioaccumulation potential of *Aspergillus niger* and *Aspergillus flavus* for removal of heavy metals from paper mill effluent. J. Environ. Biol. 33, 1063-1068.

United Nation Environment Programme (UNEP), 2013. Global Mercury Assessment 2013: Sources, Emissions, Releases and Environmental Transport. Available at:

http://www.unep.org/PDF/PressReleases/REPORT_Layout11.pdf. Date accessed: 12 January 2014.

Wang, J., Feng, X., Anderson, C.W.N., Xing, Y., Shang, L., 2012. Remediation of mercury contaminated sites – A review. J. Hazard. Mater. 221–222 (2012), 1– 18.

CHAPTER 2
LITERATURE REVIEW

2.1 Mercury

Mercury (Hg) in the environment is having high toxicity, high mobility and long persistence in the environment. The Agency for Toxic Substances and Disease Registry ranked mercury as the third priority hazardous substance, after arsenic and lead. Its presence in the atmosphere derives from natural and anthropogenic activities (Selin, 2009) and is able to be retained for 6 to 24 months while transporting over thousands of kilometers before redepositing on the earth's surface (Schroeder and Munthe, 1998; Dastoor and Larocque, 2004). The presence of mercury in the biosphere appears in similar ways, with additional amounts from the redeposition process.

2.1.1 Chemical structure and properties

Mercury (Hg) is present in form of liquid metal at ambient temperature and pressure. Physic-chemical properties of mercury in the environment are as presented in **Table 2.1**.

Table 2.1 Physic-chemical properties of mercury in the environment (Schroeder and Munthe, 1998; ATSDR, 1999).

Properties	Elemental	Inorganic				Organic
	Hg^0	$HgCl_2$	HgO	HgS	Hg_2Cl_2	CH_3HgCl
Chemical name	Mercury	Mercuric(II) chloride	Mercuric(II) oxide	Mercuric(II) sulfide	Mercurous(I) chloride	Methylmercuric chloride
Chemical structure	Hg	Hg^{++} Cl^- Cl^-	Hg = O	Hg = S	Cl–Hg–Hg–Cl	– Hg – Cl
Molecular weight	200.59	271.52	216.59	232.68	472.09	251.1
Melting point (°C)	-38.8	277	500 (decompose-tion)	584 (sublimation)	400-500 (sublimation)	170
Water-solubility (g/l)	49.6×10^{-6} (20°C)	66 (20°C)	0.053 (25°C)	2×10^{-24} (25°C)	2×10^{-5} (25°C)	<0.1 (21°C)
Boiling point (°C)	356.7	303	-	-	384	-
Vapor tension (Pa)	0.18	0.009	9.2×10^{-12}	nd	-	1.133

In form of salts, mercury presents in two ionic states such as mercurous salts or mercury (I) and mercuric salts or mercury (II). Mercury (II) is much more common in the

environment. Once soluble in water, they are bioavailable and toxic (Boening, 2000). According to ATSDR (1999), inorganic mercury compounds the so called mercury salts are formed when mercury combines with elements such as sulfur, chlorine, or oxygen. They appears in form of white powder or crystals except mercuric sulfide (cinnabar) which is red and will turns black with exposure of light. Cinnabar (HgS) is the most common form of mercury in environment which is non-toxic (**Fig. 2.1**).

Fig. 2.1 Cinnabar: the principal ore of mercury (UNEP, 2013)

Other available forms of mercury are organometallic compounds or organomercurials used for industry and agriculture (Boening, 2000). Those formed when mercury combine with carbon. There are large numbers of organic mercury compounds; however the most common in the environment is methylmercury or monomethylmercury and the past phenylmercury used for some commercial product that was not allowed anymore caused by its harmful to people and animal. Those compounds exist as salts i.e. methylmercuric chloride or phenylmercuric acetate that is white crystalline solids when pure.

Elemental or metallic mercury (Hg^0) is liquid at room temperature. It is slightly soluble in water and volatile influenced by temperature. The colorless and odorless vapor will increase with the increase of temperature. In this form, mercury is easily transport throughout atmosphere (Boening, 2000).

Naturally, several form of mercury may occur in the environment. The commonly founded natural forms of mercury are metallic mercury, mercuric sulfide (cinnabar ore), mercuric chloride, and methylmercury. The form can be changed from one form to another by microorganisms (bacteria and fungi) and natural processes. The most common generated organic mercury compound by natural process and microorganism is methylmercury. In this form, mercury will bring to food chain of freshwater and

saltwater fish marine mammals and bring to the dangerous mercury biomagnification (the Minamata case).

2.1.2 Environmental fate

Mercury is mined in form of cinnabar ore containing mercuric sulfide that is refined to have liquid metallic form. Liquid metallic mercury uses for many purposes such as chlorine and caustic soda production, gold extraction, thermometer, barometers, batteries, electrical switches, and dental amalgams. The major pathways for transformation of mercury and various mercury compounds in air, water, and soil can be seen at **Fig.2.2**

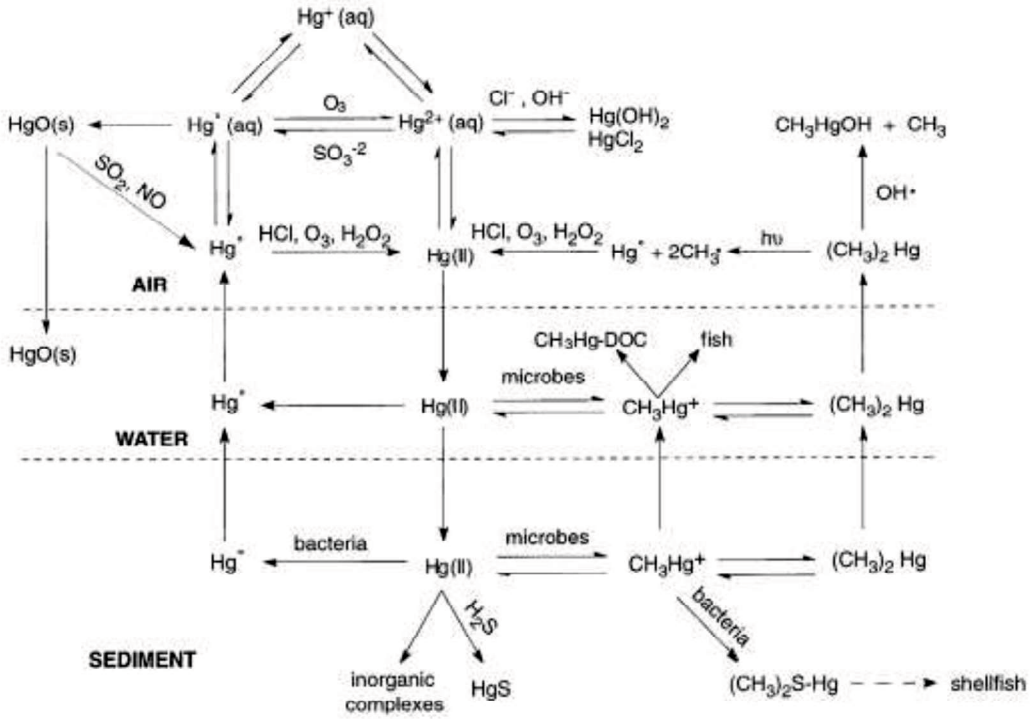

Fig. 2.2 Transformation of mercury in air, water and sediment (Stein et al., 1996)
(Note: Dashed lines represent the boundary between environment compartments; aq=aqueous; DOC=dissolved organic carbon; s=solid)

There are two cycles describe the environmental transport and distribution of mercury that is the global scope (involves atmospheric circulation) and the local scope (methylation of inorganic mercury mainly from anthropogenic sources). The UNEP (2013) reported that anthropogenic activities, especially mining and the burning of coal have increased the mobilization of mercury into the environment, raising the amounts in the atmosphere, soil, fresh water, and oceans. The recently estimated global mercury

emission ranges from 5500 to 8900 tons of mercury, which is contributed from natural (10%), anthropogenic (30%), and re-emission and re-mobilization sources (60%). The anthropogenic activities emit 1960 tons of mercury to the atmosphere, mostly contributed from coal burning for energy (85%), mining, smelting, and production (10%), cement production (9%), artisanal and small-scale gold mining (37%). The minor contributors such as oil and natural gas burning, ferrous metal primary production, large-scale gold production, mercury mining, oil refining, contaminated sites, chlor-alkali industry, consumer product waste and cremation (dental amalgam) are also important. The other human activities also responsible for the Hg concentration in the environment include mining and smelting activities (Fernandez-Martinez et al., 2005), industrial production processes, waste incineration, application of fungicides and land spreading of sewage sludge and water (Steinnes, 1995).

The global mercury cycle and budgets in the environment can be seen on **Fig. 2.3**. Mercury cycles occur between air, land, water and atmosphere. In removed from the system if it buried in deep ocean or lake sediments and entrapped in stable mineral compound.

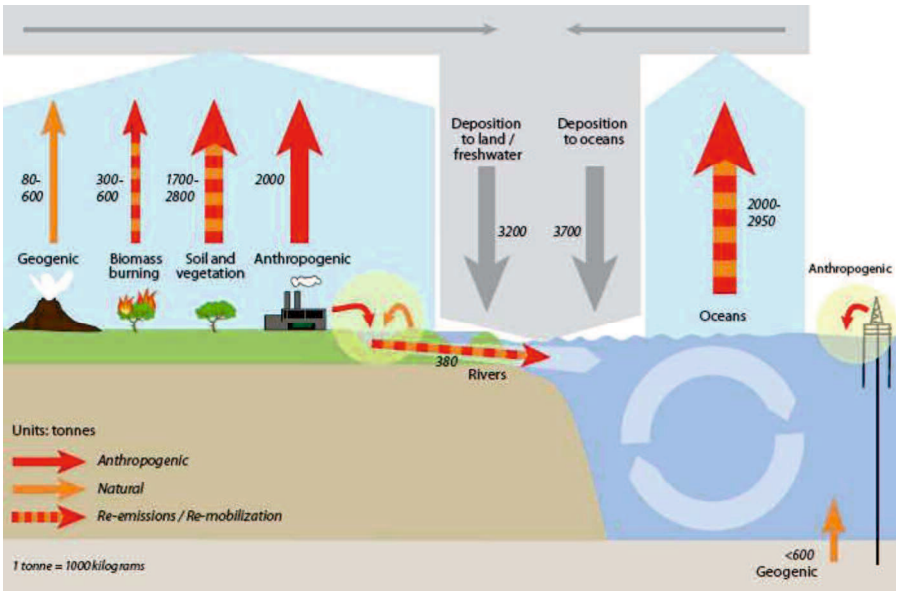

Fig. 2.3 Global mercury cycle and budgets in the environment (UNEP 2013)

Mercury contamination in agricultural soil is generally due to application of municipal wastewater and industrial effluent for crop irrigation, which often occurs in developing countries such as India (Thippeswamy et al., 2012). The other sources

mercury in agriculture fields also come from fertilizers, fungicides and pesticides, although the use of mercury in these products has been greatly reduced (UNEP, 2013). Mercury in soil is firmly bound to organic matter or precipitated as sulfide, and is found in trace concentrations in soil solutions (Schuster, 1991). At this point in the mercury cycle, the transport ends and the metal form persistent deposits in soil as long as no trigger for re-emission and re-mobilization occurs.

Re-emission of mercury contributes about 60% of mercury emission sources to air. The process is a result of natural process that converts inorganic and organic form of mercury to elemental mercury that was usually volatile and readily returns to the air. The deposited mercury on plant surfaces are possible to re-emitted during forest fire or biomass burning. This cycle can occur many times in the environment. Re-mobilization of mercury usually occurs in the aquatic environment when the deposited and accumulated mercury in soil or sediment are mobilized by rain or flood. Re-suspension of aquatic sediment by wave or storm is also one way of remobilization. However, it is very difficult to estimate the re-emission and re-mobilization rates (UNEP, 2013).

Aquatic environments are important in the pathways and fate of mercury, because it is in waters, sediments, and wetland soils that inorganic mercury is converted into methylmercury, which is toxic and concentrated in animals (UNEP, 2013). Inorganic mercury in dissolved or particulate form is the dominant type in most marine and fresh water. Total mercury in water may contain dissolved gaseous elemental mercury (less than 30%) and methyl mercury at trace levels, which may reach 30% of total mercury in some settings. The transformation of inorganic mercury to methyl mercury itself primarily occurs in sediment. Since the re-emission and re-mobilization of mercury is the greatest contributor to the mercury cycle in the environment, its management is urgently needed.

2.1.3 Toxicity

Mercury occurs naturally in the environment, so that everyone is exposed to very low levels of mercury in air, water, and food. Mercury ranged between 10 and 20 nanograms of mercury per cubic meter (ng/m^3) of air has been measured in urban outdoor air. These levels are hundreds of times lower than levels still considered to be "safe" to breathe. Background levels in nonurban settings are even lower, generally about 6 ng/m^3 or less. Mercury levels in surface water are generally less than 5 parts of

mercury per trillion parts of water (5 ppt, or 5 ng per liter of water), about a thousand times lower than “safe” drinking water standards. Normal soil levels range from 20 to 625 parts of mercury per billion parts of soil (20–625 ppb; or 20,000–625,000 ng per kilogram of soil). A part per billion is one thousand times bigger than a part per trillion (ATSDR, 1999).

Additional ways with more risk of mercury exposure are come from such sources as the slow release of mercury element from dental amalgam; the metallic mercury used in variety of household and industrial items (for example spills from broken thermometers); the metallic mercury vapors from breathing contaminated air around hazardous waste sites; the vapors from the use of fungicides or direct dermal contact with product that contain mercury (ex. Skin-lightening creams, topical antiseptic or disinfectant agents); and the most concerned mercury in form of methylmercury from food (seafood).

It is important to know which form of mercury where human has been exposed. When metallic mercury enter the human body, it can retain for weeks or months in parts of human body, trap if it enter the brain, enter to infant of pregnant mother, but mostly accumulated in kidney and leaves the body through urine and feces. Inorganic mercury is having the same path as metallic mercury. Methylmercury is the most easily absorbed form of mercury by the body. Methylmercury can be changed by the body to become inorganic mercury and possible to leave the body slowly over period of several months as inorganic mercury in the feces.

Toxicity of mercury to human body occurs if it attacks the nervous system. This drive to permanent damage of brain which is means that affect the brain and their associated function such as personality changes (irritability, shyness, nervousness), tremors, changes in vision (constriction (or narrowing) of visual field, deafness, muscle incoordination, loss sensation and difficulties with memory (ATSDR, 1999).

Soil and all its content play an important role in the earth life cycle as agent sustainability through decomposition and nutrient mobilization of many substrates to become useful for creatures and the environment. Therefore, it function should be maintained. Soil microbes are main agent of soil function that should be retained so that the soil could function as it is. In the mercury cycle, soil deposited mercury so that

prevent re-emission of mercury to harm level. Degradation of mercury to harmless form is done by soil microbes while depositing in soil.

Long term mercury pollution from anthropological activities such as field application of mercury containing sewage sludge, various industrial activities, and disposal of waste product may affect all groups of organism and ecosystem processes (Babich and Stotzky, 1985; Baath, 1989; Giller et.al, 1998). The size of bacterial and protozoan population of contaminated sites was reduced; whereas there was no significant reduce in fungal biomass (Muller, 2001) and only remaining the tolerant microbial species (Crane, 2011). Gudbrandsen et al. (2007) reported that the 28-day 50% lethal concentrations (LC_{50}) for earthworms (*Eisenia fetida*) exposed to mercury(II) was 170 mg/kg. Lock and Janssen (2001) found that the 42-day LC50 for white worm (*Enchytraeus albidus*) exposed to mercury(II) was 22 mg/kg in a soil mixed with 70% sand, 20% kaolinite clay and 10% finely ground sphagnum peat.

2.1.4 Mercury utilization microbial

Remediation technologies for mercury contaminated sites including physical, chemical, or biological technology. The remediation technology is depending on the mercury species in the soil. The term use of total mercury for understanding the biogeochemical cycle of the metal is insufficient mainly for establishing appropriate remediation method.

Microorganisms are capable for chemical reduction and removal of mercury salts from wastewater (Horn et al., 1992; Hansen and Stevens, 1992). Microorganism activities contribute to the biological cycle of mercury in the environment. Some bacteria are capable to transform mercury into harmless form shown a positive correlation between the presences of resistant microorganism with the distribution of mercury compounds in contaminated sediments. The detoxification mechanism of mercury by microorganism may be represented with methylation process which is conducted by bacteria (Robinson and Touvinen, 1984).

The research regarding the use of fungi for bioremediation of heavy metal contaminated sites is stimulated by the study of metal toxicity to fungi in term of fungicide research. Subsequently, observation on the ability of fungi to resist and adapt to toxic metals leads to further study of its physiological, biochemical and genetical explanations (Gadd, 1986). It was then, the information from the result seemed to be

useful in term with accelerating pollution in the natural environment by metalloids, toxic metals, radionuclides, and organometal(loid)s. The interest is increased when it is known that fungi are ubiquitous and sometimes dominant in metal polluted habitat, capable to uptake and translocate the toxic metals and radionuclides on fruit bodies of edible fungi and mycorrhizal fungi (Gadd, 1986; Brown and Hall, 1990).

Fungal tolerance to heavy metals demonstrates its promising to control and reduce heavy metal contamination. Even though the mechanism of the tolerance is not fully understood, the approach method using a particular strategy of suppression subtractive hybridization technique on of *Trichoderma harzianum* toward mercury shown that a possible of hydrophobin that is an ability to dissolve hydrophobic molecules into aqueous media. The tolerance was expressed with the similar growth rate with the growth rate on control culture (Puglisi et al., 2012).

Toxicity of mercury to plants may follow such processes as: (1) affected the oxidative system (Israr et al., 2006), (2) affected the photosynthesis system (Patra et al., 2004), (3) inhibited the plant growth and yield production through nutrient uptake and homeostasis (Patra and Sharma, 2000), and (4) induced the genotoxicity (Sharma et al., 1990). Mercury can bind with DNA thus causes damage on chromosomes (Chenki, 2009). The presence of mercury affect all groups of organism and ecosystem processes including microbial and macro/mezzo mediated process in soil. Mercury affects the genetic structure and functional diversity of bacterial that are sensitive to mercury; however it increases the mercury resistance organism such as mercury resistance bacteria (HgR) (Ranjard et al., 1997).

2.2 Fungi

According to Encyclopedia of Soils in the Environment (2004), Fungi are ubiquitous and dominate in soil with high organic matter. Fungi have a great ecological and economic significant to the environment. Fungi are belonging to eukaryotic and cover four phyla such as the Chytridiomycota, the Zygomycota, the Ascomycota and the Basidiomycota plus an informal group mitosporic fungus (formerly the fungi imperfecti or Deuteromycota).

2.2.1 Habitat

Fungi are distributed worldwide and have capability to grow in wide range of habitat as well as extreme environment such as desert or deep sea sediments. Most fungi grow in terrestrial though several species live in part or solely in aquatic environment. The aquatic fungi include those living in hydrothermal areas of the ocean. Organism always mentioned in term of wide microbial community in the soil environment.

In the natural habitat, fungi are capable to associate with other microbes such as algae or cyanobacteria in form of lichens. This association plays a role in soil genesis via rock weathering. Fungal association with plants coming in form of mutualism (mycorrhizas) and parasitism (plant pathogenic fungi). In association with soil fauna comes in the same form with its associated with plants.

2.2.2 Characteristics

The fundamental characteristic is the growth form which consists of a thread like hypha which grows by apical extension and periodic branching to form mycelium that permeates the environment in which fungus is growing. The diameter of hyphae varies according to age, species, and nutritional condition but typically is 3-10 μm. All fungi are obligate heterotrophs, i.e. they utilize fixed (organic) C sources as substrate. Respiration can be aerobic or anaerobic, and obligate or facultative type. The nutrition might come from other living organism through parasitic or mutualistic associations whilst others are saprotrophs. Reproduction can be sexual or asexual. Fungal propagules generally take the form of asexual or sexual spore, sclerotia (dense, heavily pigmented hyphal aggregation) or hyphal fragments.

2.2.3 Ecological adaptability and importance

Fungi could stay in inactive resting stages called rhizomorphs, conidia and spores. This form enables fungi to survive in unfavorable condition such as winter, dry season, or low density of host populations. The double wall on spores is thought to enhance their resistance to environmental extremes. When the condition is favorable enough (temperature, water and nutrient availability), the restoring spore germinated.

Fungi contribute to nutrient cycling mainly in terrestrial systems using their primary role as decomposer. Fungi involve in cycling of C, N, and P and roles the most on soil elemental cycles. The main role of fungi is decompose cellulose involve the polyphenol oxidases (mainly laccases). Fungi enzymatically mediate degradation of polymer and some possible to produce a variety of compounds such as organic acids

(siderophores) which solubilize or immobilize essential or toxic metals. The immobilization occurs through sequestration in mycelia which are further released following death and lysis or attack by patogens. Eucarpic (filamentous) fungi play a significant role in physically transporting nutrients through the soil fabric through translocation within mycelia. The influence on soil structure dynamics through mechanisms such as: (1) enmeshing soil particle together using its hypha, (2) decomposing organic matter and bind the soil particle together using its degradative enzyme.

Fungi produce large quantities of a verse array of hydrophobic proteins (**hydrophobin**), which serve to insulate hyphal walls. However, these compounds also coat soil particles and can strongly influence he water sorptivity and repellency characteristic of the soil. As hyphae extend through soil, they may also cause physical restructuring of soil.

2.2.4 *Aspergillus flavus*

Aspergillus is recognized as active agent in decay processes as causes of human and animal diseases and as fermenting agents capable of producing valuable metabolic products (Raper and Fennel, 1965). Like other *Aspergillus* species, *A. flavus* has a worldwide distribution. It grows better with water activity between 0.86-0.96 (Vujanovic et.al., 2001). The optimum temperature for grow is 37°C, however the fungal growth can be observed at range temperature from 12 to 48°C. High optimum temperature contribute to its pathogenicity in human (Hedayati et al., 2007). *A. flavus* spent most of its life growing as a saprophyte in the soil. It plays an important role as nutrient recycler, supported by plant and animal debris (Scheidegger and Payne, 2003). This fungus has ability to survive in extreme condition so that shown it competitiveness with other organism for substrate in the soil or plant (Bhatnagar et al., 2000). The fungus also capable to overwinter in form of mycelium or sclerotia (resistant structures) that is then either germinates to produce additional hyphae or produce conidia (asexual spores) which further dispersed in the soil and air.

A. flavus is genetically almost identical to *A. oryzae*. Comparative genomics will be particularly interesting as *A. flavus* is a common environmental organism whilst the sequence strain of *A. oryzae* is a ‘domesticated’ fungus, having been used in soy fermentation for thousands of years, and rarely causes disease (Hedayati et al., 2007).

The systematics of Aspergillus has been based primarily on differences in morphological and cultural characteristics (Raper and Fennel, 1965; Samson et al., 2000). *A. flavus* grouped in nine species and two varieties including *A. flavus*, *A. flavus* var. *columnaris*, *A. parasiticus*, *A. oryzae*, *A. oryzae* var. *effusus*, *A. zonatus*, *A. clavato-flavus*, *A. tamarii*, *A. flavo-furcatis*, *A. subolivaceus* and *A. avenaceus*. Accurate species identification within Aspergillus flavus complex remains difficult due to overlapping morphological and biochemical characteristics. In general, *A. flavus* is known as a velvety, yellow to green or brown mould with a goldish to red-brown reverse (**Fig. 2.4**). The conidiophores are variable in length, rough, pitted and spiny. They may be either uniseriate or biseriate. They cover the entire vesicle, and phialides point out in all direction (**Fig. 2.5**). Conidia are globose to subglobose, conspicuously echinulate, varying from 3.5 to 4.5 mm in diameter.

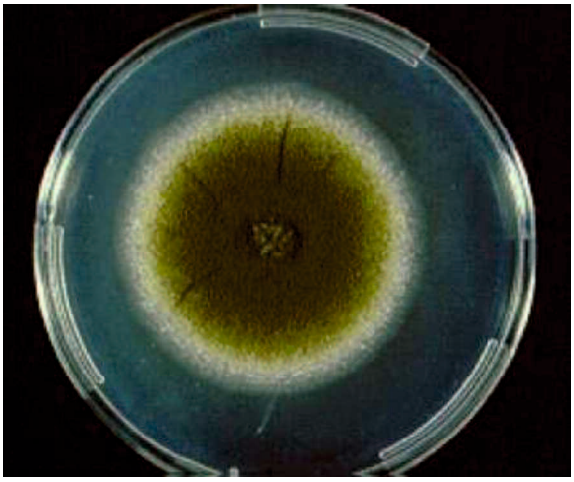

Fig. 2.4. Macroscopic features of *A. flavus* on Czapek's agar (Hedayati et al., 2007)

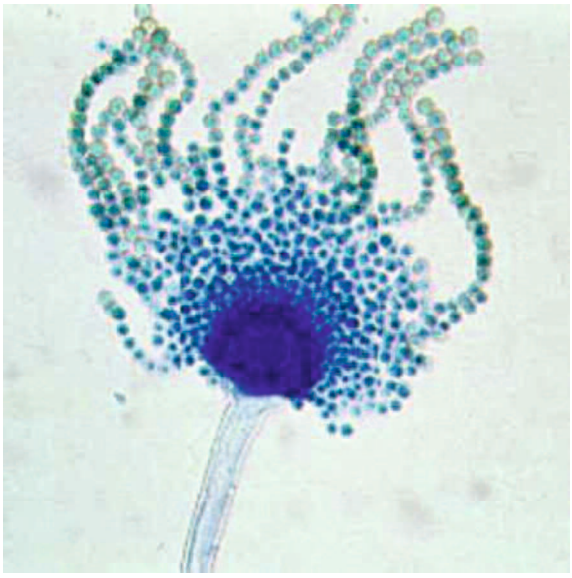

Fig. 2.5. Microscopic features of *A. flavus* (Hedayati et al., 2007)

2.3 Bioremediation of mercury contamination using fungi

Fungi are known to tolerate and detoxify metals by several mechanisms including valence transformation, intra and extracellular precipitation and active uptake (Gadd, 1993). Their high surface to volume ratio and ability to detoxify metals are among the reasons they have been considered as potential alternatives to synthetic resins for bioremediation of dilute solutions of metals and solid wastes (Joo and Hussein, 2012; Li et al., 2009). Fungal metal transformations were divided into mobile and immobile phase. Fungal mobilization of metal occur through heterotrophic (chemoorganotrophic) leaching such as what *Aspergillus niger* done by solubilize stable lead material, pyromorphite ($Pb_5(PO_4)_3Cl$) and methylation of metalloids to yield volatile derivatives (selenium) that could provide one means of removal. Metal immobilization process includes biosorption or metal binding in cell (Gadd**,** 2001). Others utilization of *A. niger* was by using its pretreated biomass in removal of inorganic (Hg^{2+}) and methyl mercury (CH_3Hg^{+}) from aqueous solution which was resulted the potential use for removal of inorganic mercury and methyl mercury ions from polluted aqueous effluent (Kapoor et al.**,** 1999)) as well as *A. versicolor* (Spry and Wiener, 1991). The other *Aspergillus* species such as *A. fumigatus* and *A. flavus* was also proven to have high tolerance to heavy metal such as Zn contaminant on textile wastewater (Moneke et al., 2010) as well as Pb, Zn, Cu, and Ni from paper mill effluent (Tamer and Tunali, 2006). Metal was proven to be accumulated in the fungal biomass (Zafar et al., 2007)).

Total mercury gives lack of information concerning its reactivity, bioavailability and toxicity (Issaro, 2009). Therefore the mercury speciation process is important. For example, soil with low bioavailable mercury concentration should be first increased up its metal bioavailability before remediated using bioremediation method and if it has low efficiency, then others remediation technics should be conducted. Some remediation technologies for mercury-contaminated soil have been developed. In general, the critical point of mercury concentration in soil for the application of remediation technologies is at 260 mg/kg (Wang et al., 2012). Extraction methods are required to remove mercury greater than 260 mg/kg, while stabilization methods are available to treat mercury concentrations less than 260 mg/kg. Biological roles in remediation of mercury-contaminated soil that involve plants (phyto) as bioremediation agents, namely phytoremediation, are continuously being developed. Phytotechnologies such as phytostabilization, phytoextraction, and phytovolatilization have been explored but this area needs more effort to become worthwhile in mercury remediation.

2.4 Reference

Agency for Toxic Substances and Disease Registry (ATSDR), 1999. Toxicological profile for mercury. U.S. Department of Health and Human Services. USA

Boening D.W., 2000. Ecological effects, transport, and fate of mercury: a general review. Chemosphere 40: 1335-1351.

Baath, E. (1989) Effects of heavy metals in soil on microbial processes and population. Water Air Soil Pollut. 47, 335-379.

Babich, H. and Stotzky, G. (1985) Heavy metals toxicity to microbe mediated ecological processes: A review and potential application to regulatory policies. Environ. Res. 36, 111-137.

Bhatnagar, D., Cleveland, T.E., Payne, G.A., 2000. Encyclopedia of food microbiology. Academic Press. London, pp. 72-79.

Brown M.T. and Hall I.R., 1990. Metal tolerance in fungi. In Shaw J, ed. *Heavy metal tolerance in plants: evoltitionary aspects.* Boca Raton: CRC Press, 95-104.

Cenkci S., Ildiz M.Y., Cigerci I.H.I., Konuk M., Bozdag A., 2009. Toxic chemicals induced genotoxicity detected by random amplified polymorphic DNA (RAPD) in bean (*Phaseolus vulgaris* L.) seedlings. Chemosphere 76: 900–906.

Crane, S., Husar M, Dighton J, Barkay T., 2011. The response of saprotrophic fungi from forest soil to mercury tolerance and community diversity. Forest and mercury: Defining the connection. Halifax 2011 Prosiding. Canada.

Dastoor, A.P., Larocque, Y., 2004. Global circulation of atmospheric mercury: a modelling study. Atmosphere Environ. 38, 147-161.

Fernandez-Martinez, R., Loredo, J., Ordonez, A., Rucandio, M.I., 2005. Distribution and mobility of mercury in soils from an old mining area in Mieres, Asturias (Spain). Sci. The Total Environ. 346(1-3), 200-212.

Gadd G.M., 1986. The response of fungi toward heavy metals. In: Herbert RA, Codd GA, eds. Microbes in extreme environments. London: Academic Press, 83-110.

Gadd, G.M., 1993. Interaction of fungi with toxic metals. New Phytology. 124, 25-60.

Gadd, G.M., 2001. Microbial metal transformations. The J. Microbiol. 39(2), 83-88.

Giller, K.E., Witter, E. and McGrath, S.P. (1998) Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: A review. Soil Biol. Biochem. 30, 1389-1414.

Gudbrandsen M., Sverdrup L.E., Aamodt S., Stenersen J., Short-term preexposure increases earthworm tolerance to mercury, Eur. J. Soil Biol. 43 (2007) 261–267.

Hansen, C.L. and Stevens, D.K. (1992), "Biological and Physio-Chemical Remediation of Mercury-Contaminated Hazardous Waste," Arsenic and Mercury: Workshop on Removal, Recovery, Treatment and Disposal, pp. 121-125.

Horn, J.M., Brunke, M., Deckwer, W.D., and Timmis, K. N. (1992), "Development of Bacterial Strains for the Remediation of Mercurial Wastes," Arsenic and Mercury: Workshop on Removal, Recovery, Treatment and Disposal, EPA/600/R-92/105, pp. 106-109.

Hedayati, M.T., Pasqualotto, A.C., Warn, P.A., Bowyer, P., Denning, D.W., 2007. *Aspergillus flavus*: Human pathogen, allergen and mycotoxin producer. Microbiology. 153, 1677-1692.

Hillel D., Hatfield JL, Powlson DS, Rosenzweig C, Scow RM, Singer MJ, Sparks DL., 2004. Encyclopedia of soils in the environment. 1st Ed. Academic Press. NY. USA

Israr M., Sahi S.V., 2006. Antioxidative responses to mercury in the cell cultures of *Sesbania drummondii*, Plant Physiol. Biochem. 44:590–595.

Joo, J.H., Hussein, K.A., 2012. Heavy metals tolerance of fungi isolated from contaminated soil. Korean J. of Soil Sci. Fert., 45(4), 565-571.

Kapoor A, Viraragavan T. Fungal biosorption-An alternative treatment option for heavy metal bearing wastewaters: A Review. *Biores Technol* 1999:**70**:95-104.

Li, P., Feng, X.B., Qiu, G.L., Shang, L.H., Li, Z.G., 2009. Mercury pollution in Asia: A review of the contaminated sites. J. Hazard. Mater., 168, 591-601.

Müller, A.K., Westergaard, K., Christensen, S., Sørensen, S.J., 2001. The effect of long-term mercury pollution on the soil microbial community. FEMS Microbiology Ecology 36 11-19.

Patra M., Bhowmik N., Bandopadhyay B., Sharma A., 2004. Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance, Environ. Exp. Bot. 52: 199‑223.

Patra M. and Sharma A., 2000. Mercury toxicity in plants, Bot. Rev. 66: 379–422.

Puglisi I., Faedda R., Sanzaro V., Lo Piero AR., Petrone G., Cacciola SO., 2012. Gene 506(2): 325-330.

Ranjard L., Richaume A., Jocteur-Monrozier L., Nazaret S., 1997. Response of soil bacteria to Hg(II) in relation to soil characteristics and cell location, FEMS Microbiol. Ecol. 24: 321–331.

Robinson J.B. and Touvinen O.H., 1984. Mechanism of microbial resistance and detoxification of mercury and organomercury compounds: Physiological, biochemical and genetic analysis. Microbiological Review. June 1984, p. 95-124.

Scheidegger, K.A., Payne, G.A., 2003. Unlocking the secret behind secondary metabolism: a review of *Aspergilus flavus* from pathogenicity to functional genomic. J. Toxicol. 22, 423-459.

Schroeder, W.H., Munthe, J., 1998. Atmospheric mercury – an overview. Atmospheric Environ. 32, 809-822.

Schuster, E., 1991. The behaviour of mercury in the soil with special emphasis on complexation and adsorption process – a review of the literature. Water Air & Soil Poll. 56, 667-680.

Selin, N.E., 2009. Global biogeochemical cycling of mercury: a review. Annual Rev. Environ. Res. 34, 43-63.

Sharma A., Talukder G., Sharma A., 1990. Advances in Cell and Chromosome Research, Aspect Publications Ltd., West Yorkshire, pp. 197–213.

Stein ED, Cohen Y, Winer AM. 1996. Environmental distribution and transformation of mercury compounds. Critical Review in Environ Sci Technol 26(1):1-43.

Steinnes, E., 1995. Heavy metals in soils: Mercury. In Alloway, B.J. (Ed.). Chapman & Hall. London. pp. 245-259.

Thippeswamy, B., Shivakumar, C.K., Krishnappa, M., 2012. Bioaccumulation potential of *Aspergillus niger* and *Aspergillus flavus* for removal of heavy metals from paper mill effluent. J. Environ. Biol. 33, 1063-1068.

United Nation Environment Programme (UNEP), 2013. Global Mercury Assessment 2013: Sources, Emissions, Releases and Environmental Transport. Available at: http://www.unep.org/PDF/PressReleases/REPORT_Layout11.pdf. Date accessed: 12 January 2014.

Wang, J., Feng, X., Anderson, C.W.N., Xing, Y., Shang, L., 2012. Remediation of mercury contaminated sites – A review. J. Hazard. Mater. 221–222 (2012), 1– 18

Zafar, S., Aqil, F., Ahmad, I., 2007. Metal tolerance and bio sorption potential of filamentous fungi isolated from metal contaminated agricultural soil. Biores. Technol. 98, 2557-2561.

CHAPTER 3
SELECTION AND IDENTIFICATION OF FUNGAL STRAIN AND ITS CAPABILITY FOR LIVING IN MERCURY CONTAMINATED MEDIA

3.1 Introduction

The response of soil microorganism to the excessive heavy metal level was coming in vary such as population loss (Knight et al., 1997), changes in population structure (Frostegard et al., 1993; Pennanen et al., 1996) and physiological activity (Valsecchi et al., 1995). Those affected the soil productivity and sustainability because soil microorganism plays a vital role in maintaining soil productivity. Moreover, the problem could become very serious as heavy metals persist in the soil and the negative effects are long lasting. Mercury (Hg) is ranked as the third amongst priority hazardous heavy metals after arsenic (As) and lead (Pb) that becoming a global concern due to its high toxicity, high mobility and long persistence in the environment.

Soil microbial community contains saprotrophic fungi that are important members to contribute to soil processes such as bulk decomposition and nutrient mobilization. This make them having potential role in ecosystem response to mercury and its mobilization in terrestrial ecosystem. Fungi are known to tolerate and detoxify metals by several mechanisms including valence transformation, intra and extracellular precipitation and active uptake (Gadd, 1993). Fungal metal transformations can be divided into mobile and immobile phase types. Fungal mobilizations of metals occur through heterotrophic (chemoorganotrophic) leaching. Metal immobilization processes include biosorption or metal binding in cells (Gadd, 2001). Long term exposure of mercury affect the microbial community shown by reducing of the size of bacterial and protozoan population whereas no significant difference on fungal biomass (Muller, 2001). Due to the importance of these fungi in the ecosystem and their tolerance to the mercury contamination on soil so is needed to be more understood their response to mercury for further potential develop use such as in bioremediation of contaminated sites of substrate. Therefore, the objective of this chapter was to select, identify and observe the fungal capability to grow in contaminated media.

3.2 Material and method

3.2.1 Chemical and media

Mercury stock solution was made as a concentrated solution of Hg^{2+} (referred to as mercury(II)) by dissolving 0.677 g $HgCl_2$ powder in 100 mL sterile distilled water by aseptic technique. The media potato dextrose agar (PDA) was made from 24 g potato dextrose broth (PDB) (Difco; Becton Dickenson and Company, USA) powder and 20 g standard agar per liter adjusted to pH 5.7 and autoclaved at 121°C for 15 minutes. When the temperature of the media reached about 45°C, it was mixed thoroughly with the mercury(II) solution (*V/V*) to give the desired concentration before being poured into petri plates using aseptic techniques.

3.2.2 Selection of fungal strain

The fungal strains namely FRP1, FRP2, GRP1, KRP1, KRP2, and KRP3 were obtained from the fungal strains collection of the Hygiene and Sanitation Laboratory, Yamaguchi University, Japan. These collections were the result of screening and isolation of tropical forest soil and plantation soil at Malang, East Java, Indonesia, carried out by Arfarita *et al*. (2011). The F codes represent that the fungal isolates were come from forest soil. The K codes represent that the isolates were come from soil sample of agriculture plantation at Karangploso area. While, the G code represents that the fungal isolate was come from agriculture plantation soil that intensively applied herbicide. There was no particular reason for choosing the fungal strains.

The fungal strains were refreshed two times on non-contaminated PDA plates by placing a small piece containing fungal mycelia in the center of the plates and incubating at 30°C for 5 days. The second refresh process used a 7 mm plug of mycelia obtained from the edge of the fungal colony and incubated at for 30°C for 5 days. The 5 day old fungal colony was then used for the experiments.

The fungal strain selection experiment was done by placing a 7 mm plug of 5-day-old mycelia of each strain on the center of both mercury and non-mercury amended PDA plates in triplicate and incubated at 30°C for 7 days (Shim et al., 2005). Concentration of the media was carried out to obtain 25 mg/L Hg^{2+}. The growth diameter of the fungal strains was measured every 24 h for 7 days. The fungal strain

with the highest ratio of growth diameter at the end of observation (day 7) was selected for further study. The ratio of growth diameter (*R*) was calculated using the formula:

$R = M / S$

Where, *M* (mm) is growth diameter on treatment (Hg^{2+} contaminated media), and *S* (mm) is growth diameter on control (non-contaminated media).

3.2.3 Identification of mercury resistant fungal strain

The selected fungal strain was then identified by the morphology of the colony and cell-based taxonomic investigation as suggested by Domsch et al. (1993). Fungal identification was performed by 18S rRNA gene amplification (White et al., 1990). DNA isolation was carried out using a modified CTAB procedure as described by Cai et al. (2005). A partial sequence of the 18S rRNA gene (± 320 bp) was amplified using primer pair NS1 (5'-GTAGTCATATGCTTGTCTC-3') and GCfung (5'-CGCCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCATTCCCCGTTACCCTTG-3')).

The optimized PCR thermal cycles for the primer pair NS1 and GC-Fung followed the program as described by May et al. (2001). Initial denaturation was done at 95 °C for 4 min and 35 cycles of 95°C for 1 min, continued with annealing at 50 °C for 1 min and 10 sec, extension at 72°C for 2 min, and a final extension at 72°C for 8 min. Each PCR reaction contained 5 µL of 10× PCR buffer, 2 µL of $MgCl_2$ (25 mmol/L), 4 µL of dNTP (2.5 µmol/L) mixture, 1 µL of BSA (1 µg/µL), 0.3 µmol/L of each primer, 0.8 units *Taq* Polymerase, and 10 ng template DNA. The result of the 18S rRNA gene sequence was compared with those available in the GenBank public databases (http://www.ncbi.nlm.nih.gov/GenBank/)

3.2.4 Toleration level of fungal strain for mercury contamination

The tolerance level of the fungal strain was observed by applying the fungal strain to various concentrations of mercury to find the concentration that inhibits the growth of the fungal strain. A 7 mm mycelia plug from a 5-day-old fungal colony was placed in PDA plate media (pH 5.7) containing mercury(II) at various concentrations i.e. 5, 15, 25, 50, 75, 100 and 200 mg/L. The media was previously autoclaved in 121°C for 15 min, and cooled to 45°C before addition of the desired concentration of mercury(II) via aseptic techniques. The inoculated plates were incubated at 30°C for 5 days. Growth

diameter was observed daily to determine the growth profile of the fungal strain and ratio of growth diameter value.

3.3 Result and discussion

3.3.1 Selection of fungal strain on mercury contaminated media

The isolate of six fungal strains namely FRP1, FRP2, GRP1, KRP1, KRP2, and KRP3 were grown on PDA media containing 25 mg/L mercury(II). Media with no mercury contamination were provided as control. The ratio of growth diameter and the growth diameter of the six fungal strains on PDA media containing mercury can be seen in **Fig. 3.1 and 3.2**.

Fungal strains tolerance to the presence of mercury in the media indicates that fungal strain KRP1 had the highest on both the ratio of growth diameter and the growth diameter, thus marked it as the most tolerant strain and chosen for further study. High ratio of growth diameter showed the high tolerance of the fungal strains and gave a low effect of contamination on fungal growth. Different tolerance processes might take place for each isolate (Iram *et al*., 2009). Isolates of the same genus could even show a difference in the level of resistance to metals (Ezzouhri *et al*., 2009). However, the growth diameter could express the fungal condition whenever the contaminant is harmful or not. **Fig. 3.1** showed the ratio of fungal growth compare to the growth on non-contaminated media (control) which is describe that fungal strain KRP1 has the highest ratio. The other fungal strain KRP2 was only slightly less strength compare to strain KRP1. However, the **Fig. 3.2** showed that fungal strain KRP1 has the highest growth diameter which is means that this strain has more capability to eliminate the contaminant effect on their growth. Fungi showing high tolerance to toxic metals may be useful in metal recovery systems (Zafar *et al*., 2007). Heavy-metal-resistant microorganisms play an important role in the bioremediation of heavy-metal-contaminated soils (Ray and Ray, 2009; Abou-Shanab *et al*., 2007).

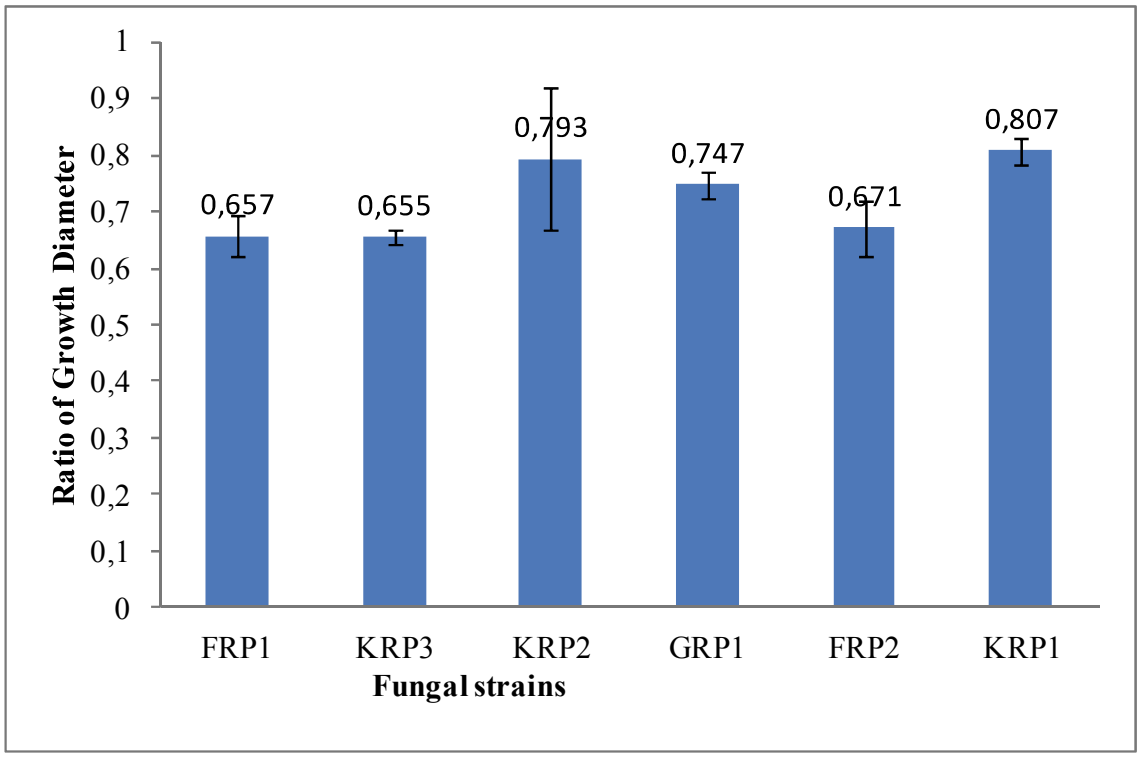

Fig. 3.1 Ratio of growth diameter of fungal strains in mercury contaminated media on the 7 day old fungal colony

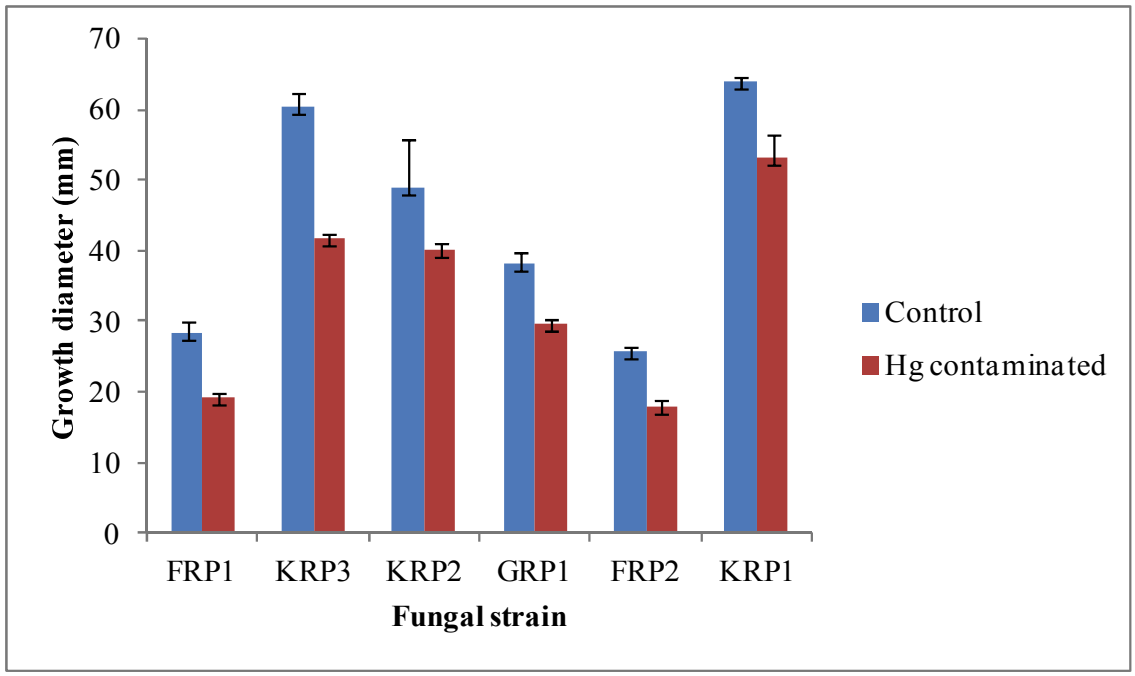

Fig. 3.2 Growth diameter of fungi on 7 days old fungal colony

Fungal survival in the presence of toxic metals mainly depends on intrinsic biochemical and structural properties, physiological and/or genetics adaptation, including morphological changes and environmental modification of metal speciation, availability and toxicity[1]. This fungus was showed highest growth diameter in mercury contaminated media as an indication of fungus hardiness. The ratio of growth diameter of fungal strain KRP1 was also showing highest ratio comparing to other strains. This indicates that the presence of mercury contaminant inhibit the growth of fungal strain KRP1 less than others. The other strain such as KRP2 showed high tolerance as well, however this strain has lower growth diameter showing lower capability to grow in mercury contaminated media. According to these two reasons, it can be said that fungal strain KRP1 showed the resistance or tolerance to mercury contaminant.

An organism may directly and/or indirectly rely on several survival strategies for example, methallothienein synthesis mechanism in *Saccharomyces cerevisiae* to Cu^{2+} by binding or precipitating it around the cell wall and intracellular transport[12]. In terms of bioremediation, fungi are able to process the target compound through enzymatic breakdown (cometabolism), uptake and concentrate within its body (accumulation), and even used the target compound as carbon source. However, fungi are often more proficient at cometabolism and accumulation process. Oxidative enzyme that play a major role and the excreted organic acids and chelators by fungus are involved on cometabolism process and made many toxic chemicals mineralized by fungi already highly oxidized[13].

3.3.2 Identification of mercury resistant fungal strain

Fungal strain KRP1 was identified based on the morphology of colonies and cell-based taxonomic investigation as *Aspergillus* sp. strain KRP1. According to partial 320 bp sequences of 18S rRNA amplicon of KRP1 strain and comparison in the GenBank databases, the result showed that the strain had 98% nucleotide base homology to *A. flavus* strain KRP1 (**Figure 3.3 and 3.4**). The obtained fungal strain cell and colony morphology from the screening and isolation process are shown in **Fig. 3.5**.

A. flavus spends most of its life growing as a saprophyte in the soil and plays an important role as a nutrient recycler, supported by plant and animal debris (Scheidegger

and Payne, 2003). The ability to survive in harsh conditions and overwinter allows it to easily out-compete other organism for substrates in the soil or plants (Bhatnagar et al., 2000). *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., and *Rhizopus* sp. were present in soil contaminated with heavy metals in agricultural fields exposed to heavy metals and other pollutants of untreated wastewater from industrial effluents (Iram *et al*., 2009). Thus, Aspergillus sp. appeared to be the most commonly occurring strain in the heavy-metal-contaminated agricultural soil as reported at Faisalabad and Rawalpindi, Pakistan (Zafar et al., 2007), Aligarh (Ahmad et al., 2005) or Gujranwala and Sialkot, India (Akhtar et al., 2013). *A. niger* was also found in a mining area as the second dominant genus after *Penicillium* sp. (Joo and Hussein, 2012).

Aspergillus sp. is known as a fungal type resistant to heavy metals as well as *Penicillium* sp. *A. niger* was proved to be able to resist high concentrations of various heavy metals such as Cd, Co, Cr, Cu, Ni, Pb and Zn (Iqbal et al., 2010). As a biological leaching agent for heavy metals from contaminated soil, *A. niger* exhibits good potential for generating a variety of organic acids effective for metal solubilization. These acids were effective in removing the exchangeable, carbonate and oxide fraction of Cu, Cd, Pb and Zn (Pandey et al., 2013).

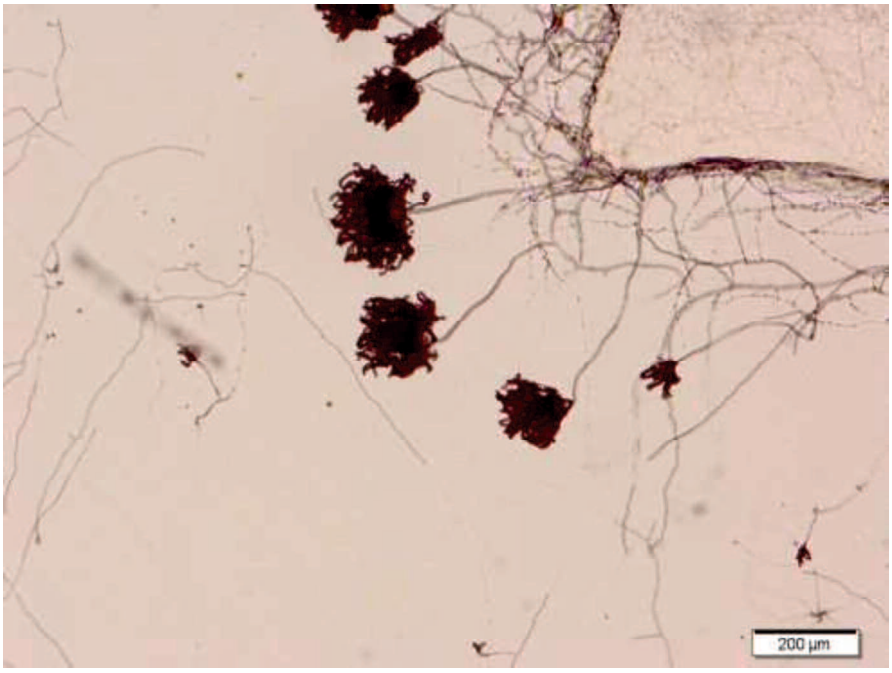

Fig. 3.3 Cell morphology of *A. flavus* strain KRP1

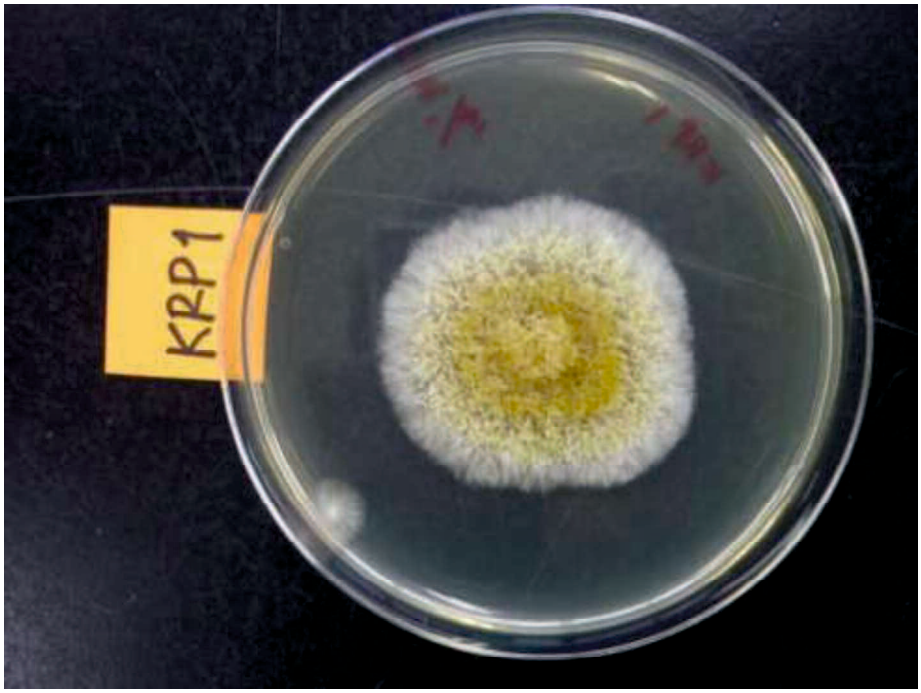

Fig. 3.4 Colony of *A. flavus* strain KRP1

Research development regarding bioremediation using *A. flavus* has also begun to be considered. Heat-inactivated (killed) *A. flavus* biomass was suitable for use as a biosorbent for the removal of As (III) from aqueous solution (Maheswari and Murugesan, 2012). Both fungus *A. niger* and *A. flavus* showed the capability to accumulate Pb, Zn, Cu, and Ni from paper mill effluent (Thippeswamy et al., 2012). Fungal species *Aspergillus cervinus* appears as one of representative colony on mercury contaminated enumeration plate MEA media of long term mercury contaminated soil besides *Umbelopsis spp*. (Crane *et al*., 2011).

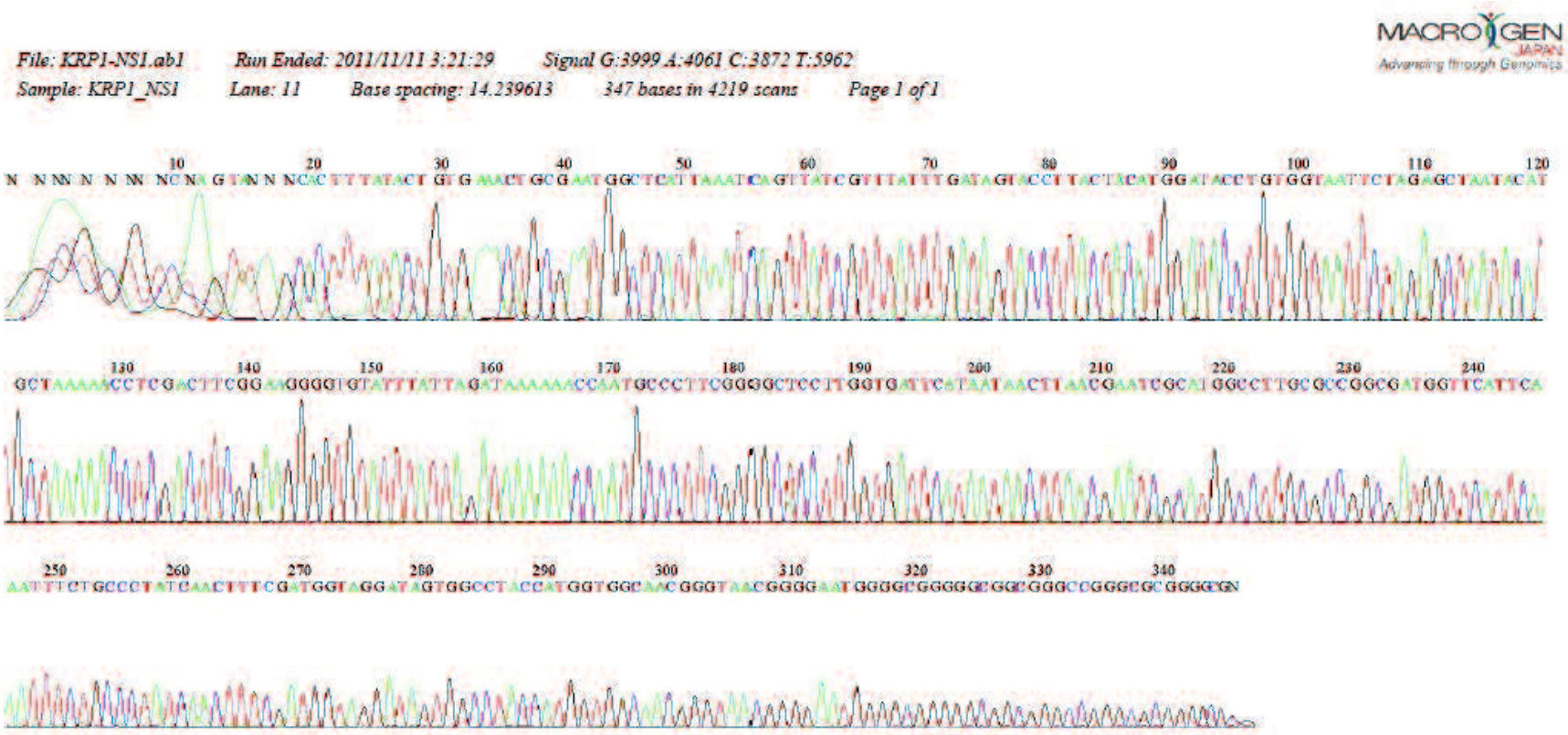

Fig 3.5 Nucleotide sequence of 18 rRNA gene of Aspergillus flavus strain KRP1

3.3.3 Toleration level of fungal strain for mercury contamination

The fungal *A. flavus* strain KRP1 tolerance to the presence of mercury contamination reached 100 mg/L (**Fig. 5**). Mercury seems to be toxic to this fungal strain, however at certain levels they are still able to grow. Fungi are known to be good at accumulating heavy metals onto their mycelium and spores (Bennet *et al*., 2002) as shown by *Phanerochaete chrysosporium,* which is both tolerant and has the ability to accumulate mercury (Dhawale *et al*., 1996). Hence, those fungi were considered as potential metal biosorbents for use in emerging bioengineering technologies for treating industrial effluent and contaminated waters and soil. This mechanism will decrease the risk of heavy metals absorption by cultivated crops in contaminated agricultural soil, preventing their consumption by humans.

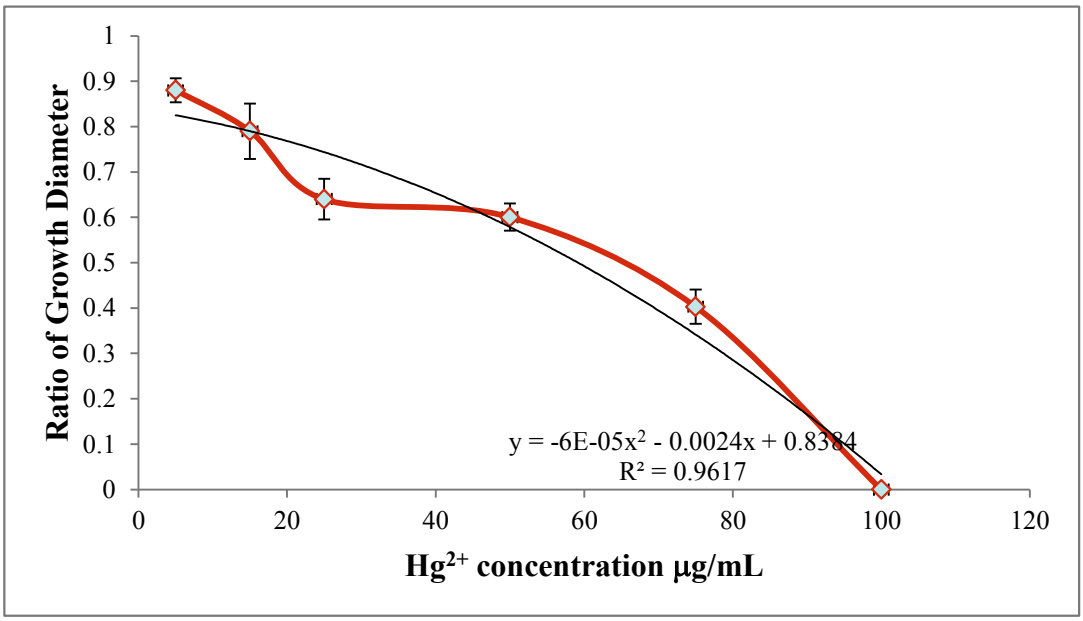

Fig. 3.6 Tolerance level of *A. flavus* strain KRP1 in mercury contaminated media

The tolerance value of *A. flavus* strain KRP1 as amount as 100 mg/L is seems to limit its capability to be applied in hard contaminated soil with mercury such as tailing of gold mining which could reach 327 mg mercury/kg soil (Muddarisna *et al*., 2013). However, this fungal strain is possible to be used for rather lower contaminated soil such as contaminated agricultural soil as described previously at Chapter I.

3.4 Conclusion

Fungal strain KRP1 showed its capability to grow in mercury contaminated media thus chosen for further study for its potential use in bioremediation of mercury contaminated soil. The fungal strain was identified as *Aspergillus flavus* strain KRP1. Tolerance level of this fungus to mercury is 100 mg/L.

3.5 References

Ahmad, I., Zafar, S., Ahmad, F., 2005. Heavy metal biosorption potential of *Aspergillus* and *Rhizopus* sp. isolated from wastewater treated soil. J. App. Sci. and Environ. Man. 9(1), 123-126.

Abou-Shanab, R.A., Van-Berkum, I.P., Angle, J.S., 2007. Heavy metal resistance and genotypic analysis of metal resistance genes in gram-positive and gram-negative bacteria present in Ni-rich serpentine soil and in the rhizosphere of *Alyssum murale*. Chemosphere. 68, 360-367.

Akhtar, S., Mahmood-ul-Hassan, M., Ahmad, R., Suthor, V., Yasin, M., 2013. Metal tolerance potential of filamentous fungi isolated from soils irrigated with untreated municipal effluent. Soil Environ. 32(1), 55-62.

Arfarita, N., Imai, T., Kanno, A., Higuchi, T., Yamamoto, K., Sekine, M., 2011. Screening of soil–born fungi from forest soil using glyphosate herbicide as the sole source of phosphorous. J. Water and Environ. Tech. 9(4), 391-399.

Agency for Toxic Substances and disease Registry (ATSDR), Priority List of Hazardous Substances 2013. Available at: http://www.atsdr.cdc.gov/SPL/index.html. Date accessed: 12 January 2014.

Bennet, J.W., Wunch, K.G., Faison, B.D., 2002. Manual of environmental microbiology: Use of Fungi Biodegradation. 2nd Ed. ASM Press. Washington D.C., pp. 960-971.

Bhatnagar, D., Cleveland, T.E., Payne, G.A., 2000. Encyclopedia of food microbiology. Academic Press. London, pp. 72-79.

Cai, L., Jeewon, R., Hyde, K.D., 2005. Phylogenetic evaluation and taxonomic revision of Schizothecium based on ribosomal DNA and protein coding Genes. Fungal Diversity. 19, 1-21.

Crane, S., Husar M, Dighton J, Barkay T., 2011. The response of saprotrophic fungi from forest soil to mercury tolerance and community diversity. Forest and mercury: Defining the connection. Halifax 2011 Prosiding. Canada.

Dhawale, S.S., Lane, A.C., Dhawale, W., 1996. Effect of mercury on the white rot fungus *Phanerochaete chrysosporium*. Bull. En. Contam. Toxicol. 56, 825-832.

Domsch, K.H., Gams, W., Anderson, T., 1993. Compedium of soil fungi Vol.2, Academic Press, London.

Ezzourhi, I., Castro, E., Moya, M., Espinola, F., Lairini, K., 2009. Heavy metal tolerance of filamentous fungi isolated from polluted sites in Tangier, Morocco. J. Microbiol. Research. 3, 35-48.

Frostegard, A., Tunlid, A., Baath, E., 1993. Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals**.** Appl. Environ. Microbiol., 59 (1993), pp. 3605–3617.

Gadd, G.M., 1993. Interaction of fungi with toxic metals. New Phytology. 124, 25-60.

Gadd, G.M., 2001. Microbial metal transformations. The J. Microbiol. 39(2), 83-88.

Iqbal, Z., Pervez, M.A., Ahmad, S., Iftikhar, Y., Yasin, M., Nawaz, A., Ghazanfar, M.U., Dasti, A.A., Saleem, A., 2010. Determination of minimum inhibitory concentrations of fungicides against fungus *Fusarium mangiferae*. Pak. J. of Botany. 42(5), 3525-3532.

Iram, S., Ahmad, I., Stuben, D., 2009. Fungal tolerance to heavy metals. Pak. J. of Botany. 41(2), 885-895.

Joo, J.H., Hussein, K.A., 2012. Heavy metals tolerance of fungi isolated from contaminated soil. Korean J. of Soil Sci. Fert., 45(4), 565-571.

Knight, B., McGrath, S.P., Chaudri, A.M., 1997. Biomass carbon measurements and substrate utilization patterns of microbial populations from soils amended with cadmium, copper, or zinc. Appl. Environ. Microbiol., 63 (1997), pp. 39–43

May, L.A., Smiley, B., Schmidt, M.G., 2001. Comparative denaturing gradient gel electrophoresis analysis of fungal communities associated with whole plant corn silage. Canadian J. Microbiol. 47(9), 829-841.

Muddarisna, N., Krisnayanti B.D., Utami S.R., Handayanto, E., 2013. The potential of wild plant for phytoremediation of soil contaminated with mercury of gold

cyanidation tailings. IOSR J. En. Sci., Toxicology and Food Technology (IOSR-JESTFT) 4(1), 15-19.

Müller, A.K., Westergaard, K., Christensen, S., Sørensen, S.J., 2001. The effect of long-term mercury pollution on the soil microbial community. FEMS Microbiology Ecology 36 11-19.

Pandey, A.K., Jamaluddin, Awasthi, A.K., Pandey, A., 2013. Biosorption potential of indigenous fungal strains for municipal solid waste leachate management in Jabalpur city. J. Environ. Sci. Comp. Sci. Eng. Technol. (JECET); March - May 2013; 2(2), 385-393.

Pennanen, T., Frostegard, A., Fritze, H., Baath, E., 1996. Phospholipid fatty acid composition and heavy metal tolerance of soil microbial communities along two heavy metal-polluted gradients in coniferous forests. Appl. Environ. Microbiol., 62 (1996), pp. 420–428

Ray, S., Ray, M.K., 2009. Bioremediation of heavy metal toxicity-with special reference to chromium. J. Medical Sci. 2, 57-63.

Scheidegger, K.A., Payne, G.A., 2003. Unlocking the secret behind secondary metabolism: a review of *Aspergilus flavus* from pathogenicity to functional genomic. J. Toxicol. 22, 423-459.

Shim, S.M., Oh, Y.H., Lee, K.R., Kim, S.H., Im, K.H., Kim, J.W., Lee, U.Y., Shim, J.O., Shim, M.J., Lee, M., Ro, H.S., Lee, H.S., Lee, T.S., 2005. Culture Conditions Affecting the Optimal Mycelial Growth of *Cystoderma amianthinum*. Mycobiol. 33(1), 15-18.

Thippeswamy, B., Shivakumar, C.K., Krishnappa, M., 2012. Bioaccumulation potential of *Aspergillus niger* and *Aspergillus flavus* for removal of heavy metals from paper mill effluent. J. Environ. Biol. 33, 1063-1068.

Valsecchi, G., Gugliotti, C., Farini, A., 1995. Microbial biomass, activity, and organic matter accumulation in soils contaminated with heavy metals. Biol. Fertil. Soils 20, 253–259.

Zafar, S., Aqil, F., Ahmad, I., 2007. Metal tolerance and bio sorption potential of filamentous fungi isolated from metal contaminated agricultural soil. Biores. Technol. 98, 2557-2561.

CHAPTER 4
CHARACTERISTIC OF FUNGAL STRAIN ON MERCURY CONTAMINATED MEDIA

4.1 Introduction

Information on characteristics of live fungi can help in the identification of critical points important for fungal development. The fungal characteristic information is used as a base of further study on the utilization of the fungi in bioremediation. The information of preferable environment for fungi to live such as pH, temperature and water activity were played role as controlling factors mainly for fungal growth. The other information such as fungal tolerance to contaminant was also need to be observed so that can gives information about the efficiency of the fungal strain application for bioremediation.

Aspergillus genera are having high capability to grow in highly aerobic environment and can be found in oxygen-rich environment (loose soil) demonstrate its oligotrophic characteristic which is capable to grow in less-nutrient environment. Less-nutrients mean less organic matter referring to non-acidic environment. That kind of environment was often found in heavy metals contaminated environment, thus the identified *Aspergillus flavus* strain KRP1 have a possibility to be used for recovery of the contaminated site.

This chapter studied about the characteristic *A. flavus* strain KRP1 on mercury contaminant environment. The study was to found optimum pH, optimum temperature, and the fungal growth profile based on optimum condition.

4.2 Material and Method

4.2.1 Optimal growth condition

The fungal characteristics evaluated were optimum pH and temperature in mercury-contaminated media, tolerance level to concentration of contaminant and growth profile. The optimum pH observation for the mycelia growth was conducted by growing the fungal strain in 25 mg/L mercury(II)-contaminated PDA media adjusted to pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0 with 1 mol/L NaOH or HCl. The non-contaminated media was also provided as control. A 7 mm mycelia plug was removed from the edge of the 5-

day-old colony culture of the fungal strain grown on PDA media. Each plug was placed on the center of the plates and then incubated for 5 days at 30°C. The average values were obtained from three replicates.

To investigate the optimum temperature, a 7 mm mycelia plug was removed from the edge of 5-day-old refreshed culture on the optimum pH PDA media and placed in the center of 25 mg/L mercury(II)-contaminated and non-contaminated media and incubated for 5 days at 25, 27.5, 30, 32.5, and 35°C. The mycelia growth measurement was performed through growth diameter expression according to the method described by Shim *et al*. (2005). The average values were obtained from three replicates.

4.2.2 Growth profile of fungal strain on mercury contaminated media and mercury removal from the media

Growth profiles observation was examined in 250mL Erlenmeyer flask containing 100mL Potato dextrose Broth (PDB) medium maintained to have 10 mg/L mercury(II) concentration and inoculated with $2x10^7$ spore/mL. The pH of the medium was maintained at 5.7 – 6 during study using 1 mol/L NaOH or HCl. All glassware was washed with 5% HNO_3 and rinsed with deionized water to remove contaminating metals on glassware. The culture was incubated at 30°C with shaking at 100rpm (EYELA, Japan) for 28 days. Every 4 days, the culture was filtered and the dry weight of the mycelium was determined. The average values were obtained from three replicates.

4.3 Result and discussion

4.3.1 Optimal growth condition of fungal strain on mercury contaminated media

Information on characteristics of live fungi can help in the identification of critical points important for fungal development. The optimum pH and temperature for *A. flavus* strain KRP1 to grow in mercury-contaminated PDA *in vitro* can be seen in **Fig. 4.1**.

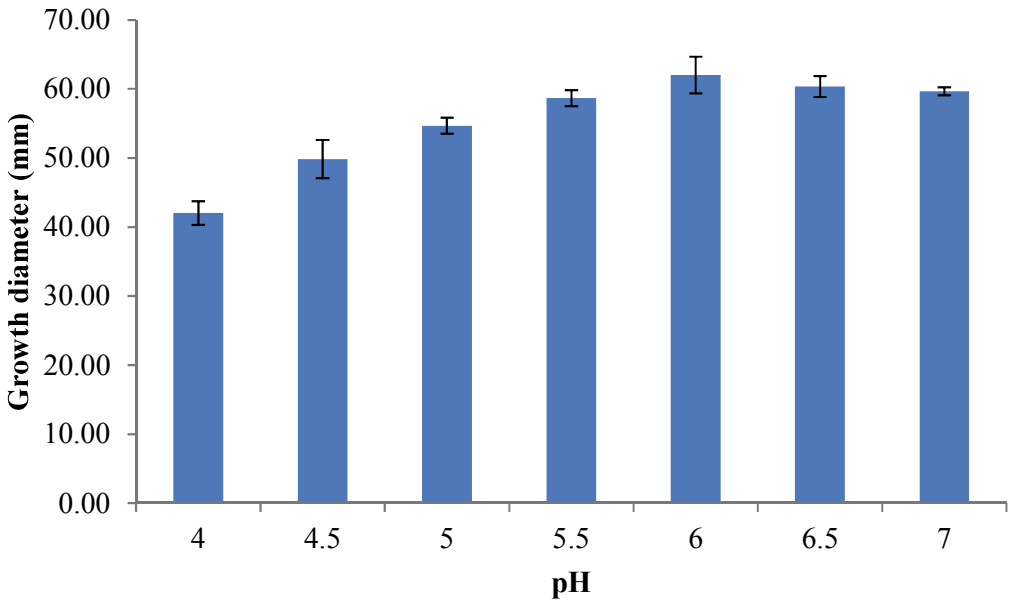

Fig. 4.1 Effect of pH on the mycelial growth of *A. flavus* strain KRP1 *in vitro*.

The strain was able to grow well in pH ranging from 5 to 7. This means that the range of pH should be considered in the application of *A. flavus* strain KRP1 for bioremediation of mercury contamination. *Aspergillus* genera have a high capability to grow in highly aerobic environments and can be found in oxygen-rich environments (loose soil) and demonstrate oligotrophic characteristics, being capable of growth in low-nutrient environments. pH has not been significantly studied in the growth of fungi since their cells are capable to produce acid during metabolism (Bekada et al., 2008) however it still a controlling factor for the growth of *A. flavus* along with temperature and water activity (Gibson et al., 1994). In the use of *Aspergillus* for biosorption of heavy metals in wastewater, pH is an important parameter in affecting the biosorption capacity of *A. niger*. At low pH (less than 4), heavy metal removal was inhibited, probably caused by a positive charge density on metal binding sites due to the high concentration of protons in solution (Joo and Hussein, 2012).

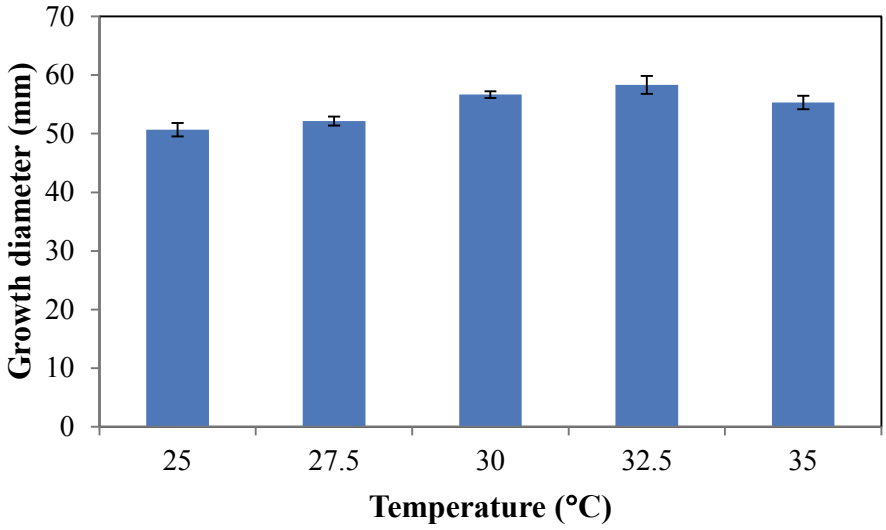

Fig. 4.2 Effect of temperature on the mycelial growth of *A. flavus* strain KRP1 *in vitro*.

The capability of *A. flavus* strain KRP1 to grow in a wide range of temperature can be seen in **Fig. 4.2**. This strain had better growth at the quite high temperatures of 27.5-35°C. The fungal growth rate in situ had an optimum temperature of 25-30°C and became lower at higher temperature (Pietikäinen et al., 2005). Another report stated that *A. flavus* has optimum growth temperature of 37°C, however its growth can be observed at temperatures ranging from 12-48°C (Hedayati *et al*., 2007). The ability to survive in harsh conditions shows its competitiveness with other organisms (Bhatnagar *et al*., 2000). The high optimum temperature may contribute to its pathogenicity to creatures.

4.3.2 Growth profile of fungal strain on mercury contaminated media and mercury removal from the media

The growth profile of *A. flavus* strain KRP1 was further monitored on 28 days based on mycelia dry mass using potato dextrose broth medium (see **Fig. 4.3**). Growth phase includes "lag" phase, the acceleration phase that occur at the "exponential" phase and then "decelerate" phase, followed by stationary phase and lyses (cell death) (British Mycological Society). The "exponential" phase of this fungal strain was during 8 days where there was progressive increase of dry mass weight to be called as growth on up to the first 8 days of cultivation. A "decelerate" phase followed by "stationary" phase occurs between 12 and 20 days of cultivation; and then a slight increase on next days.

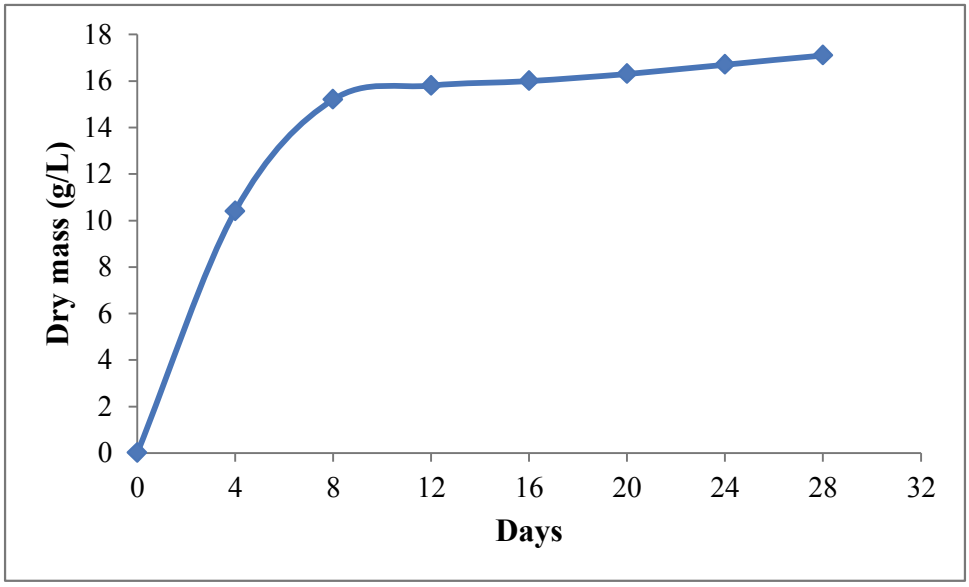

Fig. 4.3 Growth kinetics of *A. flavus* KRP1 on Potato Dextrose Broth medium containing 10mg/L mercury(II).

The fungal growth was further studied for deeper understanding of the tolerance expression. The growth is terminated immediately after depletion of the first essential nutrient, irrespective of the nature of this essential nutrient. Physiological properties of filamentous fungi are strongly dependent on the nutritional status and thus the growth phase (Vrable *et al*., 2009). The growth rate of fungi in mercury-contaminated media appeared to be disturbed during the lag phase of the growth. A high concentration of mercury was able to delay the lag phase by a certain time. However, this expression did not appear at concentration 100 mg/L, when the fungal growth was totally inhibited (**Fig. 4.4**).

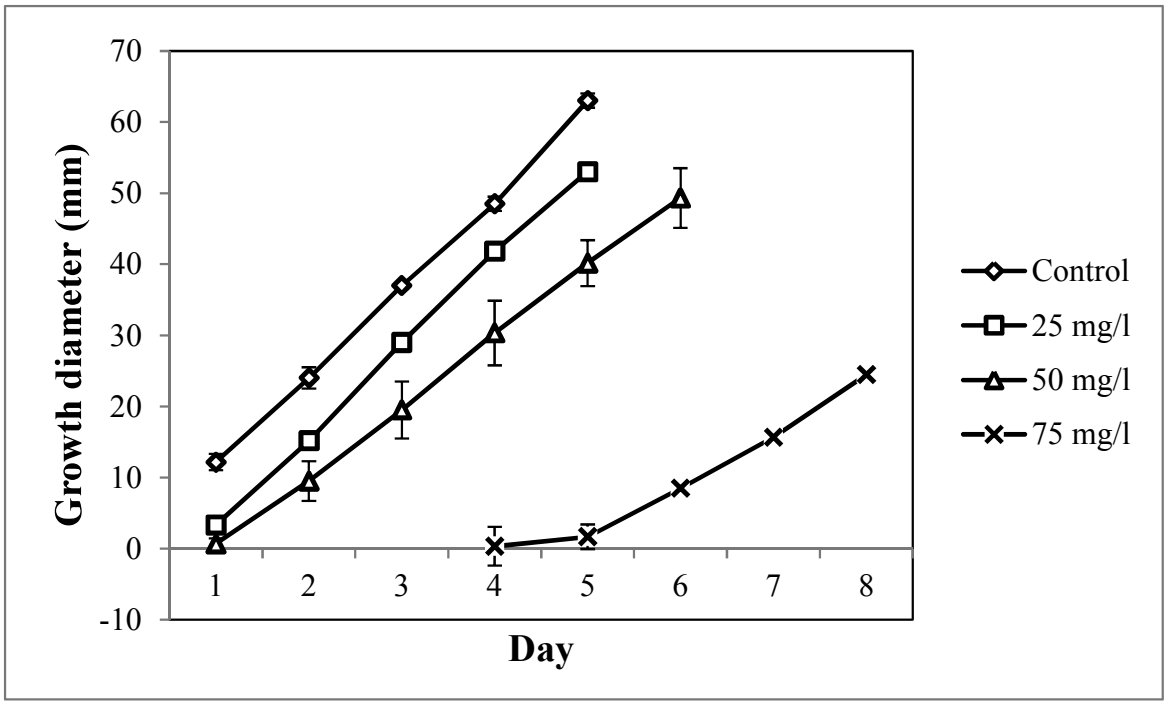

Fig. 4.4 Effect of mercury concentration to the growth of *A. flavus* strain KRP1. Control cultures did not contain metal ion.

The toxicity of heavy metals was found to vary with exposure time and fungal type. The fungal growth phase in the presence of heavy metals reflected the tolerance development by the rates of growth during lag, retarded, similar and enhanced phases (Valix *et al*., 2001).

4.4 Conclusion

The fungal characteristics for optimum growth in mercury-contaminated media were pH range 5.5-7 and temperature from 25-35°C. The presence of mercury(II) contamination disturbs the lag phase of fungal growth, causing the delay of mycelium growth.

4.5 References

Bekada, A.M.A., Benakriche, B., Hamadi, K., Bensoltane, A., 2008. Modelling of effect of water activity, pH and temperature on the growth rate of *Mucor racemousus* isolated from soft camembert cheese. World J. Agri. Sci. 4(6), 790-794.

Bhatnagar, D., Cleveland, T.E., Payne, G.A., 2000. Encyclopedia of food microbiology.

Academic Press. London, pp. 72-79.

Gibson, A.M., Baranyi, J., Pitt, J.I., Eyles, M.J., Robert, T.A., 1994. Predicting fungal growth: the effect of water activity on *Aspergillus flavus* and related species. Intl. J. Food Microbiol. 23, 419-431.

Hedayati, M.T., Pasqualotto, A.C., Warn, P.A., Bowyer, P., Denning, D.W., 2007. *Aspergillus flavus*: Human pathogen, allergen and mycotoxin producer. Microbiology. 153, 1677-1692.

Joo, J.H., Hussein, K.A., 2012. Heavy metals tolerance of fungi isolated from contaminated soil. Korean J. of Soil Sci. Fert., 45(4), 565-571.

Pietikäinen, J., Petterson, M., Bååth, E., 2005. Comparison of temperature effect on soil respiration and bacterial and fungal growth rates. FEMS Microbiol. Ecol. 52,49–58.

Valix, M., Tang, J.Y., Malik, R., 2001. Heavy metal tolerance of fungi. Mineral Eng. 14(5), 499-505.

Vrable, P., Schinagl, C., Burgstaller, W., 2009. Growth kinetics of filamentous fungi in batch culture: textbook knowledge and reality. Mycoses. 52(S), 430.

CHAPTER 5
POTENTIAL USE OF FUNGAL STRAIN FOR BIOREMEDIATION OF MERCURY

5.1 Introduction

Bioremediation technologies are divided into biodegradation, biotransformation, biodeterioration, bioaugmentation, bioaccumulation and cometabolism which are specified with each meaning. Biodegradation is biologically mediated breakdown of chemical compounds which is implies series of biochemical reaction and completed with mineralization. Biotransformation or so called bioconversion refers to a single step in biochemical pathway in which a molecule is catalytically converted into different molecule with consideration on the water solubility, easiness to be excreted by the cell, toxicity, and the hazardous level. Biodeterioration is the breakdown of economically useful substances. Bioremediation is the use of biological system to transform and/or degrade toxic compounds to become harmless involving indigenous microbes with or without nutrient supplementation. When involving exogenous organism into sites then it called bioaugmentation. Bioaccumulation, which is synonymously with biosorption, is the concentration of the substances without any metabolic transformation. This techniques usually used for metals and certain radionuclides from aqueous environment which is then recycle or contain the loaded biomass. Cometabolism describes the situation where an organism is able to biotransform a substrate but is unable to grow on it. The other meaning is the degradation of a given compound by the combined effort of several organisms through mutual efforts.

The use of fungi in bioremediation has been limited comparing to bacteria. In term of fungal-metal relationship, fungi are good at bioaccumulation of metals. Many species have been proven capable to absorb cadmium, copper, lead, mercury and zinc onto their mycelium and spores. The wall of dead fungi sometimes binds metals better than the living ones (Bennet *et al*., 2002). Rhizopus arrhizus have been use in a system for treating uranium and thorium (Tieen-Sears *et al*., 1984). Fungal biomass resulted from industrial fermentation can be used for concentration of heavy metal contamination (Gadd, 1986; Gadd, 1992; Ross, 1975).

Aspergillus flavus strain KRP1 on this study is observed on its potential in bioremediation of mercury contamination both in liquid and soil. For that purposes, the objective on this chapter was divided as follows:

1. To know the utilization of mercury by means of total mercury in conjunction with fungal growth in broth media.
2. To know the potential use of the fungal strain for bioremediation in contaminated liquid media.
3. To know the potential use of the fungal strain for bioremediation in contaminated soil.

5.2 Material and method

5.2.1 Utilization of mercury by means of total mercury

The utilization of mercury by *Aspergillus flavus* strain KRP1 was examined in 250 mL Erlenmeyer flask containing 100 mL PDB medium. The medium was contaminated with Hg^{2+} solution and maintained to have 10 mg/L of Hg^{2+}. The observation was done as in growth profile. The mercury removal was determined on the basis of total mercury concentration on the liquid medium. Every 4 days a 6 mL sample of each broth culture was pipette into centrifugation tube and centrifuged for 15 min at 6000rpm. One milliliter of the supernatant was pipette into 200 mL Erlenmeyer flask and diluted with deionized water into 100 mL to be measured the mercury concentration using CVAAS Hiranuma 200. The average values were obtained from three replicates.

5.2.2 Potential use of the fungal strain for bioremediation in contaminated liquid media

The potential use of the fungal strain for bioremediation in contaminated liquid media was conducted in liquid PDB medium made from 24 g PDB powder (Difco; Becton Dickenson and Company, USA) dissolved in 1 L distilled water and adjusted to optimum pH. It was carried out in two types of systems, namely shaken and static systems, as approaches for aqueous and solid states. The capacity of the fungal strain to remove mercury contamination was evaluated in liquid PDB media 100 mL of the media was then put into 300 mL Erlenmeyer flasks and autoclaved at 121°C for 15 min. The media was allowed to cool down to room temperature (25°C) before being amended to contain 10 mg/L mercury(II) by aseptic technique. Media without

contaminant was also provided as a baseline. Control flasks containing medium and mercury(II) but no inoculated spore suspension were also processed the same way as the treatment. Each treatment was carried out in triplicate.

The fungal strain was provided in the form of a spore suspension made from a 5-day-old mycelial plug grown on PDA and suspended in sterile distilled water. The concentration of spores was measured and calculated using a haemocytometer. Spore suspension in the amount of 10^8 spore/mL was inoculated into each flask by aseptic technique.

The inoculated flasks were incubated on an orbital shaker (130 r/min) at optimum temperature for 7 days for the shaken system. After incubation, the fungal biomass was harvested from the growth medium by filtering through Whatman #1 filter paper. The fungal biomass was then dried in an oven at 80°C overnight. Filtrate and liquid from the control treatment was centrifuged at 6000 r/min for 15 min before analysis. The mercury content in the filtrate was analyzed using a Cold Vapor Atomic Absorption Spectrophotometer (CVAAS) Hiranuma HG-200. The detection limit for mercury was or 5 ng/L.

Observation data were final pH of the media, fungal dry mass weight and mercury concentration on the filtrate. Mercury(II) removal from the filtrate by the fungal strain was obtained by simply dividing the reduction in concentration by the initial concentration and multiplying by 100 to yield the percentage of removal. The obtained data were analyzed statistically using statistical data analysis in SPSS Statistic 13.0 using Two Way ANOVA with replication for final pH and dry weight biomass data, while single factor One Way ANOVA was used to determine the significance of the percentage of mercury removal compared to control.

5.2.3 Potential use of the fungal strain for bioremediation on contaminated soil.

Potential use of *A. flavus* strain KRP1 in bio-augmentation term was observed. For soil preparation, a soil taken from garden was then characteristics checked including texture and field capacity (FC). The soil that used in this study is having a silt clay texture and reached field capacity at 0.31 gram water/gram soil. This information was used for watering the soil during treatment. The water content of the soil was kept on field capacity level by using balancer. The soil was 2 mm sieved before using it for experiment. The soil was thoroughly mixed with $HgCl_2$ solution to have 0 and 25

mg/L/kg soil and remain in closed bag for 24 h. in amount of 250 g of the soil was then put in pots (3 replicates). The water content of the soil was managed in field capacity during experiments.

The tested plant was prepared by first seedling it on a seedbed for 2 days until emerging shoots. The seed was then moved to a sand media which is spray with NPK fertilizer first and cultivated for 2 weeks (3-4 leaf was formed). Similarly weight of the plant (50 g) was chosen and cultivate on the treated media. The initial weight was noted to be compared with final weight at the end of experiment.

Fungi was cultivated on bottles (250 ml) containing 100g of sterile rice husk and then inoculate aseptically with five mycelial plug (7 mm in diameter) taken from 5 day old culture fungi on potato dextrose agar (PDA), and incubate for 14 days. The conidia will be harvested by scrapping the surface and mixed to sterile DW (**Fig. 5.1**). The number of conidia per ml suspension was determined by dilution method with Haemocytometer.

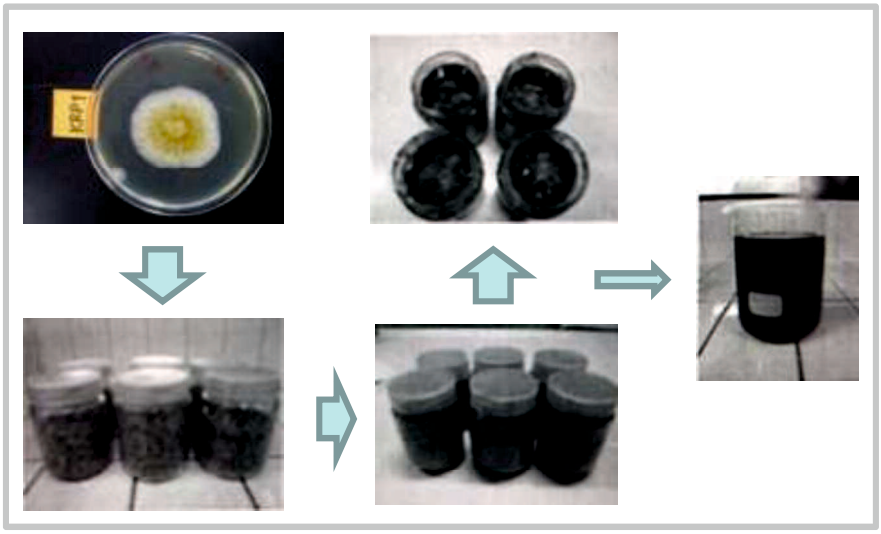

Fig. 5.1 Fungal preparation

There were eight combinations for the treatment and all in 3 replicates (**Table 5.1**). The active grow in amount of 10^9 spore/ml spore suspension (50 ml each) was sprayed to the pot, while the non-fungal treatment were sprayed with DW. The cultivated plant (2 weeks old) was transplanted in plastic pot (contaminated and non-contaminated soil in triplicate). Watering was conducted daily in amount of initial water content (using balancer). No fertilizer applied during experiment.

Table 5.1 Experimental treatments design

M	C	P
0 : No Fungi	0 : No contamination	0 : No Plant
1 : With Fungi	1 : With contamination	1 : With plant

Observation was conducted for 14 days. On day-0 and day-14, 10 gram soil media was taken for soil microbial analysis (CFU) using Rose Bengal Agar (RBA) media using spread method. RBA media is a specific media use for fungal colony enumeration. The fungal colony (CFU) was counted after incubation at 27°C for 48 h (**Fig. 5.2**). Data provided from the plant's dry weight represented as plant growth, total Hg concentration in plant, and available and total Hg in the soil. The hg was measured using CVAA of Mercury F732-5 at Soil Science Department, Brawijaya University.

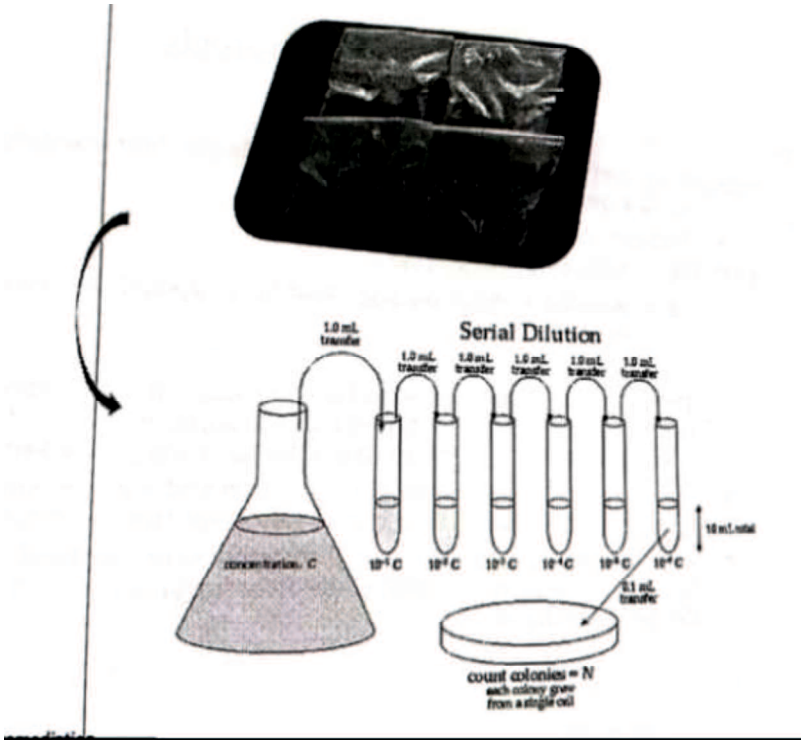

Fig. 5.2 Soil microbial analysis method

5.3 Result and discussion

5.3.1 Utilization of mercury by means of total mercury

The growth profile of *A. flavus* strain KRP1 was observed on the fungal growth in potato dextrose broth (PDB) medium containing 10 mg/L of mercury. The progressive increase of mycelial dry mass during the first days of incubation was accompanied by decreasing of total mercury from the medium. Degradation of mercury from Potato Dextrose Broth culture medium was occur during progressive increase of mycelia dry mass in the first 8 days of incubation. The rate of increasing of mycelia dry mass was coupled with the decreasing of the mercury concentration in the culture medium (see **Fig. 5.3**). This indicates a utilization process was occurred and could be said that a mechanism of degradation possessed.

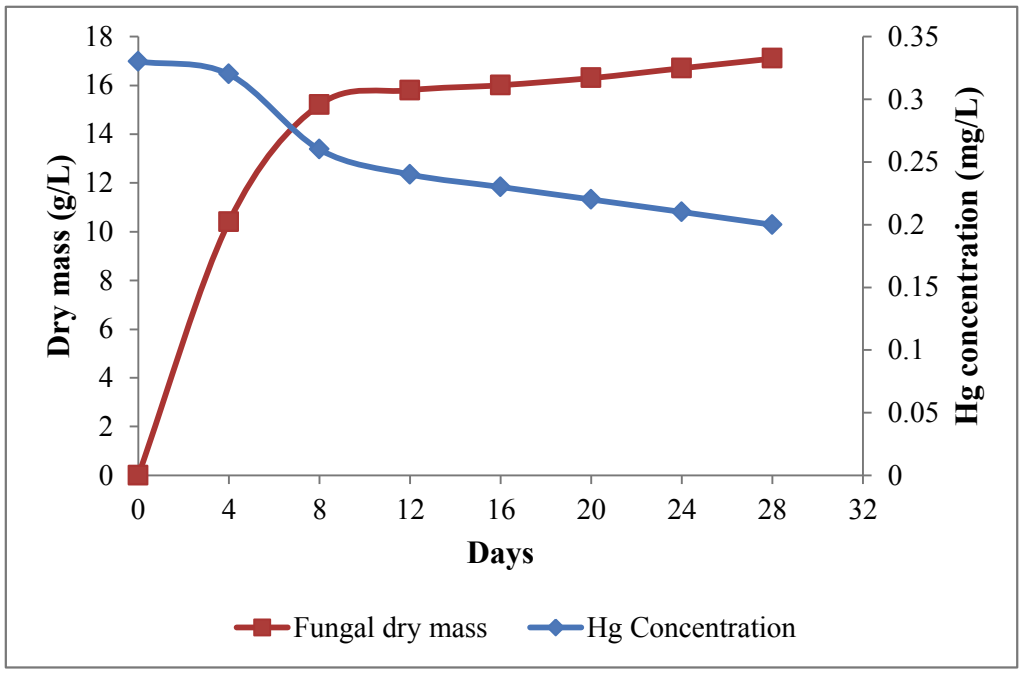

Fig. 5.3 Growth kinetics of *A. flavus* strain KRP1 on potato dextrose broth medium containing 10mg/L of mercury(II)

The total mercury concentration dropped most notably on 8th days of cultivation and continued until 28 days of cultivation. This result indicates that the utilization process for mercury takes place and perhaps shows that the studied fungal strain may possess a mechanism for degradation of mercury contaminant.

5.3.2 Potential use of the fungal strain for bioremediation in contaminated liquid media

The capability of *A. flavus* strain KRP1 to remove mercury(II) from the media

showed that the shaken system was able to remove 97.50% of 10 mg/L mercury(II). The capability of the fungal strain in the static system was significantly higher at 98.73% removal (**Table 5.2**). The pH of the culture media tended toward acidic during incubation. The presence of fungi naturally lowered the pH of the media as their naturally plays role as fermenter and decomposer in the natural life cycle. Even though the difference was not significant, the static system tended to have lower pH than the shaken system. The shaken culture system provided more dry mass than the static system. This is because in the orbital shaking enabled the fungal spore for having more contact with the media, thus higher nutrient uptake by the fungal spores that was then used it for growth. Mercury removal from the culture media in the static system was significantly ($p < 0.05$) higher than that in the shaken system. Similar phenomena were also observed to prevail in Cd removal by *Trichoderma koningii*, *Aspergillus terreus* Thom, *Gliocladium roseum* and *Talaromyces helices* (Messaccesi *et al*., 2002).

Table 5.2 Removal capacity of *A. flavus* strain KRP1 for mercury contamination in culture media

System	Hg(II) (mg/L)	pH	Dry mass (g/L)	Hg removal (%)	Removal capacity (Hg (mg/L)/g dry weight)
Shaken	10	4.13	14.9	97.50a	6.55a
	0	4.12	15.4		
Static	10	4.01	14.3	98.73b	6.91b
	0	4.18	14.6		
HSD				0.49	0.26

a and b showed significant differences based on One way ANOVA statistical analysis. HSD: Honestly Significant Difference.

A. flavus strain KRP1 was able to remove mercury(II) from the medium in 7 days incubation time, thus showing potential for bioremoval of mercury. The removal mechanism that might occur is biosorption or bioimmobilization based on comparison with mercury removal in the control (**Fig. 5.4**). Almost no mercury(II) removal occurred in the control media. Only about 28% mercury(II) was removed from the control shaken system. This might be caused by the shaking process triggering utilization by the

remaining oxygen inside flasks. However, further tests should be conducted to determine the cause with certainty. In general, this result pointed to the possible application of *A. flavus* KRP1 as a metallic bio-absorbent for contaminated sites. The static system was an approach to the general case for solid or contaminated site treatment.

Fungi are known to be able to resist and detoxify heavy metals. In terms of fungal bioremediation strategies, there are three general categories such as: (1) using the target compound as a carbon source, (2) enzymatically attacking the target compound but not using it as a carbon sources (cometabolism), and (3) taking up and concentrating the target compound within the organism (bioaccumulation). Fungi were able to participate in all categories but were more proficient at cometabolism and bioaccumulation (Bennet *et al*., 2002). Oxidative enzymes that play a major role and the organic acids and chelators excreted by fungus are involved in the cometabolism process and result in many toxic chemicals mineralized by fungi being highly oxidized. This mechanism was thought to be occurring in this research based on the comparison of removal between the two systems and the control.

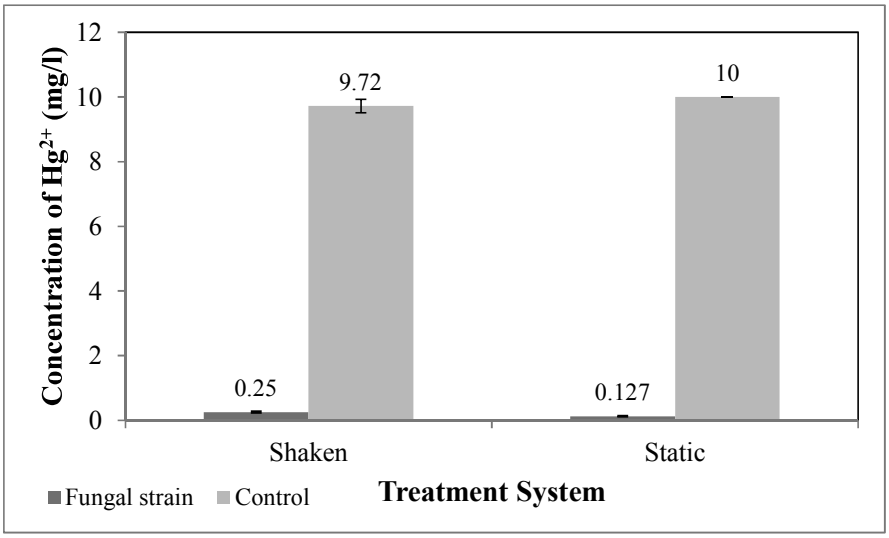

Fig. 5.4 Mercury processing ability of *A. flavus* strain KRP1 in different system. The isolate were grown with 10 mg/l of mercury(II). The control medium contained heavy metal but no organism.

Uses of fungi for bioremediation of mercury contamination both as living or dead fungal biomass have been reported. The mechanisms involved are biosorption, bioaccumulation, and reduction (Arica *et al*., 2003). Mercury(II) immobilization or

biosorption using immobilized fungal cells of both inactivated and live fungus of *Phanerorochaete chrysosporium* showed high promise (Kaçar *et al*., 2002). The immobilized fungus performed better than the live fungus. Amongst 14 fungal species such as *Aspergillus flavus* I-V, *Aspergillus fumigatus* I-II, *Helminthosporium* sp., *Cladosporium* sp., *Mucor rouxii* mutant, *M. rouxii* IM-80, *Mucor* sp. 1 and 2, and *Candida albican* that have been used in biosorption of mercury(II) in aqueous solution, it was found that the fungal biomass of *Mucor rouxii* mutant, *M. rouxii* IM-80, *Mucor* sp 1 and 2 were effective in removal of mercury(II) (Martinez-Juarez *et al*., 2012).

5.3.3 Potential use of the fungal strain for bioremediation on contaminated soil.

Bioaugmentation technology has been practiced intentionally in number of areas including agriculture and forestry (Jasper, 1994) and wastewater treatment (Rittman and Whiteman, 1994). Determination of potential success of bioaugmentation needs an understanding of the bioavailability of the pollutant, the survival activity of added microorganism and the general environment condition that control soil bioremediation rates (Vogel, 1996).

The presence of Hg in the soil affected the soil microbial existence expressed by the number colony forming unit (CFU/g of soil), however it had better existence with the presence of plant (**Fig. 5.5**). The presence of plant may bring all together with its micro environment like mycorhiza which is not possible to be found on the no-plant soil environment.

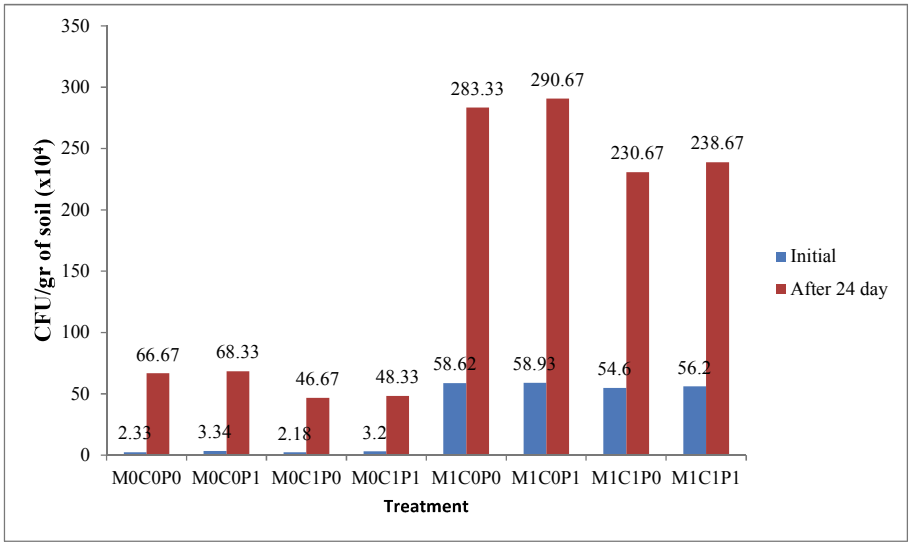

Fig. 5.5 The CFU of soil microbes as initiation and after 24 days.

From the **Fig. 5.5**, it can be seen in general that the number of microbe in the soil is higher both in contaminated or non-contaminated pots with the presence of plant. This finding is supported by the fact that plant microcosm is possible to support more diver microorganism through the plant-soil relationship. Therefore, the number of microbes is higher than that which has no plant. The microbes were tending to survive in mercury contaminated soil and have more chance of survival with the present of plant even not significantly differ.

The removal of Hg from the soil can be seen on **Fig. 5.6**.

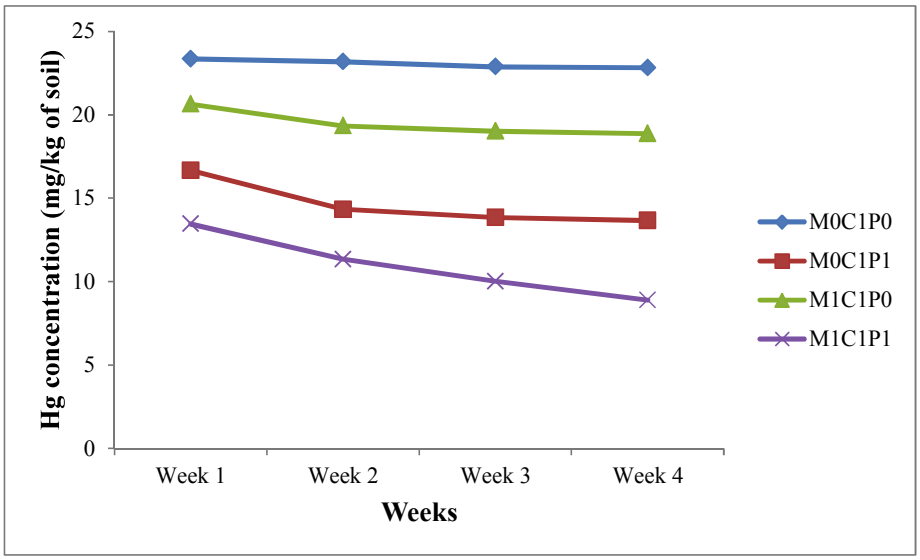

Fig. 5.6 The Hg concentration in the soil

The presence of Hg contaminant affected the total number of microbe yet tends to decrease the Hg contaminant from soil. The presence of plant could intend to support the microbial environment and also brings a micro system including soil microbial in the plant root zone (rhizosphere). This will lead to better soil condition and at the end can recover the soil quality. The combination between plant and fungal strain could perform better in Hg removal.

5.3.4 Bioremediation mechanism of mercury contaminated soil using fungi

Based on the result on sub chapter 5.3.2, it seems that the possible mechanism of *A. flavus* strain KRP1 in mercury contaminated liquid media is biosorption. Thus, the same mechanism could be occurring in application of this fungal strain in mercury

contaminated soil. Tobin *et al*., (1994) studied about adsorption of heavy metal in microorganism showed that the process is considered to be a two-phase process: (i) initial rapid phase of metabolism-independent binding on the cell wall followed by (ii) relatively slower energy-dependent active uptake or intracellular accumulation. Das et al. (2007) study on the adsorption mechanism of mercury on *Aspergillus versicolor* biomass in liquid media showed a Transmission electron microscopic (TEM) result that provide information regarding the metal binding location with reference to the individual cell (**Fig. 5.7**) and thereby help to obtain a better understanding of the adsorption phenomenon. It seems that the cell retains the mercury, therefore it is stabilized.

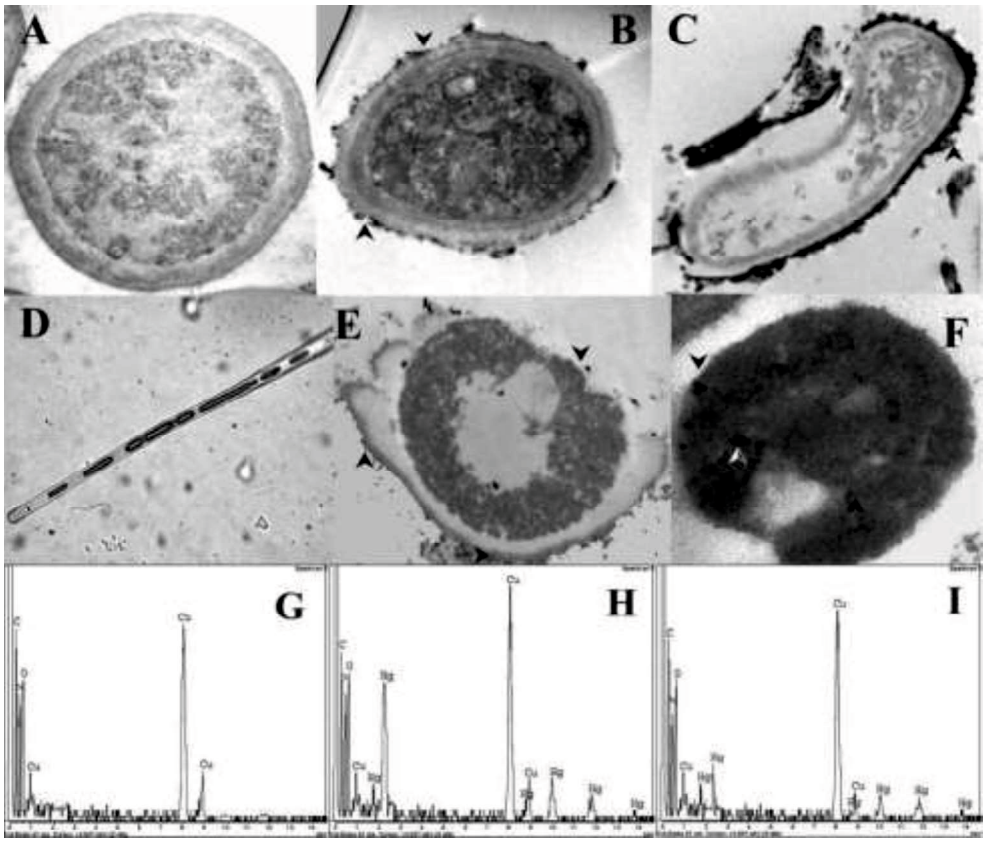

Fig. 5.7 Transmission electron micrographs of thin section of *A. versicolor* (Das *et al*., 2007) where: (A) control cell; (B) and (C) indicate accumulation of mercury on the cell surface (50 and 500 mg/L, respectively, (D) phase contrast micrograph of *A. versicolor* protoplast. Thin section of (E) spheroplast and (F) protoplast after adsorption of mercury from a solution containing 50 mg/L mercury. EDXA spectrum of (G) pristine biomass, (H) mercury-adsorbed biomass and, (I) mercury-adsorbed protoplast and spheroplast. Arrows indicate the location of mercury.

Basic thinking about how to maintain the contaminated sites after bioremediation could be described as follow:

Since, the fungal strain is inhabit in soil and the metal is bind in the cell, the mechanism of how to maintain the contaminated is by simply maintain the pH of the soil for not drop below pH 5 and in aerobic state. In this state, it is possible for other contaminant maintaining process occurs for example are volatilization, contaminant maintaining by bacteria, or enzymatical process that change the contaminant into more available form for plant absorption. Since mercury in form of volatile Hg^0 in the atmosphere is common, very dilute and consider as non-toxic, so that the danger of mercury is becoming less. If it is combined with known mercury accumulated plant, so that the mercury can be removed from contaminated land by phytoremediation.

5.4 Conclusion

Fungal strain *Aspergillus flavus* strain KRP1 has potential use for bioremediation of mercury contamination in both in substrate and soil. In term of bioremediation of mercury contaminated soil, the use of *A. flavus* strain KRP1 could be played a role as; (1) biosorbent in either living or dry mass form which is detoxify the toxic effect of mercury to other organism; (2) bioremediator, when it is applied through bioaugmentation.

5.5 References

Arica, M.Y., Arpa, C., Kaya, B., Bektas, S., Denizli, A., Genc, O., 2003. Comparative biosorption of mercuric ions from aquatic systems by immobilized live and heat-inactivated *Trametes versicolor* and *Pleurotus sajur-caju*. Biores. Tech. 89,145-154.

Bennet, J.W., Wunch, K.G., Faison, B.D., 2002. Manual of environmental microbiology: Use of Fungi Biodegradation. 2nd Ed. ASM Press. Washington D.C., pp. 960-971.

Das, S.K., Das A.R., Guha A.K., 2007. A study on adsorption mechanism of mercury on *Aspergillus versicolor* biomass. Environ. Sci. Technol. 41, 8281–8287.

Gadd G.M., 1986. The response of fungi toward heavy metals. In: Herbert RA, Codd GA, eds. Microbes in extreme environments. London: Academic Press, 83-110.

Gadd, G. M. 1992. Microbial control of heavy metal pollution, p. 59-88. In J.C. Fry, G.M. Gadd, R.A. Herbert, C.W. Jones, and I.A. Watson-Craik (ed.), Microbial control of Pollution. Cambridge University Press, Cambridge, UK

Jasper DA: Bioremediation of agricultural and forestry soils with symbiotic micro-organisms. Aust J Soil Res, 32:1301-1319.

Kaçar, Y., Arpa, C., Tan, S., Denizli, A., Genç, O., Arıca, M.Y., 2002. Biosorption of Hg(II) and Cd(II) from aqueous solutions: comparison of biosorptive capacity of alginate and immobilized live and heat inactivated *Phanerochaete chrysporium*. Process Biochem. 19(37), Issue 6, 601-610.

Martinez-Juarez, V.M., Cardenas-Gonzalez, J.F., Torre-Bouscoulet, M.E., Acosta-Rodriguez, I., 2012. Biosorption of mercury (II) from aqueous solution onto fungal biomass. Bioinorganic Chem. and Appli. Vol. 2012. DOI:10.115/2012/156190.

Messaccesi, G, Romero, M.C., Cazau, M.C., Bucsinszky, A.M., 2002. Cadmium removal capacities of filamentous soil fungi isolated from industrially polluted sediment, in La Plata (Argentina). World J. Microbiol. Biotechnol. 18, 817-820.

Rittmann BE, Whiteman R., 1994. Bioaugmentation: a coming of age. Wat. Int, 1:12-16.

Ross, I. S. 1975. Some effects of heavy metals on fungal cells. Br. Mycol. Soc. 64:175-193.

Tieen-Sears, M.E., Martin, S.M., and Volesky, B., 1984. Propagation of Rhizopus javanicus biosorbent. Appl. Environ. Microbiol. 48:137 -141.

Vogel, T..M., 1996. Bioaugmentation as soil bioremediation approach. Current Opinion iin Biotechnology, 7:311-316.

CHAPTER 6
GENERAL CONCLUSION AND FUTURE WORKS

6.1 General conclusion

General conclusion of this thesis can be written as follow:

Fungal strain *Aspergillus flavus* strain KRP1 has potential use for bioremediation of mercury contamination in both in liquid and soil. In order to use this fungal strain, some notification should be concerned such as:

(1) The tolerance level of this fungus to mercury is 100 mg/l.

(2) The optimum growth in mercury-contaminated media was pH range 5-7 and temperature from 27.5-35°C. The presence of mercury(II) contamination disturbs the lag phase of fungal growth, causing the delay of mycelium growth.

(3) The use of *A. flavus* strain KRP1 could be played a role as; (1) biosorbent in either living or dry mass form which is detoxify the toxic effect of mercury to other organism; (2) bioremediator, when it is applied through bioaugmentation.

6.2 Future works

Future works are still need to be done in order to use this fungal strain for mercury remediation. The future works are as follows:

1. Observation for more mercury types other than inorganic mercury such as the methyl mercury as a kind of organo-mercury.
2. Observation for more bioremediation mechanism that shown most effective and efficient in remediating mercury from soil.
3. Application in contaminated field both as single organism or symbiotic with bacteria or plant.
4. Cost analysis for mass production.

REFERENCES

Abou-Shanab, R.A., Van-Berkum, I.P., Angle, J.S., 2007. Heavy metal resistance and genotypic analysis of metal resistance genes in gram-positive and gram-negative bacteria present in Ni-rich serpentine soil and in the rhizosphere of *Alyssum murale*. Chemosphere. 68, 360-367.

Agency for Toxic Substances and Disease Registry (ATSDR), 1999. Toxicological profile for mercury. U.S. Department of Health and Human Services. USA

Ahmad, I., Zafar, S., Ahmad, F., 2005. Heavy metal biosorption potential of *Aspergillus* and *Rhizopus* sp. isolated from wastewater treated soil. J. App. Sci. and Environ. Man. 9(1), 123-126.

Akhtar, S., Mahmood-ul-Hassan, M., Ahmad, R., Suthor, V., Yasin, M., 2013. Metal tolerance potential of filamentous fungi isolated from soils irrigated with untreated municipal effluent. Soil Environ. 32(1), 55-62.

Arfarita, N., Imai, T., Kanno, A., Higuchi, T., Yamamoto, K., Sekine, M., 2011. Screening of soil–born fungi from forest soil using glyphosate herbicide as the sole source of phosphorous. J. Water and Environ. Tech. 9(4), 391-399.

Arica, M.Y., Arpa, C., Kaya, B., Bektas, S., Denizli, A., Genc, O., 2003. Comparative biosorption of mercuric ions from aquatic systems by immobilized live and heat-inactivated *Trametes versicolor* and *Pleurotus sajur-caju*. Biores. Tech. 89, 145-154.

Baath, E. (1989) Effects of heavy metals in soil on microbial processes and population. Water Air Soil Pollut. 47, 335-379.

Babich, H. and Stotzky, G. (1985) Heavy metals toxicity to microbe mediated ecological processes: A review and potential application to regulatory policies. Environ. Res. 36, 111-137.

Bekada, A.M.A., Benakriche, B., Hamadi, K., Bensoltane, A., 2008. Modelling of effect of water activity, pH and temperature on the growth rate of *Mucor racemousus* isolated from soft camembert cheese. World J. Agri. Sci. 4(6), 790-794.

Bennet, J.W., Wunch, K.G., Faison, B.D., 2002. Manual of environmental microbiology: Use of Fungi Biodegradation. 2nd Ed. ASM Press. Washington D.C.,

pp. 960-971.

Bhatnagar, D., Cleveland, T.E., Payne, G.A., 2000. Encyclopedia of food microbiology. Academic Press. London, pp. 72-79.

Boening D.W., 2000. Ecological effects, transport, and fate of mercury: a general review. Chemosphere 40: 1335-1351.

Brown M.T. and Hall I.R., 1990. Metal tolerance in fungi. In Shaw J, ed. *Heavy metal tolerance in plants: evoltitionary aspects.* Boca Raton: CRC Press, 95-104.

Cai, L., Jeewon, R., Hyde, K.D., 2005. Phylogenetic evaluation and taxonomic revision of Schizothecium based on ribosomal DNA and protein coding Genes. Fungal Diversity. 19, 1-21.

Cenkci S., Ildiz M.Y., Cigerci I.H.I., Konuk M., Bozdag A., 2009. Toxic chemicals induced genotoxicity detected by random amplified polymorphic DNA (RAPD) in bean (*Phaseolus vulgaris* L.) seedlings. Chemosphere 76: 900–906.

Crane, S., Husar M, Dighton J, Barkay T., 2011. The response of saprotrophic fungi from forest soil to mercury tolerance and community diversity. Forest and mercury: Defining the connection. Halifax 2011 Prosiding. Canada.

Das, S.K., Das A.R., Guha A.K., 2007. A study on adsorption mechanism of mercury on *Aspergillus versicolor* biomass. Environ. Sci. Technol. 41, 8281–8287.

Dastoor, A.P., Larocque, Y., 2004. Global circulation of atmospheric mercury: a modelling study. Atmosphere Environ. 38, 147-161.

Dhawale, S.S., Lane, A.C., Dhawale, W., 1996. Effect of mercury on the white rot fungus *Phanerochaete chrysosporium*. Bull. Environ. Contam. Toxicol. 56, 825-832.

Domsch, K.H., Gams, W., Anderson, T., 1993. Compedium of soil fungi Vol.2, Academic Press, London.

Evanko, C.R. and Dzombak, D.A., 1997. Remediation of metals-contaminated soil and groundwater. http://www.cluin.org/download/toolkit/metals.pdf. Pittsburg, PA.

Ezzourhi, I., Castro, E., Moya, M., Espinola, F., Lairini, K., 2009. Heavy metal tolerance of filamentous fungi isolated from polluted sites in Tangier, Morocco. J. Microbiol. Research. 3, 35-48.

Fernandez-Martinez, R., Loredo, J., Ordonez, A., Rucandio, M.I., 2005. Distribution and mobility of mercury in soils from an old mining area in Mieres, Asturias (Spain). Sci. The Total Environ. 346(1-3), 200-212.

Frostegard, A., Tunlid, A., Baath, E., 1993. Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals**.** Appl. Environ. Microbiol., 59 (1993), pp. 3605–3617.

Gadd G.M., 1986. The response of fungi toward heavy metals. In: Herbert RA, Codd GA, eds. Microbes in extreme environments. London: Academic Press, 83-110.

Gadd, G. M. 1992. Microbial control of heavy metal pollution, p. 59-88. In J.C. Fry, G.M. Gadd, R.A. Herbert, C.W. Jones, and I.A. Watson-Craik (ed.), Microbial control of Pollution. Cambridge University Press, Cambridge, UK

Gadd, G.M., 1993. Interaction of fungi with toxic metals. New Phytology. 124, 25-60.

Gadd, G.M., 2001. Microbial metal transformations. The J. Microbiol. 39(2), 83-88.

Gibson, A.M., Baranyi, J., Pitt, J.I., Eyles, M.J., Robert, T.A., 1994. Predicting fungal growth: the effect of water activity on *Aspergillus flavus* and related species. Intl. J. Food Microbiol. 23, 419-431.

Giller, K.E., Witter, E. and McGrath, S.P. (1998) Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: A review. Soil Biol. Biochem. 30, 1389-1414.

Gudbrandsen M., Sverdrup L.E., Aamodt S., Stenersen J., Short-term preexposure increases earthworm tolerance to mercury, Eur. J. Soil Biol. 43 (2007) 261–267.

Hansen, C.L. and Stevens, D.K. (1992), "Biological and Physio-Chemical Remediation of Mercury-Contaminated Hazardous Waste," Arsenic and Mercury: Workshop on Removal, Recovery, Treatment and Disposal, pp. 121-125.

Hedayati, M.T., Pasqualotto, A.C., Warn, P.A., Bowyer, P., Denning, D.W., 2007. *Aspergillus flavus*: Human pathogen, allergen and mycotoxin producer. Microbiology. 153, 1677-1692.

Hillel D., Hatfield JL, Powlson DS, Rosenzweig C, Scow RM, Singer MJ, Sparks DL., 2004. Encyclopedia of soils in the environment. 1st Ed. Academic Press. NY. USA

Horn, J.M., Brunke, M., Deckwer, W.D., and Timmis, K. N. (1992), "Development of Bacterial Strains for the Remediation of Mercurial Wastes," Arsenic and Mercury: Workshop on Removal, Recovery, Treatment and Disposal, EPA/600/R-92/105, pp. 106-109.

Iqbal, Z., Pervez, M.A., Ahmad, S., Iftikhar, Y., Yasin, M., Nawaz, A., Ghazanfar, M.U., Dasti, A.A., Saleem, A., 2010. Determination of minimum inhibitory concentrations of fungicides against fungus *Fusarium mangiferae*. Pak. J. of Botany. 42(5), 3525-3532.

Iram, S., Ahmad, I., Stuben, D., 2009. Fungal tolerance to heavy metals. Pak. J. of Botany. 41(2), 885-895.

Israr M., Sahi S.V., 2006. Antioxidative responses to mercury in the cell cultures of *Sesbania drummondii*, Plant Physiol. Biochem. 44:590–595.

Jasper DA: Bioremediation of agricultural and forestry soils with symbiotic micro-organisms. Aust. J. Soil Res., 32:1301-1319.

Joo, J.H., Hussein, K.A., 2012. Heavy metals tolerance of fungi isolated from contaminated soil. Korean J. of Soil Sci. Fert., 45(4), 565-571.

Kaçar, Y., Arpa, C., Tan, S., Denizli, A., Genç, O., Arıca, M.Y., 2002. Biosorption of Hg(II) and Cd(II) from aqueous solutions: comparison of biosorptive capacity of alginate and immobilized live and heat inactivated *Phanerochaete chrysporium*. Process Biochem. 19(37), Issue 6, 601-610.

Kapoor A, Viraragavan T. Fungal biosorption-An alternative treatment option for heavy metal bearing wastewaters: A Review. *Biores Technol* 1999:**70**:95-104.

Karunasagar, D., Arunachalam, J., Rashmi, K., Naveena-Lavanya-Latha, J., Maruthi-Mohan, P., 2003. Biosorption of inorganic and methyl mercury by a biosorbent from *Aspergillus niger*. World J. Microbiol. Biotechnol. 19, 291-295.

Knight, B., McGrath, S.P., Chaudri, A.M., 1997. Biomass carbon measurements and substrate utilization patterns of microbial populations from soils amended with cadmium, copper, or zinc. Appl. Environ. Microbiol., 63 (1997), pp. 39–43

Li, P., Feng, X.B., Qiu, G.L., Shang, L.H., Li, Z.G., 2009. Mercury pollution in Asia: A review of the contaminated sites. J. Hazard. Mater., 168, 591-601.

Martinez-Juarez, V.M., Cardenas-Gonzalez, J.F., Torre-Bouscoulet, M.E., Acosta-Rodriguez, I., 2012. Biosorption of mercury (II) from aqueous solution onto fungal biomass. Bioinorganic Chem. and Appli. Vol. 2012. DOI:10.115/2012/156190.

May, L.A., Smiley, B., Schmidt, M.G., 2001. Comparative denaturing gradient gel electrophoresis analysis of fungal communities associated with whole plant corn silage. Canadian J. Microbiol. 47(9), 829-841.

Means, J.L. and Hinchee, R.E., 1994, Emerging technology for bioremediation of metals. Lewis Publishers. Boca Raton. FL.

Messaccesi, G, Romero, M.C., Cazau, M.C., Bucsinszky, A.M., 2002. Cadmium removal capacities of filamentous soil fungi isolated from industrially polluted sediment, in La Plata (Argentina). World J. Microbiol. Biotechnol. 18, 817-820.

Müller, A.K., Westergaard, K., Christensen, S., Sørensen, S.J., 2001. The effect of long-term mercury pollution on the soil microbial community. FEMS Microbiology Ecology 36 11-19.

Pandey, A.K., Jamaluddin, Awasthi, A.K., Pandey, A., 2013. Biosorption potential of indigenous fungal strains for municipal solid waste leachate management in Jabalpur city. J. Environ. Sci. Comp. Sci. Eng. Technol. (JECET); March - May 2013; 2(2), 385-393.

Patra M., Bhowmik N., Bandopadhyay B., Sharma A., 2004. Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance, Environ. Exp. Bot. 52: 199‑223.

Patra M. and Sharma A., 2000. Mercury toxicity in plants, Bot. Rev. 66: 379–422.

Pennanen, T., Frostegard, A., Fritze, H., Baath, E., 1996. Phospholipid fatty acid composition and heavy metal tolerance of soil microbial communities along two heavy metal-polluted gradients in coniferous forests. Appl. Environ. Microbiol., 62 (1996), pp. 420–428

Pietikäinen, J., Petterson, M., Bååth, E., 2005. Comparison of temperature effect on soil respiration and bacterial and fungal growth rates. FEMS Microbiol. Ecol. 52,49–58.

Puglisi I., Faedda R., Sanzaro V., Lo Piero AR., Petrone G., Cacciola SO., 2012. Gene 506(2): 325-330.

Ranjard L., Richaume A., Jocteur-Monrozier L., Nazaret S., 1997. Response of soil bacteria to Hg(II) in relation to soil characteristics and cell location, FEMS Microbiol. Ecol. 24: 321–331.

Ray, S., Ray, M.K., 2009. Bioremediation of heavy metal toxicity-with special reference to chromium. J. Medical Sci. 2, 57-63.

Rittmann BE, Whiteman R., 1994. Bioaugmentation: a coming of age. Wat. Int, 1:12-16.

Robinson J.B. and Touvinen O.H., 1984. Mechanism of microbial resistance and detoxification of mercury and organomercury compounds: Physiological, biochemical and genetic analysis. Microbiological Review. June 1984, p. 95-124.

Ross, I. S. 1975. Some effects of heavy metals on fungal cells. Br. Mycol. Soc. 64:175-193.

Scheidegger, K.A., Payne, G.A., 2003. Unlocking the secret behind secondary metabolism: a review of *Aspergilus flavus* from pathogenicity to functional genomic. J. Toxicol. 22, 423-459.

Schroeder, W.H., Munthe, J., 1998. Atmospheric mercury – an overview. Atmospheric Environ. 32, 809-822.

Schuster, E., 1991. The behaviour of mercury in the soil with special emphasis on complexation and adsorption process – a review of the literature. Water Air & Soil Poll. 56, 667-680.

Selin, N.E., 2009. Global biogeochemical cycling of mercury: a review. Annual Rev. Environ. Res. 34, 43-63.

Sharma A., Talukder G., Sharma A., 1990. Advances in Cell and Chromosome Research, Aspect Publications Ltd., West Yorkshire, pp. 197–213.

Shim, S.M., Oh, Y.H., Lee, K.R., Kim, S.H., Im, K.H., Kim, J.W., Lee, U.Y., Shim, J.O., Shim, M.J., Lee, M., Ro, H.S., Lee, H.S., Lee, T.S., 2005. Culture Conditions Affecting the Optimal Mycelial Growth of *Cystoderma amianthinum*. Mycobiol. 33(1), 15-18.

Stein ED, Cohen Y, Winer AM. 1996. Environmental distribution and transformation of mercury compounds. Critical Review in Environ Sci Technol 26(1):1-43.

Steinnes, E., 1995. Heavy metals in soils: Mercury. In Alloway, B.J. (Ed.). Chapman & Hall. London. pp. 245-259.

Thippeswamy, B., Shivakumar, C.K., Krishnappa, M., 2012. Bioaccumulation potential of *Aspergillus niger* and *Aspergillus flavus* for removal of heavy metals from paper mill effluent. J. Environ. Biol. 33, 1063-1068.

Tieen-Sears, M.E., Martin, S.M., and Volesky, B., 1984. Propagation of Rhizopus javanicus biosorbent. Appl. Environ. Microbiol. 48:137 -141.

United Nation Environment Programme (UNEP), 2013. Global Mercury Assessment 2013: Sources, Emissions, Releases and Environmental Transport. Available at: http://www.unep.org/PDF/PressReleases/REPORT_Layout11.pdf. Date accessed: 12 January 2014.

Valix, M., Tang, J.Y., Malik, R., 2001. Heavy metal tolerance of fungi. Mineral Eng. 14(5), 499-505.

Valsecchi, G., Gugliotti, C., Farini, A., 1995. Microbial biomass, activity, and organic matter accumulation in soils contaminated with heavy metals. Biol. Fertil. Soils 20, 253–259.

Vogel, T.M., 1996. Bioaugmentation as soil bioremediation approach. Current Opinion iin Biotechnology, 7:311-316.

Vrable, P., Schinagl, C., Burgstaller, W., 2009. Growth kinetics of filamentous fungi in batch culture: textbook knowledge and reality. Mycoses. 52(S), 430.

Wang, J., Feng, X., Anderson, C.W.N., Xing, Y., Shang, L., 2012. Remediation of mercury contaminated sites – A review. J. Hazard. Mater. 221–222 (2012), 1– 18.

Zafar, S., Aqil, F., Ahmad, I., 2007. Metal tolerance and bio sorption potential of filamentous fungi isolated from metal contaminated agricultural soil. Biores. Technol. 98, 2557-2561.

RESPONSE TO COMMITTEE MEMBERS REVIEWS

1. Prof. Masahiko Sekine

No.	Slide	Comment	Answer	Revised slide/thesis
1.		Try to be more focus on the reason why bioremediation method is chosen comparing to other method or other microorganism (bacteria).	It has been done as suggested	Slide 5
2.	Slide 4, 28	The mercury problem is too general. Brings up the Indonesia problem of mercury and how harmful it is for human and how potential of using this fungus to solve such problem.	It has been done as suggested	Slide 12-13
3.	Slide 19	Find the better reason of why *Aspergillus* is used for mercury problem	It has been done as suggested	Slide 16
4.		Make a clear explanation of the figure (title, axis) so that easier to understand.	It has been done as suggested	
5.	Slide 46	Put the caption like on slide 44	It has been done as suggested	Slide 48
6.	Slide 47	Simplify the explanation about the mechanism (biosorption) supported by sufficient reference	It has been done as suggested	Slide 49

2. Prof. Masakazu Niinae

No.	Slide	Comment	Answer	Revised slide/thesis
1.		Show only the biosorption mechanism using fungi or possibility of the mechanism	It has been done as suggested	Slide 49
2.	Slide 44	Put the CFU explanation here.	It has been done as suggested	Slide 46

3. Assoc. Prof. Eiichi Toorisaka

No.	Slide	Comment	Answer	Revised slide/thesis
1.		Explain about the basic thinking about how to maintain the contaminated sites after bioremediation.	It has been done as suggested	Slide 50

4. Assoc. Prof. Takaya Higuchi

No.	Slide	Comment	Answer	Revised slide/thesis
1.	Slide 11	Put the reference source.	It has been done as suggested	
2.		Explain about the HSD	It has been done as suggested	Slide 43
3.		Use the standard unit in all slides	It has been done as suggested	