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HEAVY METAL TOLERANCE AND OXALATE CRYSTALLIZATION BY WOOD-ROTTING FUNGI

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Industrial Microbiology

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Field of study	Industrial Microbiology
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เบญจวรรณ แก้วด้วง: การทนต่อโลหะหนักและการเกิดผลึกออกซาเลตโดยรา่อยสลายเนื้อไม้ (HEAVY METAL TOLERANCE AND OXALATE CRYSTALLIZATION BY WOOD-ROTTING FUNGI) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.ประกิตติ์สิน สีหนนทน์, 219 หน้า.

งานวิจัยนี้ได้คัดเลือกหารา่อยสลายเนื้อไม้ที่มีความสามารถในการทนต่อโลหะหนักและสามารถกำจัดโลหะหนักโดยการตกตะกอนให้อยู่ในรูปของผลึกโลหะออกซาเลตคริสตัล จากการศึกษาพบว่าจากจำนวนราทั้งหมด 60 ไอโซเลต มีเพียง 5 ไอโซเลต โดยเป็นราบราวน์รอท 3 ไอโซเลต คือ MR40, KYO และ WR4 และราไวท์รอท 2 ไอโซเลต คือ WR3 และ WR5 ที่สามารถทนต่อโลหะหนักและสร้างผลึกของโลหะหนักได้บนอาหารเลี้ยงเชื้อที่มีการเติมโลหะหนักที่ความเข้มข้นแตกต่างกัน โดยเฉพาะอย่างยิ่งราบราวน์รอท ไอโซเลต KYO สามารถที่จะเจริญและผลิตผลึกได้เป็นจำนวนมากบนอาหารเลี้ยงเชื้อที่มีโลหะหนักความเข้มข้นสูง โดยผลึกที่สร้างโดยรา่อยสลายเนื้อไม้นั้นจะมีองค์ประกอบหลักสำคัญคือกรดออกซาลิค ซึ่งเป็นการบ่งชี้ว่ากรดออกซาลิคนั้นเป็นสารเมตาโบไลท์สำคัญที่รากลุ่มนี้ใช้ในการตอบสนองต่อความเป็นพิษของโลหะหนัก การผลิตกรดออกซาลิคโดยรา่อยสลายเนื้อไม้นั้นสามารถทำให้เกิดการเปลี่ยนรูปของโลหะหนักของ ซิงค์ซัลเฟต เปลี่ยนรูปเป็น ซิงค์ออกซาเลตไฮเดรต ($C_2O_4Zn.2H_2O$), คอปเปอร์ซัลเฟต เปลี่ยนรูปเป็น คอปเปอร์ออกซาเลตไฮเดรต ($C_2CuO_4.xH_2O$), แคดเมียมซัลเฟต เปลี่ยนรูปเป็น แคดเมียมออกซาเลตไฮเดรต ($C_2CdO_4.2.5H_2O$ และ $C_2CdO_4.3H_2O$), และ เลดไนเตรต เปลี่ยนรูปเป็น เลดออกซาเลต (PbC_2O_4) โดยโลหะหนักที่อยู่ในรูปนี้จะไม่ละลายน้ำและส่งผลกระทบเพียงเล็กน้อยต่อการเจริญของรา

ผลกระทบของโลหะหนักต่อการเจริญของราและจากการสะสมโลหะหนักโดยรา่อยสลายเนื้อไม้พบว่าโลหะหนักไม่ได้ทำให้เกิดการลดลงของพีเอชหรือการผลิตกรดอินทรีย์ นอกจากนี้ยังพบว่าการเพิ่มความเข้มข้นของโลหะหนักเป็นสาเหตุทำให้การเจริญของเส้นใยลดลงในขณะที่มวลชีวภาพของรามีค่าเพิ่มขึ้นอย่างมีนัยสำคัญ และจากการวิเคราะห์หาปริมาณโลหะหนักในเส้นใยราที่เจริญบนอาหารเลี้ยงเชื้อที่เติมโลหะหนัก พบว่าการสะสมโลหะหนักในเส้นใยราจะเพิ่มมากขึ้นเมื่อความเข้มข้นของโลหะหนักที่เติมลงในอาหารเลี้ยงเชื้อนั้นเพิ่มมากขึ้น งานวิจัยนี้ได้แสดงให้เห็นว่ารา่อยสลายเนื้อไม้ที่นำมาใช้ในงานวิจัยนี้สามารถที่จะทนต่อโลหะหนักที่ความเข้มข้นสูงและสามารถกำจัดโลหะหนักโดยการตกตะกอนให้อยู่ในรูปของผลึกโลหะออกซาเลตได้ นอกจากนั้นงานวิจัยนี้ยังแสดงให้เห็นว่าเชื้อชนิดนี้สามารถที่จะดูดซับสะสมโลหะหนักไว้ในส่วนของเส้นใยได้ ดังนั้นจึงสมควรเป็นอย่างยิ่งที่จะนำมวลชีวภาพของราชนิดนี้ไปประยุกต์ใช้เป็นตัวดูดซับโลหะหนัก ซึ่งนอกจากจะช่วยในการบำบัดโลหะหนักแล้วยังช่วยในการดึงโลหะหนักที่มีค่ากลับมาได้อีกด้วย

ภาควิชา.....จุลชีววิทยา..... ลายมือชื่อนิสิต.....เบญจวรรณ แก้วด้วง.....

สาขาวิชา.....จุลชีววิทยาทางอุตสาหกรรม..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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BENJAWAN KAEWDOUNG: HEAVY METAL TOLERANCE AND OXALATE CRYSTALLIZATION BY WOOD-ROTTING FUNGI. THESIS ADVISOR: ASSOC. PROF.PRAKITSIN SIHANONTH, Ph. D., 219 pp.

This study was conducted to select wood-rot fungal strains which showed high metal tolerance and were able to remove heavy metals by precipitation as metal oxalate crystals. The result showed that among sixty fungal isolates, only five isolates which included three brown-rot fungal isolate MR40, KYO and WR4, and two white-rot fungal isolate WR4 and WR5, were able to tolerate and produce the metal crystals in the agar medium amended with various concentrations of heavy metals. Especially brown-rot fungal isolate KYO was able to grow at high concentration of heavy metals and produced numerous metal crystals. Metal crystals formed by wood-rotting fungi consist mainly of oxalic acid which indicated that oxalic acid as an important metabolite elaborated in the response of wood-rotting fungi to toxic metal stress. Oxalate production could result in the transformation of zinc sulfate into zinc oxalate hydrate ($C_2O_4Zn.2H_2O$), copper sulfate into copper oxalate hydrate (moolooite)($C_2CuO_4.xH_2O$), cadmium sulfate into cadmium oxalate hydrate ($C_2CdO_4.2.5H_2O$ and $C_2CdO_4.3H_2O$), and lead nitrate into lead oxalate (PbC_2O_4), which were resistant to further solubilization and less inhibitory effect on the fungal growth.

An experiment was conducted to determine the effect of heavy metals on fungal growth and heavy metal accumulation by wood-rotting fungi. The result showed that the presences of the metal compounds did not stimulate the pH reduction and/or acid production. Moreover, increasing concentrations of heavy metal compounds in the culture medium caused a significant decrease in the radial growth of the most fungal isolates, whereas the fungal biomasses were significantly increased. The analysis of metal content in the fungal mycelia cultivated on media containing heavy metals showed that the accumulated heavy metals significantly increased when the concentrations of heavy metals increased. These results provide the evidence that these wood-rotting fungi could tolerate high concentrations of heavy metals and able to remove heavy metals from the metal-amended media by precipitation as metal oxalate crystals. Moreover, these fungi were capable to accumulate heavy metals within their biomass during immobilization of the soluble metal compounds therefore fungal biomasses should be applied as biosorbent for heavy metals which may provide potential for metal removal and recovery of valuable elements.

Department: .....Microbiology..... Student's Signature : Benjawan Kaewdoung

Field of study: ....Industrial Microbiology..... Advisor's Signature : Prakitsin Sihanonth

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CHAPTER I

INTRODUCTION

Heavy metal pollution represents an important environmental problem because of the toxic effects of metals, and their accumulation throughout the food chain that could lead to serious ecological and human health problems. The problems with metal contamination were particularly pronounced in localities where industrial exploitation had lead to accumulation of extreme concentrations of these substances, like the surroundings of smelters, tanneries, waste treatment plants, or mining sites. The concentrations of heavy metals in such places were high, and their influence on ecosystems obvious (Nordgren et al., 1983; Griller et al., 1998). This was confirm from previous studies which reported that the upper parts of the Pilcomayo River, South America were strongly affected by the release of mine tailings from the Potosi mines where mean concentrations of lead, cadmium, copper, and zinc in water, filtered water, sediment, and chironomid larvae were up to a thousand times higher than the local background levels (Smolders et al., 2003). Moreover, Lim et al. (2009) found that mine tailings from an abandoned metal mine in Korea contained much higher concentrations of arsenic and heavy metals [e.g., arsenic: 67,336, Fe: 137,180, copper: 764, lead: 3,572, and zinc: 12,420 (mg/kg)] than the Korean soil contamination criteria. In Thailand, the problem of heavy metal contamination is found in various region of country such as In Mae-Sot District, Tak Province was contaminated with high levels of cadmium. Through, the studies found that the rice fields and rice grains in this area had the high concentrations of cadmium (Simmons et al., 2005). Moreover, people that lived in this area had the high levels of cadmium in their urine and blood, suspected to have come from eating cadmium-contaminated rice (Swaddiwudhipong et al., 2007). Unlike degradable organic contaminants and even short-lived radionuclides that could become less toxic over time, metals could be considered “conservative” because they were not decomposed in the

environment (Fomina et al., 2005b). In recent years, research on heavy metal remediation focused on bioremediation technologies rather than physical and chemical methods of metal remediation such as vitrification, ion exchange, chemical precipitation and solidification/stabilization, which were expensive, inefficient and eco-unfriendly. One environmentally-friendly option of dealing with such metal contamination was through bioremediation using metal-tolerant wood-rotting fungi, which were of high efficiency and low cost compared to chemical extraction processes (Kartal and Imamura, 2003). Many wood-rotting fungi were known for their remarkable ability to withstand stress induced by toxic metals and could employ numerous mechanisms and strategies in detoxification, including enhanced metal efflux, suppressed influx, extracellular metal sequestration and precipitation, metal binding to cell walls, intracellular sequestration, and complexation that could alleviate the toxicity of heavy metals (Gadd,1993). However, certain mechanisms were generally based on metal immobilization, e.g. through the production of intracellular and extracellular chelating compounds such as organic acids and metal-binding peptides (Baldrian, 2003; Gadd, 2007a). Metal immobilization by insoluble metal oxalate formation was a process of marked environmental significance both regarding fungal survival and metal detoxification (Dutton and Evans, 1996; Gadd, 1999). The formation of oxalates containing potentially toxic metals might provide a mechanism whereby oxalate producing fungi could tolerate environments containing high concentrations of toxic metals (Sayer and Gadd, 1997; Fomina et al., 2005c; Gadd, 2007b). Moreover, most metal oxalates produced by wood-rotting fungi were immobile and resistant to further solubilization, so its not released heavy metals to the environment (Morris and Allen, 1994). However, the study of heavy metal remediation by wood-rotting fungi in Thailand has not been studied yet.

Therefore, the main objectives of this study are as follow:

1. To select fungal strains which show high metal tolerance and able to remove heavy metals from the metal-amended media by precipitation as metal oxalate crystals (biominerals).
2. To study the effect of heavy metals on fungal growth and determine heavy metal accumulation by wood-rotting fungi.

CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Heavy metals

Heavy metals were defined as metals having density more than 5 g/cm^3 (Elmsley, 2001). They may include both essential and non-essential metals. For organisms, essential metals such as copper, zinc, iron, cobalt, manganese, molybdenum, selenium etc., play vital role as co-factor in enzyme functions, essential to the activity of antioxidant enzymes like glutathione peroxidase, required for several enzymes such as carboxypeptidase and required for component of many redox enzymes, including cytochrome c oxidase (Voet and Voet, 2004), while non-essential metals such as cadmium, mercury, nickel, lead, antimony, tin and titanium were not at all required by organisms, instead they interfere with the function of essential metals and enzymes. In human, for example, ingestion of lead can interfere with the absorption of iron in the intestine thereby causing an iron deficiency. The lead can also increase copper and calcium nutrient deficiencies that already exist (Fergusson, 1990).

The supraoptimal level of essential heavy metals and higher levels of non-essential heavy metals, both cause toxicity and their increasing concentration in environment pose threat to living systems. There were ranges of concentrations of bioavailable, yet potentially toxic, essential metals/metalloids that were needed in micro-quantities by critical organs and for biochemical processes. When there is an excess or a deficiency in the diet of one or more than one of these elements over time, an organism can develop an abnormal condition, disease, or even die. For example, a deficiency of Se in human, especially young children, may be the cause of congestive heart failure (Keshan disease). On the other hand, ingestion of excess amounts of Se can result in acute vascular disruption and hemorrhaging and chronic dermatitis, hair loss, jaundice and caries (Fergusson, 1990). There were

threshold concentrations of metals in critical organs or biochemical systems of organisms that cause health of problems. In humans, for example, concentrations > 10 μg/g mercury in the liver or > 6 μg/g mercury in the brain can cause death whereas > 20 μg/dl mercury in the blood can cause chromosome damage (Fergusson, 1990). The build-up of a metal in an organism could be from ingestion of increase in available amounts in a food web or from bioaccumulation of low contents over a period of time. The result was the same chronic illness, incapacitation or death. Table 2.1 reviews the essential and non-essential roles of the focus metals.

Table 2.1 Toxic effected on ingestion of excess or deficiency amounts over time of essential micronutrient or non-essential potentially toxic metals in living populations. Compiled from Fergusson (1990) and Merian (1991)

Heavy metal	Essential/Non-essential	Toxic effection
Arsenic	Non-essential	Carcinogen
Beryllium	Non-essential	Toxic by inhalation of dust of or compounds.
Cadmium	Non-essential	Toxic in soluble and respirable forms; interferes with zinc in enzyme catalysis and key metabolic processes, and zinc bioavailability. Bioaccumulated at all toxic levels. Toxic to some plants at concentrations much lower than zinc, lead and copper. Is a carcinogen and is also teratogenic and embryoidal (Smith, 1999).
Cobalt	Essential	Located in active site of cobaltamine (vitamin B_{12}). Played an important role in biochemical reactions essential for life.

Table 2.1 (continued) Toxic effects from ingestion of excess or deficiency amounts over time of essential micronutrient or non-essential potentially toxic metals in living populations. Compiled from Fergusson (1990) and Merian (1991)

Heavy metal	Essential/Non-essential	Toxic effection
Chromium	Essential	Hexavalent via anthropogenic activities toxic. Essential at low concentrations but toxic at elevated levels. Chromium (VI) very soluble, toxic and a carcinogen. 50 µg/l in drinking water. Chromium (III) sparingly soluble and relatively non-toxic. Ilton (1999).
Copper	Essential	Necessary nutrient but excesses can produce toxicity.
Iron	Essential element	Could exert a strong influence on biological reactions.
Mercury	Non-essential	Poisoning over time gives neuropathy.
Manganese	Essential	-
Molybdenum	Essential	Molybdenosis in cattle
Nickel	Essential	Essential to plants, possibly essential to animal and humans.
Lead	Non-essentail	Toxic
Antimony	Non-essentail	-
Selenium	Essentail at trace concentrations	Toxic at elevated concentration. Selenate is more toxic to plants than selenite.
Zinc	Essential trace element	Essential for growth development and reproduction but excesses level could produce toxicity.

2.2 Sources of heavy metals

The United States Environmental Protection Agency (U.S. EPA) included 13 metals in their priority pollutants list: silver, arsenic, beryllium, cadmium, chromium, copper, mercury, nikel, lead, antimony, selenium, thallium, and zinc. Their natural origins were contrasted with their more diverse anthropogenic sources. While the natural sources were dominated by parent rocks and metallic mineral, the main anthropogenic sources were agricultural activities (fertilizers, animal manures, pesticides, etc.), metallurgical activities (mining, smelting etc.), energy production and transportation (leaded gasoline, battery manufacture, power plants, ect.), microelectronic products, and finally waste disposal. The heavy metals from anthropogenic sources were released into the environment by many human activities. They posed potential hazards to people, animals, plants and other organisms when contact the metal contaminated soil and surface or ground water. Table 2.2 gives the example of the multiple uses and products, which contain heavy metals.

Table 2.2 Anthropogenic sources and uses of heavy metals, through which they can be introduced into the environment

Heavy metal	Use of heavy metal
Silver	Mining, photographic industry.
Arsenic	Additive to animal feed, wood preservative (copper chrome arsenate), special glasses, ceramics, pesticides, insecticides, herbicides, fungicides, rodenticides, algicides, sheep dip, electronic components (gallium arsenate semiconductors, integrated circuits, diodes, infra-red detectors, laser technology), non-ferrous smelters, metallurgy, coal-fired and geothermal electrical generation, texile and tanning, pigments and anti-fouling paints, light filters, fireworks, veterinary medicine.
Beryllium	Alloy (with copper), electrical insulators in power transistors, moderator or neutron deflectors in nuclear reactors.
Cadmium	Nickel/cadmium batteries, pigments, anti-corrosive metal coatings, plastic stabilizers, alloys, coal combustion, neutron absorbers in nuclear reactors.
Chromium	Manufacturing of ferro-alloys (special steels), plating, pigments, texiles and leather tanning, passivation of corrosion of cooling circuits, wood treatment, audio, video, and data storage.
Copper	Good conductor of heat and electricity, water pipes, roofing, kitchenware, chemicals and pharmaceutical equipment, pigments, alloys.
Mercury	Extracting of metals by amalgamation, mobile cathode in the chloralkali cell for the production of sodium chloride and chloride ion from brine, electrical and measuring apparatus, fungicides, catalysts, pharmaceuticals, dental fillings, scientific instruments, rectifiers, oscillators, electrodes, mercury vapour lamps, X-ray tubes, solders.

Table 2.2 (continued) Anthropogenic sources and uses of heavy metals, through which they can be introduced into the environment

Heavy metal	Use of heavy metal
Nickel	As an alloy in the steel industry, electroplating, nickel/cadmium batteries, arc-welding, rods, pigments for paints and ceramics, surgical and dental protheses, molds for ceramic and glass containers, computer components, catalysts.
Lead	Antiknock agents, tetramethyllead, lead-acid batteries, pigments, glassware, ceramics, plastic, in alloys, sheets, cable sheathings, solder, ordinance, pipes or tubing.
Antimony	Type-metal alloy (with lead to prevent corrosion), in electrical applications, Britannia metal, pewter, Queen's metal, sterline, in primers and tracer cells in munition manufacture, semiconductors, flameproof pigments and glass, medicines for parasitic diseases, as a nauseant, as expectorant, combustion of fossil fuels.
Selenium	In the glass industry, semiconductors, thermoelements, photoelectric and photocells, and xerographic materials, inorganic pigments, rubber production, stainless steel, lubricants, dandruff treatment.
Thallium	Used for alloys (with lead, silver, or gold) with special properties, in the electronics industry, for infrared optical systems, as a catalyst, deep temperature thermometers, low melting glasses, semiconductors, supraconductors.
Zinc	Zinc alloy (bronze, brass), anti-corrosion coating, batteries, cans, PVC Stabilizers, precipitating gold from cyanide solution, in medicines and chemical rubber industry, paints, soldering and welding fluxes.

Reference: Bradl (2005)

2.2.1 Agricultural activities

2.2.1.1 Phosphatic fertilizers

Fertilizers were added to the soil in order to provide additional nutrients to crops or by changing soil condition such as pH to make nutrients more bioavailable. However, there contained various amounts of zinc, cadmium, and other heavy metals that originated from parent rock. The differences in heavy metal content were caused by impurities co-precipitated with the phosphates. Therefore, cadmium input into agricultural soils varies considerably according to the cadmium concentration of fertilizer used (Hutton and Symon, 1986; Rothbaum et al., 1986).

Li et al. (2001) reported that the application of cadmium containing phosphate fertilizers may be an important source of higher cadmium content in Hong Kong urban park soils.

2.2.1.2 Pesticides

Pesticides (i.e., herbicides, insecticides, fungicides, rodenticides) were widely used in high-production agriculture for the control of insects and diseases in fruit, vegetable, and other crops. Although metal-based pesticide were no longer in use, their former application lead to increased accumulation of heavy metals, especially of mercury from methyl mercurials, of arsenic and of lead from lead arsenate into soils and groundwater (Frank et al., 1976).

Gilpin et al. (2007) studied soils from historic orchards in four counties in Virginia and West Virginia, USA, which lead arsenate pesticides were widely used in apple orchards from1925 to 1955. They found that soils from these orchard sites had elevated arsenic and lead concentrations relative to reference sites, consistent with trends expected from lead-arsenate pesticide contamination. Moreover, these orchard soils also contained elevated

mercury and copper concentrations relative to reference sites, consistent with the co-use of other metal-based pesticides and mercury-based fungicides in orchards during this time period. Orchard and reference soils, and stream bottom sediments in the area, show an association of arsenic and heavy metals with both iron and aluminium concentrations, consistent with their retention in soils and sediments by sorption to clay and fine-grained Fe-Mn oxyhydroxide minerals

2.2.1.3 Biosolids

"Biosolids" refers to sewage sludge, animal wastes, dredged sediments from harbours and rivers, and some industrial wastes such as paper plup sludge (Adriano, 2001). These biosolids were an important group of soil amendments that used for soil enhancement due to their increased content in nutrients and organic matter. They contained nutrients, heavy metals, and pathogens such as *Escherichia coli.* The main heavy metals of concern in sewage sludge and dredged sediment were cadmium, zinc, copper, lead, selenium, molybdenum, mercury, chromium, arsenic, and nickel, which may depress plant yield or degrade the quality of food or fiber produced when applied to soils in excessive amounts. Concentrations of these elements depend on the type and amount of urban and industrial discharges into the sewage treatment system and on the amount added in the treatment system.

In a survey the concentration of arsenic, copper, nickel, lead, tin and zinc in sediments from Gulf of Mexico, east coasts of the United States, it was concluded that high levels of these metals were found at a variety of sites located near big cities (Daskalakis and O' Conner, 1995)

. Singh (1999) supports the concept that urban effluents have a great influence on the concentration and distribution of toxic heavy metals in river sediments. Urban activities were associated with the higher concentration of heavy metals such as the Yamuna River

sediments were classified as moderately to very highly pollute with chromium, nickel, copper, zinc, lead and cadmium in Delhi and Agra.

Adaikpoh et al. (2005) found that the sediment samples from River Ekulu in Enugu presence the high concentration of toxic elements such as manganese, chromium, cadmium, arsenic, nickel, and lead at concentration 0.256-0.389, 0.214-0.267, 0.036-0.043, 0.016-0.018, 0.064-0.067 and 0.013-0.017 mg/kg, respectively.

Marcussen et al. (2008) reported that the sediments of the To Lich and Kim Nguu rivers were polluted with potentially toxic elements (PTEs) with maximum concentrations of arsenic, cadmium, chromium, copper, nickel, lead, antimony and zinc were 73, 427, 281, 240, 218, 363, 12.5 and 1240 mg kg^{-1} d.w, respectively.

2.2.2 Industrial Activities

2.2.2.1 Mining

Most metal occurring in ore deposits had only low concentration. During the extraction process, large amount of waste rock were produced, which still contain traces of heavy metals that had not been picked out of the ore-bearing rock. The waste rock was usually disposed of in mine tailings or rock spoils. In the case of pyrite , this mineral will weather in the tailing due to oxidizing environmental conditions and thus create acid mine drainage. The acid conditions also mobilize heavy metals form the waste rock. This mobilization could cause fatal environmental and health problems through respiration, drinking and cooking contaminate water, and eating food grown on soils influenced by irrigation. Numerous examples were known especially for the heavy metals arsenic, cadmium, copper, mercury and lead (Siegel, 2002).

Smolders et al. (2003) reported that the upper parts of the Pilcomayo River, South America were strongly affected by the release of mine tailings from the Potosı´ mines where

mean concentrations of lead, cadmium, copper, and zinc in water, filtered water, sediment, and chironomid larvae were up to a thousand times higher than the local background levels.

Lim et al. (2009) found that mine tailings from an abandoned metal mine in Korea contained much higher concentrations of arsenic and others heavy metals [e.g., arsenic: 67,336, iron: 137,180, copper: 764, lead: 3,572, and zinc: 12,420 (mg/kg)] than the Korean soil contamination criteria.

2.2.2.2 Coal combustion

Coal combustion was used to generate electric power in coal–burning power plants, contribute heavily to the release of heavy metals in the environment especially into the atmosphere. Heavy metals in coal residues that could bioaccumulate and, therefore, could be critical in the food chain were arsenic, cadmium, boron, molybdenum, zinc and selenium (Adriano et al., 1980; Carlson and Adriano, 1993).

Vaisman and Lacerda (2003) reported that the present use of fossil fuel combustion for energy production is projected to increase from the present 14.5% to 29.6% of the total energy generation in Brazil in 2005. Most of this increase will be based on coal- and natural-gas-burning plants. The changes will result in an increase of about 100% in the average emissions (in tons $year^{-1}$) of arsenic (9.4 to 17.7), chromium (7.0 to 16.6) and mercury (2.4 to 4.1), 50% of cadmium (1.2 to 1.8), and 20% of nickel (101 to 123) and lead (23.3 to 29.9).

Keegan et al. (2006) determined the concentrations of arsenic, zinc, lead, copper, chromium, nickel and cadmium in the coal samples which were taken from the coal-burning power station in central Slovakia, and soil samples (n =113) were taken up to 12 km from the plant. They found that the concentrations of arsenic in coal were high (518 mg/g). Those of other heavy metals in general were low. Concentrations of soil arsenic were substantially raised in the near vicinity of the power station but decreased rapidly with distance from the source and ore, on average, over the guideline limit for residential soils in the United

Kingdom. Soil concentrations of other heavy metals were higher in the vicinity of the plant but none, overall was raised.

McConnell and Edwards (2008) reported that coal burning in North America and Europe was the likely source of toxic heavy metals in the Arctic after 1860. Although these results showed that heavy-metal pollution in the North Atlantic sector of the Arctic was substantially lower today than a century ago, contamination of other sectors might be increasing because of the rapid coal-driven growth of Asian economies.

2.2.2.3 Petroleum combustion

Large amounts of lead stemming from gasoline additives were released into the atmosphere from fuel burning for transportation. The greatest single source of air pollution in many countries is automobile exhaust. For example, nearly all the lead in the air in the United Kingdom comes from the exhaust gases of petrol engines (Thornton, 1991). Roadside soils and vegetation have been shown to be contaminated with various heavy metals primarily from auto emission, which include lead, zinc, cadmium, copper, and nickel (Motto et al., 1970). Contaminated zones may extend up to several hundred meters from the road depending on traffic intensity and location.

Li et al. (2001) found that the street dust in Hong Kong has highly elevated Zn concentration, particularly along the main trunk roads, which may come from the traffic sources, especially the vehicle tyres.

Akbar et al. (2006) reported that the contamination of lead, cadmium, copper, and zinc in the soils from the roadside verges in the northern England was higher as compared to their natural background levels, however, were below the critical maximum levels above which toxicity is possible. The highest concentrations were detected in the samples collected from the border zone of the verges and there was a trend of gradual decrease in the metal contents with the increasing distance from the paved roads.

2.2.2.4 Solid waste Disposal

Solid wastes were produced worldwide in immense amounts of thousands of millions of tons annually. The most important sources of heavy metals stem from wastes from industrial activities, especially energy generation, from mining, agricultural activities (animal manure), and domestic waste (e.g. batteries, tires, appliances, junked automobiles). These wastes were often disposed of without proper treatment at waste disposal sites, so the metals from these wastes can enter to the environment. (Bradl, 2005). Moreover, landfilling of municipal solid waste could lead to several metals including cadmium, copper, lead, tin, zinc being dispersed into soil, ground waters and surface waters in leachates if the landfill was not managed properly. Incineration of wastes could also lead to the emission of metal aerosols such as lead, cadmium and mercury if appropriate pollution control equipment was not installed (Alloway, 1995). So, the most toxic and concentrated metal-bearing solid wastes should be disposed of in a secure disposal site.

Samuding et al. (2009) reported that groundwater system at solid waste disposal site in Taiping, Perak showed significantly high levels of lead, manganese, zinc, iron, Cadmium, which was exceeded the maximum permissible concentration (MPC) as specified World Health Organization, WHO Standards for Drinking Water.

Karnchanawong and Limpiteeprakan (2009) evaluated the contents of heavy metals leached from spent household batteries in the municipal solid waste (MSW). They found that the direct disposal of spent household batteries into a MSW landfill in the acidogenic phase could increase the heavy metal contents in the landfill leachate.

2.3 Pathways of Heavy metal Access

In order to cause any effect in a living organism, heavy metals had to get into contact with this organism. There were three principal ways, through which this might happen. The first pathway was through the atmosphere or through atmospheric deposition to water and soil, the second was through drinking contaminated water or using it for cooking and crop irrigation, and the third was through accumulation in food web.

2.3.1 Respiration

Heavy metals could enter organism by respiration of natural and anthropogenic emissions. The most important sources of atmospheric emissions were from coal, smelters, and mineral processing, industrial manufacturing processes (e.g., chemicals, pulp and paper) and automobile exhaust emissions (Siegel, 2002). The respiration of metal pollutants through dust was one of the most serious threads to humans working in industrial workplaces. The most recognized health problems were " black lung " disease, silicosis, and radiation sickness in miners, and lung cancer in asbestos workers (Siegel, 2002). Table 2.3 lists some health problems arising from the respiration and contact of heavy metals with humans in the workplace.

2.3.2 Water

The second pathway of entering organisms was through drinking water contaminated with heavy metals, which could be ingested directly by drinking, or indirectly by using this water for cooking and irrigation. These contaminations could be both of natural and of anthropogenic origins. One should be aware that to date more than 30%of the world population does not have access to clean water for drinking, cooking, personal hygiene, and sanitation, which was threatening especially for infants and children (Bradl, 2005).

Das et al. (1996) reported that in West India and Bangladesh, more than 20 million people were estimated to be exposed to arsenic-contaminated drinking water. The source of arsenic was due to the geological condition of the underground strata.

Ivanova et al. (2003) reported that the waters from the region of the town of Panagjurishte (South Bulgaria), where copper ore mining and processing works were located, were mainly polluted with lead, copper and cyanides. The level of these metals in this region exceeded their maximum permissible concentrations for the country.

2.3.3 Food

The third direct route into the food web was through foods that have natural high contents of potentially toxic metals or that have bioaccumulated them in a growth environment. Plant uptake was one of the main path-ways through which metals enter the food chain. If soils contain high natural metal contents, were amended with metal-bearing sludges (e.g., cadmium, copper, lead and zinc), or irrigated with water contaminated with metals, then some plants will hyperaccumulate those metals, which results in polluted food crop and animal forage. Then the heavy metals were transferred through higher trophic levels to humans (Bradl, 2005).

Khan et al. (2008) studied the heavy metals in contaminated food crops which irrigated with wastewater in Beijing, China. They found that heavy metal concentrations in plants grown in wastewater-irrigated soils were significantly higher ($P \leq 0.001$) than in plants grown in the reference soil, and exceeded the permissible limits set by the State Environmental Protection Administration (SEPA) in China and the World Health Organization (WHO).

Zhuang et al. (2009) studied soils and food crops were contaminated with lead and cadmium in the vicinity of Dabaoshan mine (Guangdong, southern China). They found that the concentrations of heavy metals in the soils exceed the corresponding maximum

allowable concentration levels for agricultural soils in China. Rice grain could accumulate unacceptably high concentrations of lead and cadmium. The estimated dietary intakes of lead and cadmium from food crops grown in the contaminated soils exceeded the tolerable daily intake limits. Therefore, the crops which were grown in the vicinity of the Dabaoshan mine present a potentially serious health risk to the local inhabitants.

Similary, aquatic life form that bioaccumulate heavy metals from polluted waters and from contaminated sediments in a water column, or bottom sediments, could pass pollutants up the food chain. This can harm aquatic animal, predator mammals, and ultimately a human population (Siegel, 2002).

Javed (2005) found that the bed sediments in the River Ravi contaminated with iron, zinc, lead, nickel and manganese which related effluent discharging tributaries have been studied. The suspended matter and the soluble metallic ions had affected the quality of bed sediments significantly that resulted in the accumulation of metals in fish organs (gill, liver and kidney).

Ozturk et al. (2009) studied heavy metal levels in water, sediment and fish samples from Avsar Dam lake, in Turkey. The obtained results showed that the average values of iron in water samples were higher than the respective reference values for fresh water (national and international water quality guidelines). The analysis of heavy metals in sediments indicated that among the six heavy metals teste, iron was maximally accumulated, followed by nickel, copper, chromium, lead and cadmium. In the fish samples, cadmium, chromium, nickel and lead concentrations exceeded the tolerable values provided by international institutions.

Table 2.3 Heavy metals cause human diseases

Heavy metals	Diseases : Sources
Carcinogens which may occur in the workplace	
- Arsenic, cadmium, Chromium	- Human carcinogens
- Cobalt, lead, nickel	- Possible human carcinogens
- Arsenic, beryllium, cadmium, chromium	- Lung carcinogens include
Some reported cancers caused by or associated with certain occupations and industries	
- Arsenic	- Lung, skin: Pesticides, other
- Chromium	- Lung: Metals, welders
- Nickel	- Lung, nose: Metals, smelters, engineering
Substances linked to occupations liver disease	
- Arsenic	- Cirrhosis, angiosarcoma, hepatocellar carcinoma: Pesticide, wood, vinters, smelters
- Beryllium	- Granulomatous disease: Ceramics
Substances reported to have damaged the kidney in the workplace	
- Cadmium	- Nephrotoxicity: Welding
- Lead	- Nephrotoxicity: Chemicals, paint, batteries
- Mercury	- Nephrotoxicity: Chemicals, paints

Table 2.3 (continued) Heavy metals cause human disease

Heavy metals	Diseases : Sources
Possible factors influencing reproductivity outcomes based on experimental data	
- Arsenic	- Fetotoxic, teratogen, transplacental carcinogen:Agriculture, wood preserving
- Cadmium	- Spontaneous abortions, impaired implantation, teratogen, male and female damage: Engineering, chemicals, batteries, paints, smelting, mining
- Chromium	- Teratogen: Chemicals, engineering
- Lead	- Decreased fertility, fetotoxic, impaired, implantation, sperm damage, hormonal alteration
- Manganese	- Decreased fertility, impaired implantation
- Mercury	- Fetotoxic, teratogen, menstrual disorders: Chemicals, pesticides
- Selenium	- Fetotoxic
- Titanium	- Fetotoxic
Substances found in the workplace reported to have caused neurological damage	
- Arsenic	- Peripheral neuropathy: Metal production, pesticides
- Lead	- Encephalopathy and peripheral neuropathy

Table 2.3 (continued) Heavy metals cause human disease

Heavy metals	Diseases : Sources
- Manganese	- Encephalopathy, ataxia, later Parkinson disease-like symptoms occur, acute psychosis: Engineering, aircraft industry, steel, aluminium, magnesium, and cast iron production
- Mercury	- Tremor, weakness, peripheral neuropathy is uncommon, chronic exposure leads to ataxia, metal impairment: Chemicals, pharmaceuticals, dentistry, plastic, paper,
Substances known or associated with visual damage in the workplace	
- Lead	- Optical neuropathy: Foundry industry
- Mercury	- Cranial nerve palsies: chemicals
Substances reported to have caused immune system effects in the workplace	
- Nickel	- Hypersensitivity: metals engineering
Reported respiratory effects of certain workplace substances	
- Metals: Especially nickel, chromium, cobalt, and vanadium	-Occupational asthma, metal and engineering workers

Table 2.3 (continued) Heavy metals cause human disease

Heavy metals	Diseases : Sources
Substances known to be absorbed through or damaging to the skin in the workplace	
- Arsenic	- Skin cancer: agriculture, lead workers, dyers, copper smelters, brass makers, chemicals, textiles, painters, pesticide users
- Chromium	- Allergic contact dermatitis: Metals and engineering workers
- Nickel	- Allergic contact dermatitis: Metals, engineering, jewellery
Substances linked with cardiovascular toxicity	
- Arsenic	- Myocardial injury
- Antimony	- Hypertension
- Cadmium	- Hypertension
- Cobalt	- Myocardial injury
- Lead	- Myocardial injury, hypertension
Reported adverse effects of chemicals on the blood	
- Arsenic	- Aplastic anaemia: Glass, paints, enamels, pesticides, tanning agents
- Copper	- Red blood cells
- Lead	- Red blood cells, porphyria: General

Reference: Siegel (2002)

2.4 Heavy metal contamination in Thailand

In recent years, Thailand has experienced an impressive growth in population, industry and agriculture (Molle, 2002). The environmental con-sequences of this phenomenon concerned with the pollution impacts. Heavy metal pollution was one the most serious environmental problems in Thailand because of the toxic effects of metals, and their accumulation throughout the food chain that could cause serious problems to all organisms and human health. The problems with metal contamination were particularly pronounced in localities where industrial exploitation has lead to accumulation of extreme concentrations of these substances, like the surroundings of waste treatment plants, or mining sites. This evidence was agreed with the report from Pornpimol (1997) found that lead concentrations at Pattani River, Bannang Sata District were found to exceed the standards during 1986-1992 and 1996-1997 which the source of lead in this River were from tin mines in the watershed areas of Yala's Bannang Sata District. Other sources of lead were industries along Pattani Bay especially shipyards which use lead-based plumboplumbic oxide in ship building and repair work. Follow up studies investigated the high lead concentrations in the environment, such as in water sediment and marine flora, and the effect in that in children health. The condition was done by Arrykul and Kooptarnond (1993) showed high concentrations of lead over 3,333 mg in each gram of sediment which were taken from a tributary stream running through a mine dump in Ban Thamthalu, Bannang Sata District. The lead level decreased to only 32 mg per gram in sediment downstream, but increase again at a sampling station close to the Pattani Bay. Moreover, in 1994 it was found that seaweed species in Pattani Bay which was used as the local diet, Gracilaria fishery, contained at very high lead levels.

For other contaminate areas, the Map Ta Phut Industrial Estate in Rayong Province which released heavy metal industrial wastes in this areas at level beyond safety standards. A health study of villagers living in areas near the estate also found that the incidence of

cancers and leukaemia among the villagers was five times higher than the national rate. Another study showed that residents were also experiencing genetic defects (Janjira Pongrai, 2009). Rangsit University's Arpha Wangkiat has supported that water resources in the area were also contaminated by excess amounts of arsenic, cadmium, cobalt, chromium, copper, iron, manganese, nickel, lead and zinc. In short, these independent sources of data confirm that pollutants have reached a critical level, posing a serious health threat to residents (Kamol Sukin, 2007).

Cadmium is a by–product and released into the environment through zinc mining. Mae Tao River and Mae Tao Creek were the main sources of water supply used for agriculture, and has been affected from zinc mining. In Mae-Sot District, Tak Province is contaminated with high levels of cadmium. Through, studies have found high concentrations of cadmium in the rice fields and rice grains in this area (Simmons et al., 2005). People that live in this area have high levels of cadmium in their urine and have high levels of cadmium in their blood, suspected to have come from eating cadmium-contaminated rice (Swaddiwudhipong et al., 2007).

Krissanakriangkrai et al. (2009) determined the magnitude of cadmium pollutants in water, sediment, fish and shellfish collected from Mae-Toa creek which has been affected from zinc mining, and observed seasonal variations in the bioavailable cadmium concentrations in this surface water. They found that rainy months were found to have higher cadmium concentrations than during dry months. Elevated concentrations of cadmium in the rainy season may be due to agricultural and mining run off. Cadmium levels in the river sediment exceed the allowable standard. The highest concentrations of cadmium were detected in Swampeel (0.27 mg/kg wet weight). According to human health risk assessment the Hazard Quotient (HQ) from eating Swamp Eel was 1.3 which was higher than one, therefore, a health risk to humans can occur if consumption of a few large meals occurs over a very short period of time.

Prechthai et al.,2008 reported that Nonthaburi dumpsite in Thailand, was found Zn is the highest concentrated heavy metal compared to manganese, copper, chromium, cadmium, lead, nickel and mercury in the solid waste. From the sequential extraction, manganese, zinc and cadmium mostly found in reducible form, showed its susceptibility to be leached easily. The estimated individual contamination factor (*C*if), showed zinc with highest affinity to leach.

2.5 Heavy metal remediation techniques

Nowaday, a variety of methods for the remediation of both solid and liquid media such as water, groundwater, industrial wastewaters, soils, sediments and sludges were known. To give a general overview over different technologies it was most convenient to divide them into three major categories: first, physical methods that simply restrict access to the contamination through containment or removal; second chemical methods that attempted to alter contaminant speciation to either enhance mobility under various extraction scenarios or decrease mobility to reduce potential exposure hazards; and third, biological methods that attempted to use natural or enhanced biochemical processes to either increase contaminant mobility for extraction (e.g., bioleaching) or reduce mobility by altering metal speciation (e.g., bioaccumulation and biosorption) (Adriano, 2001).

2.5.1 Physical remediation

Physical remediation techniques include soil washing, encapsulation, vitrification, electrokinesis and carbon adsorption.

2.5.1.1 Soil washing

Soil washing or extraction was applied widely for the remediation of heavy metal-contaminated soils in Europe (Tuin and Tels, 1991) and this procedure was also applicable

for metal-contaminated soils. It was based on desorption or dissolution of metal from the soil inorganic and organic matrix during washing with acids and chelating agents. This method was increased in metal solubility and mobility to remove metals from contaminated soil which increase the risk for transport and redistribution of contamination to underlying soil and grown water. Moreover the high cost of chelating agents and choice of extractants makes this technology not feasible. This method is efficient in removing heavy metals from soils, despite the fact that it cannot reduce their toxicity (Mulligan et al., 2001).

2.5.1.2 Encapsulation

Encapsulation of contaminated areas was commonly used for remediation by containment or pollution prevention (Philip, 2001). This process that was employed for relatively small areas (e.g.,smelter site) and involves covering the site with an impermeable or very slowly permeable layer, such as polyethylene or compacted clay, to minimize percolation of water through the site. This technique is commonly used for remediation by containment or pollution prevention which similar to that used for closing landfills. Wastes or affected soils were covered with a polymeric substance, such as polyethylene or asphaltic bitumen (Chappell and Willitts, 1980). The limitation of this process is site specific and must be developed for each waste.

2.5.1.3 Vitrification

Vitrification was a solidification technique that involves heating of contaminated soils or mine spoil material by various means to the point of producing a melt that hardens into a glasslike material such as silica. The vitrified material had very low permeability and chemically inert, was resistant to weathering. Heating methods include natural gas-fired burners, electrical power current, and plasma centrifugal furnace where temperatures can rise to as much as 2000 ^{o}c. The vitrification process reduced the volume of the treated

material by 25 to 35% and this process can typically treat up to 1,000 tons of material in one melt setting. Vitrification was best suited for wastes that were difficult to treat, such as mixed wastes.

Disadvantages of vitrification (Freeman and Harris, 1995) were

- Vitrification were often not feasible cleanup methods due to the high costs of energy needed to complete the process
- The possible need for emission control for waste gases.
- Although vitrification process can treat soil saturated with water, however additional power is used to dry the soil prior to melting and may increase the cost of remediation by 10%.

2.5.1.4. Electrokinetics technique

Electrokinetics was an innovative technology that was aimed at separating and extracting heavy metals, radionuclides, and organic contaminants from saturated or unsaturated soils, sludges, sediment and groundwater (Cundy and Hopkinson,2005). The process in this technique involve passing a low intensity electric current between a cathode and an anode imbedded in the contaminated soil. Ions and small charged particles, in addition to water, were transported between the electrodes. Positive ions were attracted to the negatively charged cathode, and negative ions move to the positively charged anode. An electric gradient initiates movement by electromigration (charged chemicals movement), electro-osmosis (movement of fluid), electrophoresis (charged particle movement) and electrolysis (chemical reactions due to electric field). Metals as soluble ions and bound to soils as oxide, hydroxides and carbonates were removed by this method (Rodsand and Acar, 1995).

The removal of heavy metals from soils using electrokinetic technique has some limitations, which were:

- Removal efficiency is significantly reduced if soil contains carbonates and large rocks or large metal objects, rubble or other obstacles which can interfere with the process.
- The whole electrokinetic remediation process is highly dependant on acidic conditions during the application, which favours the release of the heavy metal contaminants into the solution phase.
- Acidic conditions and electrolytic decay can corrode some anode materials.
- During the electrokinetic remediation process, gaseous bubbles (O_2 and H_2) cover the electrodes. These bubbles were good insulators and reduce the electrical conductivity.
- After the electrokinetic remediation process, a white layer was observed on the cathode surface. This layer may be the insoluble salt and other impurities that were not only attracted to the cathode, but also inhibited the conductivity. (Hamed et al., 1991; Acar and Gale, 1995; Cundy and Hopkinson, 2005)

2.5.1.5 Carbon adsorption

Adsorption was the process by which the soluble substances from the water were attached to the surface of an adsorbent. Adsorbents include activated carbon, silica gel, zeolites and polymers. The most common and widely used adsorbent is activated carbon. Activated carbons were carbonaceous products, with their high surface area, high micro porous character and chemical nature of their surface, have made them potential adsorbents for the removal of heavy metals from industrial wastewater. The carbon adsorption process was controlled by the diameter of the pores in the carbon filter, which was classified by the size of the diameter of the pores, varies from micropores (2 nm), to

mesopores (2-50 nm), to macropores (greater than 50 nm). In water treatment, particles of the same size of the pores had potential to get stuck and retained by the carbon. Volatile organic chemicals, metals, and some non-polar inorganic chemicals were captured and held strongly to the surface of the carbon and were thus removed from the water (Hassler, 1974).

Disadvantage of carbon adsorption (Kleiner, 1997) were

- The adsorbent has to be regenerated after use and after the regeneration step, the activated carbon loses some 10% of its weight and 15% of its uptake capacity. These were also added costs of operation.
- Presence of other organic compounds which will compete for the available adsorption sites.

2.5.2 Chemical remediation

Chemical remediation techniques for the heavy metals removal in contaminated groundwater and wastewater include precipitation of dissolved metals, ion exchange, membrane filter processes such as micro- and ultrafiltration and reverse osmosis, and carbon adsorption. When dealing with contaminated soils, sediments, and sludges, solidification and stabilization process can be used.

2.5.2.1 Precipitation

Metals precipitation from contaminated water involves in the conversion of soluble heavy metal salt to insoluble salts that will precipitate. The precipitate could then be removed from the treated water by physical methods such as sedimentation and/or filtration. The heavy metal precipitation step is simple, involving the addition of chemical reagents to

adjust the pH of contaminated water. The desired pH was one in which the metals exhibit low (or minimum) solubility in water and therefore precipitate. This desired pH was dependent on the specific metal-reagent combination. Common reagents used include

- Alkalis such as lime, caustic soda, or magnesium hydroxide slurries to precipitate metal hydroxides (hydroxide process).
- Sulfides such as sodium sulfide or ferrous sulfide slurries to precipitate metal sulfides (sulfide process) (Freeman and Harris, 1995).

Disadvantage and some limitations of precipitation technique (Dabrowski et al., 2004) were

- The rates of precipitation were usually low in water with low metal content leading to an increase in the consumption of chemicals used.
- In precipitation systems contained more than one metal ion species, optimum removals may not occur for a given metal species when another metal has been treated for maximum removal.
- High concentration of oil and grease might result in a longer settling time for the precipitation due to a formation of emulsion.
- This technique was not economical because of in the precipitation were increase cost of land or lagoon and reagent-used.
- One major problem was the formation of large amounts of sediments containing heavy metal ions.

2.5.2.2 Ion exchange

Ion exchange is a reversible chemical reaction wherein an ion (an atom or molecule that has lost or gained an electron and thus acquired an electrical charge) in the solution is exchange with a similarly charged ion attached to an immobile solid particle (Remco, 1981). The synthetic organic resins were the predominant type used in this technique today

because an organic ion exchange resin is composed of high-molecular-weight polyelectrolytes that can exchange their mobile ions for ions of similar charge from the surrounding medium. When contaminated liquids were passed over the resins, the ions (i.e., cations and anions) in the resins and in contaminated materials were exchanged. The resins exchange hydrogen ions (H^+) for the positively charged ions (such as nickel. copper, and sodium) and hydroxyl ions (OH^-) for negatively charged sulfates, chromates and chlorides. After the resin capacity has been exhausted, resins can be regenerated for re-use (Kim et al., 2002).

Disadvantages and some limitations of ion exchange (Vilensky et al., 2002)

- Ion exchange resins were more expensive than biosorbents and high operating costs over long-term.
- Hazardous chemicals were required for regeneration of the ion exchange resin
- Treatable waste streams should not contain high solids or high organic level because solids will foul the resin column and cause treatment inefficiencies.
- Oil and grease in the groundwater might clog the exchange resin.
- The acidity or alkalinity of the incoming water might limit ion exchange capability. This can usually be controlled.

2.5.2.3 Membrane Filter Processes

Membrane filter process techniques were widely used in wastewater treatment. In this technique, wastewater were concentrated and/or purified by recover desirable byproducts from waste and allow water to be recycled. Filtration techniques were available for particle sizes between 1 mm and 0.1 μm (Cartwright, 1988). Depending on the pressure range, the following technologies can be applied: first, microfiltration in the 0.5 to 3 bar

pressure range, second, ultrafiltration in the 1 to 10 bar pressure range, third, reverse osmosis in the 20 to 100 bar pressure range, and finally, electrodialysis, which were uses an electrical field. In electrodialysis, anion or cation exchange resins were built into the polymer membranes to select positively or negatively charged ions. This method was used especially for heavy metal recovery.

Disadvantage of membrane processes (Sudilovskiy et al., 2007) were

- High energy requirement for treatment of concentrated solution
- Short life span of expensive membranes
- Affected by various chemical and pH
- Skilled operator required
- Expensive cost in wastewater treatment

2.5.2.4 Solidification and Stabilization

Solidification and stabilization were a proven technology for the treatment of metal-contaminated soils, sediments, and sludges by converting these contaminants into their least soluble, mobile, or toxic form through the use of inorganic binders such as cement, lime and fly ash, organic binders such as bitumen, and other additives. The form of the final product from S/S treatment could be a continuous solid mass such as a concrete-type material (in case of the Portland cement and fly ash, react with soil) and a hard concrete-like material (in case of the lime and silica, react with soil), which increased strength, reduce contaminant mobility and decreased the surface area exposed to leaching (U.S. EPA, 1997). However, US ACE (1994) indicated the disadvantages of this system. A disadvantage was that the solid mass resulting from lime-based solidification is porous, therefore leaching might be occurred. To prevent leaching sealed or placed in a secure landfill is required. Another one is sludge or waste containing organic could not be treated.

2.5.3 Bioremediation of heavy metals

Bioremediation was becoming the technology of choice for the remediation of hazardous organic and metallic residues or by-product contaminate in environments. This technology used in metabolic processes to degrade or transform contaminants, so that they remain no longer in harmful form. In some cases, the contaminant was the primary part of the metabolic process, acting as a main source of carbon and energy for the microbial cell. In others, it was transformed into a second substance, serves as a primary energy or carbon source. In case of metals, it is only the biotransformation process that was exploited widely as a bioremediation strategy.

The use of microorganisms for remediation of heavy metal in contaminated environments, had been receiving an increasing attention for a long time because of low cost, environmentally friendly and high efficiency compared to chemical and physical processes (Kartal and Immura, 2003). Many microorganisms including bacteria and fungi could be used effectively in removing heavy metals since some bacteria and fungi were high tolerant to toxic metals. In addition, many fungi could be extremely tolerant of toxic metals in comparison with other microbial groups (Schmidt and Ziemer, 1976; Gadd, 1993), because of several fungal species had developed a high resistance to heavy metals and developed a variety of mechanisms to remove ions, such as accumulation or biosorption of heavy metals by cell wall components and extracellular materials, chelation or precipitation by secreted metabolites such as enzyme or acid, and complexation with inner low molecular weight proteins (Kartal and Immura, 2003). Therefore, bioremediation using fungi was possible good method for removal of heavy metals from the contaminated-environment.

2.6 Interactions between toxic metals and fungi

Fungi might interact with toxic metals and metal-containing minerals in various ways depending on their tolerance, metal species and ability to influence the mobility of toxic metals. Certain mechanisms were divided into two mechanisms: (1) metal mobilization and (2) metal immobilization (Gadd, 2004).

2.6.1 Metal mobilization

Many species of fungi were capable of transforming insoluble metal compounds form rocks, minerals, soil, sediments and industrial wastes into soluble derivertives by a process of metal mobilization. This process could be achieved by chelation by excreated metabolites and siderophores, and methylation, which could result in volatilization (Gadd, 2001).

2.6.1.1 Chelation by metabolites and siderophores

1. Chelation by fungal metabolites

Several species of fungi were able to convert insoluble metal compounds, e.g. certain oxides and phosphates, into soluble forms by the excretion of protons and/or various metabolites, including organic acids (Burgstaller and Schinner, 1993). Organic acids provided a source of protons for solubilization and metal-chelating anions to complex the metal cation (Gadd, 1999, 2001; Burgstaller and Schinner, 1993; Gadd and Sayer, 2000). For example, citrate and oxalate could form stable complexes with a large number of metals. Many metal citrates were highly mobile and not readily degraded. Oxalic acid could also act as a leaching agent for those metals that form soluble oxalate complexes, including Al and Fe (Strasser et al., 1994).

Fungal solubilization of insoluble metal compounds appeared to be increasing biotechnological potential for metal recovery and reclamation from low grade ores and recycling of metals from industrial by-products (Burgstaller and Schinner, 1993). Moreover, solubilization phenomena could also have consequences for mobilization of metals from toxic metal-containing minerals, e. g. pyromorphite ($Pb_5(PO_4)_3Cl$) which can form in urban and industrially contaminated soils or solid wastes. Pyromorphite could be solubilized by phosphate-solubilizing fungi, with concomitant production of lead oxalate (Sayer et al. 1999). Some advantages of using fungi for such processes include tolerance to high concentrations of potentially toxic metals and the ability to leach metals in low or high pH environments.

There were several fungi being used for metal leaching. Humur et al. (2004) used wood-rotting fungi (brown-rot fungi) for remediation of copper, chromium and boron treated wood. The result obtained showed that oxalic acid produced by wood-rotting fungi play a key role in the leaching of metal from the treated wood.

2. Chelation by siderophores

Another process for interact with metal is siderophore-mediated metal solubilization. Siderophores were highly specific iron-binding molecules which were excreted by many microorganisms to aid iron assimilation. Although primarily produced as a means of obtaining iron, siderophores were also able to bind other metals such as magnesium, manganese, nickel, copper, chromium, gallium and radionuclides such as plutonium(IV) (Birch and Bachofen 1990). From this process, solubilized metals were adsorbed to the biomass and/or precipitated with biomass, which resulted in a complete decrease metal bioavailability (Diels et al., 1999).

2.6.1.2 Methylation

A number of microorganisms were able to generate volatile organometallic compounds by means of metal methylation reactions. These reactions were thought to be a microbial resistance mechanism to volatilize and thus remove the metal ions from their environment. Once well known example of volatilization was the methylation of selenium, in which the selenate and selenite ions were converted into the volatile selenium compounds, e.g. dimenthyl selenide, and dimethyl selenone (Bender et al., 1991). This method had also been used for insitu bioremediation of selenium-containing land and water at Kesterson Reservoir, California (Thompson-Eagle and Frankenberger, 1992).

However, it was likely that fungal solubilization activities may also release potentially toxic metal cations into the environment such as soil solution. Banks et al. (1997) reported that fungal activity in mining areas can leach significant levels of metal cations into water systems, which is hazardous to human health and the environment.

2.6.2 Metal immobilization

Toxic metal species, including radionuclide, could be bound, accumulated, and precipitated by fungi. Fungal biomass could act as a metal sink, either by metal biosorption to biomass (cell walls, pigments and extracellular polysaccharides), intracellular accumulation and sequestration (bioaccumulation), or precipitation of heavy metals as insoluble organic and inorganic compounds (e.g. oxalates, sulphides or phosphate) onto and/or around hyphae (Gadd, 1993, 2001; Sayer and Gadd, 1997). These processes might enable metals to be transformed *in situ* into insoluble and chemically inert forms and were also particularly applicable to removing metals from mobile aqueous phases (Gadd, 2001).

2.6.2.1 Metal biosorption

Biosorption comprises binding of metals (or other solutes, colloids or suspensions) to the biomass by processes which did not require metabolic energy or transport. It could therefore occur in both living and dead biomass, which acts as the biosorbent for heavy metal adsorption mechanisms (Fomina and Gadd, 2002). Several chemical processes might be involved in biosorption, including adsorption, ion exchange, co-ordination and covalent bonding with the biosorptive sites (Tobin et al., 1984). In these processes, fungal cell walls played the key role in heavy metals absorption because it contained various functional groups and various biosorptive agents that were capable to bind the heavy metals (Brady and Duncan, 1994). The main chemical groups in biomass which were able to partake in biosorption were electronegative groups such as hydroxyl or sulfhydryl groups, anionic groups such as carboxyl or phosphate groups and nitrogen-containing groups such as amino groups. However, primary interactions probably involved binding to carboxyl and phosphate groups which were considered to be important binding sites for many toxic metals (Tobin et al., 1990). The potential biosorptive agents in fungal walls that had a good capacity for heavy metals binding include chitin, chitosan, glucans, mannans, pigments such were melanin, and other compounds (Siegel et al., 1990; Fogarty and Tobin, 1996). Unlike many of the other processes for metal removal using microbes, fungal biosorption could remove suspended solids as well as solutes. *Aspergillus niger* biomass took up copper, lead and zinc sulfides onto mycelial surfaces and several fungi could adsorb solid metal compounds from acid mine waters (Wainwright et al., 1986). Major advantages of fungal biosorbent materials included their good metal uptake capacities and low anticipated price. In addition, fungal biosorbents could be regenerated for multiple uses (Plaza et al., 1996).

To date, the biosorbent from fungal biomass had received most attention, probably because waste fungal biomass could arose in quantity from several industrial fermentations.

Biomass of *Aspergillus niger* arises in substantial quantities from citric acid production while *Saccharomyces cerevisiae* is available from food and beverage industries. Other kinds of biomass arising from industrial fermentations include *Rhizopus arrhizus, Aspergillus terreus* and *Penicillium chrysogenum* (Tsezos and Volesky, 1981; Luef Prey and Kubicek, 1991).

2.6.2.2 Bioaccumulation

Bioaccumulation was uptake of metal species by means of metabolism dependent processes which may involve both transport into the cell and partitioning into intracellular components (Gadd, 1993). This process occurs in living cell and usually required the specific transport system (Humar et al., 1992). Most mechanisms of metal transport appeared to rely on the electrochemical proton gradient across the cell membrane which has a chemical component, the pH gradient, and an electrical component, the membrane potential, each of which could drive transport of ionized solutes across membranes. The membrane potential appeared to be responsible for electrophoretic mono- and divalent cation transport in fungi although other gradients, e.g. Potassium ion, may also be involved (Gadd and white, 1989). In addition, in some cases, intracellular uptake may occur by diffusion, particularly where toxic effects lead to changes in membrane permeability (Gadd, 1988). Metal resistance may often be associated with decreased uptake and/or impermeability often results in reduced toxicity.

After uptake metals into cells, metal ions might be located within specific organelles (vacuole) and/or bound to intracellular metal-binding proteins such as metallothionein and γ-glutamyl peptides (phytochelatin) to respond to the cytotoxic effects of metal ions (Howe and Merchant, 1992).

- **Vacuolar compartmentation**

Fungal vacuoles had an important role in the regulation of cytosolic metal ion concentrations and the detoxification of potentially-toxic metal ions (White and Gadd, 1986; Gadd, 1993; Gharieb and Gadd, 1998; Liu and Culotta, 1999). Metals preferentially sequestered by the vacuole include manganese (II) (Gadd and Laurence, 1996), iron (II) (Bode et al., 1995), zinc (II) (White and Gadd, 1987), cobalt (II) (White and Gadd, 1986), calcium (II) and strontium (II) (Okorokov, 1994), nickel (II) (Joho et al., 1995) and the monovalent cations potassium ion, lithium ion and cesium ion (Perkins and Gadd 1993). The absence of a vacuole or a functional vacuolar H^+-ATPase in *Saccharomyces cerevisiae* was associated with increased sensitivity and a largely decreased capacity of the cells to accumulate zinc, manganese, cobalt and nickel (Ramsay and Gadd, 1997), metals were known to be mainly detoxified in the vacuole (Gadd, 1993; Joho et al., 1995).

- **Intracellular metal-binding proteins**

In response to cellular metal stress, fungi utilized intracellular sequestration of metal by cysteine-rich molecules, namely metallothioneins and γ-glutamyl peptides (Perego and Howell, 1997).

- **Metallothioneins**

Metallothioneins were low molecular weight, cysteine-rich polypeptides that could bind essential metals, e.g. copper and zinc, as well as inessential metals such as cadmium and might have relevance to metal recovery since they can bind valuable metals, e.g, gold and silver. This protein normally functions maintained at low concentrations of intracellular copper ions (Wright et al., 1988).

- γ-Glutamyl peptides

γ-glutamyl peptides were short, cysteine-containing peptides, that encompassed by the trivial name “phytochelatins” (Winge et al., 1989; Rauser, 1990). These γ-glutamyl peptides were the main metal detoxification mechanism in algae and plants (Grill et al, 1988; Gekeler et al., 1988) as well as a range in filamentous fungi and yeasts. Metal ions were bound within a metal-thiolate cluster composed of an oligomer of peptides (Winge et al., 1989). γ-Glutamyl peptides were essential for cadmium-detoxification and clearly demonstrated biochemical linkage with glutathione metabolism.

2.6.2.3 Precipitation of metal compounds

Extracellular products migh remove metals from polluted environment by precipitate metal into insoluble form. These products were included their metabolites such as organic acid (oxalic acid and citric acid) and sulfide.

1. Organic acid

Many extracellular fungal products such as organic acid could form complex or precipitate heavy metals. Carboxylic organic acids such as oxalic acid and citric acids could interact with metal ions to form insoluble oxalate crystal around cell walls and in the external medium (Gadd, 1993). In white-rot and brown-rot fungi, the extracellular metal chelation was probably more important. One of the typical metal chelators produced by both white-rot and brown-rot fungi was oxalate. The production of oxalic acid by fungi provides a means of immobilizing soluble metal ions or complexes as insoluble oxalates, thus decreasing bioavailability, increasing tolerance to these metals and survival in contaminated environments (Sayer and Gadd, 1997). Most metal oxalates were resistant to further solubilization, with only a few species of anaerobic bacteria, aerobic actinomycetes, bacteria and fungi able to degrade them readily (Morris and Allen, 1994).

Rabanus (1931); Shimazono and Takubo (1952) suggested that metal tolerance of brown-rot fungi was linked to oxalic acid production, which presumably precipitates copper into the insoluble form of copper oxalate. A relationship between copper tolerance and oxalic acid production had been found to be due to copper oxalate crystal formation in decayed wood (Murphy and Levy, 1983). Copper oxalate crystals (moolooite) had been observed around hyphae of brown-rot fungi growing on wood treated with a copper-based preservative (Murphy and Levy, 1983; Sutter and Jones, 1983, 1984). Fungi could also produce other metal oxalates with a variety of different metals and metal bearing minerals e.g. cadmium, cobalt, copper,manganese, zinc and nickel (Gadd, 1999) which might provide a mechanism whereby fungi could tolerate toxic metal-containing environments.

2. Hydrogen sulphide

Hydrogen sulphide was the metabolic product, excreted by microorganisms to the capable of immobilizing metals ions into insoluble metal sulfide. This metabolic product could lead to precipitation of metal sulphides within and on cell surfaces. Therefore, hydrogen sulphide -producing organisms often exhibit tolerance to heavy metals. Such sulphide precipitation could also occur in algae, yeasts and fungi as well as metal precipitation as phosphates or oxalates (Gadd, 1988).

By the several mechanisms that caused by many fungi, which could tolerate and detoxify toxic metal-containing environments. Many research found that various fungi in Table 2.4 were can be tolerate to toxic metals and could be used effectively in removing heavy metals, most of these fungi were Basidiomycota that cause brown-rot and white-rot decay of wood.

Table 2.4 Some examples of fungi which could tolerate and remediate the toxic heavy metals.

Type of Heavy metals	Tolerated fungi
Lead	*Agaricus macrosporus* (Melgar et al., 2007), *Beauveria caledonica* (Fomina et al., 2005c), *Fusarium oxysporum* (Sanyal et al., 2005), *Phanerochaete chrysosporium* (Yetis et al., 1998; Yetis et al., 2000; Say et al., 2001; Huang et al., 2006; Zeng et al., 2007), *Polyporous versicolor* (Yetis et al., 1998), *Trametes versicolor* (Jarosz-Wilkolazka et al., 2002; Bayramoglu et al., 2003)
Copper	*Agaricus macrosporus* (Melgar et al., 2007), *Antrodia radiculosa* (Green and Clausen, 2003; Green and Clausen, 2005), *Antrodia vaillantii* (Green and Clausen, 2005; Humar et al., 2006; Alvarez, 2007), *Aspergillus niger* (Gharieb, 2002; Gharieb et al., 2004), *Daedalea dickinsii* (Kim et al., 2009), *Fomitopsis palustris* (Kim et al., 2009), *Gloeophyllum trabeum* (Green and Clausen, 2005; Humar et al., 2006), *Hymenoscyphus ericae* (Fomina et al., 2005a), *Laetiporus sulphureus* (Green and Clausen, 2003; Guillen and Machuca, 2008), *Leucogyrophana pinastri* (Humar et al., 2004; Humar et al., 2005), *Paxillus involutus* (Fomina et al., 2005a), *Penicillium chrysogenum* (Gharieb et al., 2004), *Phanerochaete chrysosporium* (Say et al., 2001), *Pisolithus tinctorius* (Tam, 1995), *Postia placenta* (Green and Clausen, 2005 ; Alvarez, 2007), *Trametes versicolor* (Bayramoglu et al., 2003), *Tyromyces palustris* (Green and Clausen, 2005), *Wolfiporia cocos* (Green and Clausen, 2005; Guillen and Machuca, 2008)

Table 2.4 (continuted) Some examples of fungi which could tolerate and remediate the toxic heavy metals.

Type of Heavy metals	Tolerated fungi
Cobalt	*Aspergillus foetidus* (Valix et al., 2001), *Aspergillus niger* (Sayer et al., 1995; Sayer and Gadd, 1997), *Bjerkandera fumosa* (Jarosz-Wilkolazka and Gadd, 2003), *Fomitopsis pinicola* (Jarosz-Wilkolazka and Gadd, 2003), *Penicillium funiculosum* (Valix et al., 2001), *Penicillium simplicissimum* (Sayer et al., 1995; Valix et al.,2001), *Phlebia radiate* (Jarosz-Wilkolazka and Gadd, 2003), *Trametes versicolor* (Jarosz-Wilkolazka and Gadd, 2003)
Chromium	*Antrodia vaillantii* (Humar et al., 2004; Humar et al., 2005), *Leucogyrophana pinastri* (Humar et al., 2004; Humar et al., 2005), *Poria monticola* (Humar et al., 2004; Humar et al., 2005) *Meruliporia incrassata* (Barbara et al., 1996), *Antrodia radiculosa* (Barbara et al., 1996) *Wolfiporia cocos* (Groot and Woodward, 1999) *Polypore sp.*(Kim et al., 2009), *Fomitopsis palustris* (Kim et al., 2009), *Pisolithus tinctorius* (Tam, 1995)
Arsenic	*Antrodia radiculosa* (Barbara et al., 1996), *Fomitopsis palustris* (Kim et al., 2009), *Meruliporia incrassata* (Barbara et al., 1996), *Polyporales sp.*(Kim et al., 2009), *Wolfiporia cocos* (Groot and Woodward, 1999)

Table 2.4(continuted) Some examples of fungi which could tolerate and remediate the toxic heavy metals.

Type of Heavy metals	Tolerated fungi
Cadmium	*Beauveria caledonica* (Fomina et al., 2005c), *Fusarium oxysporum* (Sanyal et al., 2005), *Hymenoscyphus ericae* (Fomina et al., 2005), *Lentinus edodes* (Bayramoglu and Arica, 2008; Chen et al., 2008), *Lentinus sajor-caju* (Bayramoglu et al., 2002), *Oidiodendron maius* (Fomina et al., 2005a), *Phanerochaete chrysosporium* (Say et al., 2001), *Pisolithus tinctorius* (Tam, 1995), *Trametes versicolor* (Arica et al., 2001)
Zinc	*Aspergillus niger* (Sayer and Gadd, 1997), *Beauveria caledonica* (Fomina et al., 2005c), *Bjerkandera fumosa* (Jarosz-Wilkolazka and Gadd, 2003), *Fomitopsis pinicola* (Jarosz-Wilkolazka and Gadd, 2003), *Hymenoscyphus ericae* (Fomina et al., 2005a), *Lentinus edodes* (Bayramoglu and Arica, 2008), *Oidiodendron maius* (Martino et al., 2003; Fomina et al., 2005a), *Paxillus involutus* (Fomina et al., 2005a), *Phlebia radiate* (Jarosz-Wilkolazka and Gadd, 2003), *Pisolithus tinctorius* (Tam, 1995), *Suillus bovines* (Fomina et al., 2005a), *Suillus luteus* (Fomina et al., 2005a), *Thelephora terrestris* (Fomina et al., 2005a), *Trametes versicolor* (Bayramoglu et al., 2003; Jarosz-Wilkolazka and Gadd, 2003)
Mercury	*Lentinus edodes* (Bayramoglu and Arica, 2008), *Pisolithus tinctorius* (Tam, 1995)
Nickel	*Aspergillus niger* (Magyarosy et al., 2002), *Pisolithus tinctorius* (Tam, 1995), *Polyporous versicolor* (Dilek et al., 2002)

2.7 Wood-rotting fungi

Wood-rotting fungi or wood-rotting basidiomycetes were the organisms that capable of utilizing sound, unaltered wood as an energy source by means of enzymatic degradation of components of wood cell walls (Gilbertson, 1980). These fungi could also be divided into two major groups according to the type of rot they caused. These were usually referred to as white-rot fungi and brown-rot fungi.

White-rot fungi utilize all components of the wood cell walls, and most of them degraded lignin, cellulose, and hemicelluloses at about the same rate (Cowling, 1961; Kirk and Highley, 1973). In the advanced stages, wood decayed by white-rot fungi was also bleached, and the white to pale tan-colored residue had a spongy, stringy, or laminated structure. Most white-rot fungi produced extracellular polyphenol oxidases and generally gave positive oxidase tests when grown on gallic or tannic acid medium (Davidson et al., 1938 and Nobles, 1948). The fungi causing white rot were presented in all the main groups of the Basidiomycetes and in some of the Ascomycetes, namely the Xylariaceae (Sutherland and Crawford, 1981).

Brown-rot fungi selectively degraded cellulose and hemicelluloses from wood substrate and leave a residue of slightly modified lignin (Rayner and Boddy, 1988; Green and Highley, 1997). In the advanced stages of decay the wood undergoes severe shrinkage and cracks extensively across the grain (cuboidal cracking), leaving a residue of brown, cubical chumps and finally crumbles into powder. The modified lignin remaining gives the decayed wood its characteristic color and consistency. These brown-rot fungi did not produce extracellular polyphenol oxidases and generally gave negative oxidase tests on gallic and tannic acid medium (Davidson et al., 1938). Most of the brown-rot fungi belonged to the family Polyporaceae. Interestingly, only 6% of the total numbers of wood-rotting basidiomycetes were now known to cause a brown rot (Gilberson, 1981).

Wood-rotting fungi were known for their remarkable ability to withstand stress induced by toxic metals and could employ numerous mechanisms and strategies in detoxification, including enhanced metal efflux, suppressed influx, extracellular metal sequestration and precipitation, metal binding to cell walls, intracellular sequestration, and complexation that could alleviate the toxicity of heavy metals (Gadd,1993). However, certain mechanisms were generally based on metal immobilization, e.g. through the production of intracellular and extracellular chelating compounds such as organic acids and metal-binding peptides (Baldrian, 2003 and Gadd, 2007).

In recent years, the use of wood-rotting fungi to remove heavy metals had been received increasing attention because these fungi could tolerate and alleviate the toxicity of heavy metals. Moreover, they were high effectively in removing heavy metals from contaminated soil, water, or sediment. In many research found that wood-rotting fungi played an important role in remediation of metals from preservative treated-wood. In some cases, fungal mycelium showed high ability to absorb heavy metals from several media containing heavy metal ions.

2.7.1 The research of heavy metal remediation by wood-rotting fungi

Yetis et al. (1998) studied the heavy metal biosorption by white-rot fungi. They found that two white-rot fungi, *Polyporous versicolo*r and *Phanarochaete chrysosporium*, which were commonly used in wastewater treatment were the most effective in removing lead (II) from aqeous solutions with maximum biosorption capacities of 57.5 and 110 mg lead (II)/g dry biomass, respectively.

Rodney et al. (1999) using copper-tolerant fungi to biodegrade wood treated with copper-based preservatives. They found that the copper-tolerant fungus *Wolfiporis cocos* had ability to decompose wood treated with copper-based preservatives and concentrate copper in the mycelium.

Yetis et al. (2000) studied the removal of lead (II) by *Phanerochaete chrysosporium*. They reported that the white-rot fungus, *Phanerochaete chrysosporium*, had the potential for application as efficient biosorbent for lead (II) in aqueous wastes. Since the resting cell of this fungus was able to uptake up to 80 mg lead (II)/g dry cell.

Say et al. (2001) studied the biosorption of cadmium (II), lead (II) and copper (II) with the filamentous fungus *Phanerochaete chrysosporium*. They found that *Phanerochaete chrysosporium* had been successfully used as the adsorbing agent for removal of metals ions from artificial wastewaters. The maximum absorption capacities for cadmium (II), lead (II) and copper (II) were 27.79, 85.86, and 26.55 mg g^{-1} dry biomass, respectively.

Arica et al. (2001) studied the cadmium removal from an aqueous solution by entrapment of white-rot fungus *Trametes versicolor* in Ca-alginate beads. They reported that the maximum experimental biosorption capacities for entrapped dead fungal mycelium of *T. versicolor* was found 120.6 $\pm$ 3.8 mg cadmium (II) g^{-1} biosorbent. The biosorbents were reused in three consecutive adsorption and desorption cycles without a significant loss in the biosorption capacity. So, the plain alginate beads entrapped dead fungal mycelium have been successfully used as the biosorbing agent for removal of cadmium (II) ions from aqueous medium.

Dilek et al. (2002) studied the nickel (II) biosorption by *Polyporous versicolor*. They found that *P. versicolor* has a high affinity to nickel (II) when compared with other microbial species. The nickel (II) biosorption capacity of this fungus increased with increasing temperature. A maximum adsorption capacity of 57 mg nickel (II)/g dry biomass was obtained at 35 °C.

Bayramoglu et al. (2002) studied the entrapment of *Lentinus sajor-caju* into Ca-alginate gel beads for removal of cadmium (II) ions from aqueous solution. They reported that the maximum biosorption capacities for entrapped live and dead fungal mycelia of *L. sajur-caju* were found to be 104.8 $\pm$ 2. 7 mg cadmium (II) g^{-1} and 123.5 $\pm$ 4. 3 mg cadmium (II) g^{-1}, respectively. The biosorbents were reused in three biosorption and

desorption cycles without significant loss in the biosorption capacity. Since the biosorption capacities were relatively high for both entrapped live and dead forms, they could be considered as suitable biosorbents for the removal of cadmium in wastewater treatment systems.

Bayramoglu et al. (2003) studied the biosorption of heavy metal ions on immobilized white-rot fungus *Trametes versicolor*. They found that the CMC beads with the immobilized heat inactivated *Trametes versicolor* had been successfully used as the biosorbing agent for removal of copper (II), lead (II) and zinc (II) ions from an aqueous medium. The maximum biosorption capacities for immobilized heat inactivated fungus were 1.84 mmol copper (II), 1.11 mmol lead (II) and 1.67 mmol zinc (II) per g of dry biosorbents. The CMC beads with the immobilized fungus can be regenerated using 10mMHCl, with up to 97% recovery of the metal ions; the biosorbents reused up to five biosorption–desorption cycles without any major loss in the biosorption capacity. So, the CMC beads with immobilized heat inactivated fungus could be used as an efficient biosorbent system for the treatment of heavy metals containing wastewater streams.

Humar et al. (2004) studied the fungal bioremediation in copper, chromium and boron treated wood as done by electron paramagnetic resonance. They reported that exposure of CCB-treated wood samples to the wood decay fungi *Antrodia vaillantii*, *Leucogyrophana pinastri* and *Poria monticola* significantly increased the leaching of copper and chromium from the treated wood. The main reason for the increased leaching of these heavy metals is their reaction with the oxalic acid produced and excreted by the fungi to copper and chromium oxalate respectively, which is soluble. Therefore, oxalic acid producing fungal strains could be used for bioremediation of waste CCA- or CCB-treated wood.

Humar et al. (2006) studied the influence of corn steep liquor and glucose on colonization of control and CCB (copper/chromium/boron)-treated wood by brown rot fungi. They found that immersion of the wood samples in glucose significantly improved the ability

of copper tolerant (*Antrodia vaillantii* and *Leucogyrophana pinastri*), as well as copper sensitive wood decay fungi (*Gloeophyllum trabeum* and *Poria monticola*) to overgrow the surface of CCB-treated specimens. The fastest overgrow of the surface and the fastest penetration to the center of the impregnated specimens was observed at copper tolerant fungus *Antrodia vaillantii*. From these results, these wood decay fungi should be used for bioremediation of treated wood wastes.

Huang et al. (2006) studied the bioremediation of lead-contaminated soil by incubating with *Phanerochaete chrysosporium* and straw. They found that incubating contaminated soil with the inoculated *P. chrysosporium*, together with the added straws as nutrient, could reduce the active lead, alleviate the lead stress, and stabilize the lead-contaminated soil. In addition, the treatment improved the remediation of the soil in comparison to the controls. All these results might be because the lead ion was absorbed by the mycelia of this fungus and chelated by the humus formed in the incubation process.

Zeng et al. (2007) studied the composting of lead-contaminated solid waste with inocula of white-rot fungus. They found that the treatment of the simulated lead-contaminated solid waste by composting with white-rot fungus *Phanerochaete chrysosporium* could be successfully processed. This process could control the phytotoxicity of lead to some extent. Reasons for these results might be as follows: (i) white-rot fungi could chelate with lead by the carboxyl, hydroxyl or other active functional groups on cell wall surface, (ii) white-rot fungi could improve the composting process, as proved by the data obtained in this study and reported previously. All these results indicated that composting lead contaminated solid waste with the inocula of white-rot fungus could improve the microbial activity, reduce the active lead, and alleviate the lead stress and the hazards of compost.

Alvarez (2007) studied the fungal bioleaching of metals in preservative-treated wood. He found that two brown-rot fungi, *Antrodia vaillantii* and *Poria placenta* displayed both a high copper tolerance and a high oxalic acid production were effectively on decayed

wood containing up to 4.4% copper and chromium salts causing corrected mass losses of up to 24.3% in 4 weeks. Fungal treatment was also found to promote extensive leaching of chromium (up to 52.4%). From the results, he indicated that the potential of biological treatment with copper tolerant, oxalate-producing brown-rot fungi to decrease metal levels in decommissioned wood treated with copper-based preservatives.

Bayramoglu and Arica (2008) studied the removal of heavy mercury (II), cadmium (II) and zinc(II) metal ions by live and heat inactivated *Lentinus edodes* pellets. The metal biosorption capacities of the live fungal pellets mercury (II), cadmium (II) and zinc (II) were 336.3 ± 3.7, 78.6 ± 2.6 and 33.7 ± 1.6 mg/g, respectively, while mercury (II), cadmium (II) and zinc (II) the biosorption capacities of the heat inactivated pellets were 403.0 ± 2.9, 274.3 ± 3.6 and 57.7 ± 1.1 mg/g, respectively. The adsorption capacities of the heat inactivated fungus for metals were markedly increased compared to native form.

Chen et al. (2008) studied the cadmium removal from simulated wastewater to biomass byproduct of *Lentinus edodes*. They found that the biosorption by the by product of this brown-rot fungus which was used as an efficient biosorbent, showed the maximum uptake of cadmium was 5.58 mmol/g in weak acid condition, which was much higher than many other biosorbents.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and Chemicals

Materials and chemicals used in this research are listed as follows:

1. Malt extract (Lab-scan, Ireland)
2. Tannic acid (Carlo erban reagent, France)
3. Copper sulfate ($CuSO_4.5H_2O$) (Merck KGaA, Germany)
4. Lead nitrate ($Pb(NO_3)_2$) (May Baker Ltd., Dagenham, England)
5. Cadmium sulfate ($3CdSO_4.8H_2O$) (May Baker Ltd., Dagenham, England)
6. Zinc sulfate ($ZnSO_4.7H_2O$) (Farmitalir Carlo erban reagent, France)
7. Sodium hydroxide (NaOH) (Merck KGaA, Germany)
8. Nitric acid (HNO_3) (Merck KGaA, Germany)
9. Orthophosphoric acid (H_3PO_4) (Mallinckrodt, U.S.A.)
10. Oxalic acid ($C_2H_2O_4 \cdot 2H_2O$) (Merck KGaA, Germany)
11. Citric acid ($HOC(COOH)(CH_2COOH)_2 \cdot H_2O$) (Ajax Finechem Pty Ltd, Australia)
12. Acetic acid (CH_3COOH) (Merck KGaA, Germany)
13. Fumaric acid (CH(COOH) : CH·COOH) (The british drug house Ltd, England)
14. Succinic acid $(CH_2COOH)_2$ (May Baker Ltd., Dagenham, England)
15. Isopropyl alcohol ($CH_3CH(OH)CH_3$ (Merck KGaA, Germany)
16. Deoxynucleoside triphosphate (dNTP Mix) (Fermentas International Inc., Canada)
17. Taq buffer (Fermentas International Inc., Canada)
18. Taq polymerase (Fermentas International Inc., Canada)
19. Primer ITS1F and ITS4 (Fermentas International Inc., Canada)

20. Magnesium chrolide ($MgCl_2$) (Fermentas International Inc., Canada)
21. Agarose gel (IUAI International Inc., Japan)
22. Filtered membrane (Advantec International Inc., Japan)
23. Ethylenediaminetetraacetic Acid (EDTA) (Sigma-Aldrich Co., Inc., Singapore)
24. 100 bp Ladder Sharp DNA Marker (Fermentas International Inc., Canada)

3.2 Instruments

1. Microscope (Model BX51, Olympus Optical Co., Ltd., Japan)
2. Stereomicroscope (Model SZ-PT, Olympus Optical Co., Ltd., Japan)
3. Hot air oven (Model UC 30, Memmert GmbH and Co. KG., Western Germany)
4. Kubota Refrigerated Microcentrifuge 6500 (Kubota Corporation, Tokyo, Japan)
5. Laminar flow 'clean' (Model V6, Lab Service Ltd., Part)
6. Autoclave (Model Autoclave ES-315, Tomy Seiko Co., Ltd., Tokyo, Japan)
7. pH meter (Mettler-Toledo International Inc., New York, U.S.A.)
8. 4-Digit precision weighting balance (Model AG 204, Mettler Toledo, Switzerland)
9. Vortex mixer (Model G-560E, Scienctific Industries, Inc., Bohemia. N.Y., 11716, U.S.A)
10. Water bath (Model WB14, Becthai Bangkok Equipment & Chemical Co., Ltd., Thailand)
11. DNA thermo cycler TP 600 (TaKaRa Bio Inc., Otsu, Shiga, Japan)
12. Electrophoresis chamber set (Mupid-ex, Bruker Biospin Inc., Fallanden, Switzerland)

3.3 Tested basidiomycetous fungi

3.3.1 Collection and isolation basidome wood-rotting fungi

3.3.1.1 Collection of basidome

Sixty basidomes of wood-rotting basidiomycetes were collected from the forest areas in Yasothron, Kanjanaburi, Nakorn srithummarat, Nan, Tak, Supunburi, Nonthaburi, Pathumtani, Nakorntpathom and Bangkok provinces.

3.3.1.2 Isolation from basidome

Fruiting body tissues (0.2–0.5 cm^3) were cut aseptically from the region located between stem and cap and transferred on to the surface of 2% malt extract agar (2%MEA) (Appendix A) in Petri dishes. The Petri dishes were incubated at room temperature for 5 days. After the fungal mycelia germinate were subcultured until obtained pure mycelia. Pure fungal mycelia were cut from the growing margin of the colony and transferred to 2% MEA medium. After 7 days at room temperature, the fungal mycelial purity with clamp connection formation was examined under light microscope.

3.3.2 Collection and isolation wood-rotting fungi from decayed-wood

3.3.2.1 Collection of decayed-wood

Ten samples of decayed-woods were collected from Nan, Tak, Supunburi, Kanjanaburi, Nonthaburi and Bangkok provinces.

3.3.2.2 Isolation from decayed-wood

Inner pieces of decayed-woods were cut and placed on 2% MEA medium in Petri dishes. The Petri dishes were incubated at room temperature for 5 days. After the fungal mycelia germinate were subcultured until obtained pure mycelia. Pure fungal mycelia were cut from the growing margin of the colony and transferred to 2% MEA medium. After 7 days at room temperature, the fungal mycelial purity with clamp connection formation was examined under light microscope.

3.3.3 Basidiomycetes mycelial isolates from culture collection

Ten Basidiomycetes fungal isolates cultures were obtained from Culture Collection, Department of Microbiology, Chulalongkorn University.

The fungal from stock cultures were cultured on 2% MEA medium in Petri dishes. The Petri dishes were incubated at room temperature for 7 days. After that the fungal mycelial purity with clamp connection formation was examined under light microscope.

3.3.4 Maintenance of fungal cultures

Pure fungal mycelia of all fungal isolates were re-grown on 2% MEA medium at room temperature for 7 days. Pieces fungal mycelium were cut by using 8 mm diameter of sterile cork borer and then transferred to sterile bottles containing with 7 ml sterile distilled water. The cultures were then kept in refrigerator and fungi were re-growth in 2% MEA medium every 3 months.

3.4 Distinguished of the type of wood-rotting fungi by using Bavendamm reaction test (Davidson et al., 1938)

White-rot fungi could be distinguished from brown-rot fungi by the Bavendamm test (phenol oxidase test) (Davidson et al., 1938). This test was carried out using malt extract agar containing tannic acid (0.5 %, w/v) (Appendix A). In this experiment, all fungal isolates were precultured on 2% MEA plates. Seven days, a mycelium growth plug (8 mm diameter) was placed onto tannic acid agar plate. The agar plates were incubated at room temperature for 7 days. The appearance of a dark brown zone in the agar medium around the fungal colony indicated extracellular phenol oxidases production which was considered a positive result. White-rot fungi should give a positive Bavendamm reaction, whereas brown-rot fungi give a negative reaction.

3.5 Screening for fungal tolerance to the heavy metal and detection of their crystals formation

In this study zinc sulfate ($ZnSO_4 \cdot 7H_2O$), copper sulfate ($CuSO_4 \cdot 5H_2O$), cadmium sulfate ($3CdSO_4 \cdot 8H_2O$) and lead nitrate ($Pb(NO_3)_2$) were used for screening fungal tolerance to the heavy metal. The tolerance of sixty wood-rotting fungal isolates to heavy metals ($ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $3CdSO_4 \cdot 8H_2O$, and $Pb(NO_3)_2$) were determined growth tolerant to heavy metals which shown in Table 4.1 by incubation onto 4% MEA media (Appendix A) (adjusted pH to 5.5 with NaOH prior autoclaving) supplemented with sterilized various concentrations of heavy metals 0 – 20,0000 ppm. The heavy metal salt solutions were sterilized by filtrate through millipore filter (0.22 mm pore size) and subsequently added to 4% MEA medium autoclaved. The medium was allowed to solidify and then it inoculated with a mycelium plug (8 mm diameter) which cut from the growing margin of 7 days old cultures. Agar plates were then incubated at room temperature for 30 days. The heavy

metal tolerant assays were done in triplicate for each tested fungi and for each metal concentration. The toxic threshold concentration was defined as the highest heavy metal concentration at which fungal growth was still observed. Fungal mycelium and the agar under the colony in tested culture and control culture were examined at the end of the incubation period by light microscopy to detect the formation of crystals. The crystal formation fungal isolates were selected for further study the effect of heavy metals on fungal growth and for analysis of the mineral transformation.

3.6 Study the effect of heavy metal on fungal growth

The crystal formation fungal isolates MR40, KYO, WR3, WR4 and WR5 were grown on 20 ml of 4% MEA medium at pH 5.5, which supplemented with various concentrations of 0 – 3000 ppm $ZnSO_4.7H_2O$, 0 – 5000 ppm $CuSO_4.5H_2O$, 0 – 13,0000 ppm $3CdSO_4.8H_2O$ and 0 - 20,0000 ppm $Pb(NO_3)_2$. Sterile cellophane membranes were placed aseptically onto the surface of solid agar media (Sayer et al., 1995). The membrane allowed the passage of nutrients or metabolites exchange between the agar and the fungus, and also provided a convenient means of removing the mycelium from the agar (Sayer and Gadd, 1997). The treatments were done in triplicate by inoculation with 8 mm diameter mycelium plug. The agar plates were incubated at room temperature for 30 days. The colony diameter was measured for every 2-4 days.

3.6.1 Determination for pH change in the agar medium

The pH changes in culture media were measured at the end of the incubation period. To obtain a pH profile of the agar surface under growing fungal colonies, pH measurements were made, in triplicate, across the agar surface, using a surface combination pH electrode.

3.6.2 Determination of mycelium radial growth rate

Four radial growths were measured for each colony along the axis passing through the centre of the fungal colony. The average radial growth measurements were calculated for growth rate in millimeters per day (mm day^{-1}).

3.6.3 Determination of biomass production

Fungal biomass production was determined through the dry weight of mycelia for each treatment at the end of incubation period. The fungal mycelia were removed from agar plates by peeling fungal mycelium mat from the cellophane membrane and then dried in hot air at 80 °C until reaching constant weight. Mycelia were then weighed and the results were expressed as biomass in milligrams (mg).

3.7 Analysis of heavy metal transformation in wood-rotting fungi

Elemental crystals analysis by wood-rotting rot fungi isolates MR40, KYO, WR3, WR4 and WR5 grew on 4% MEA media, which supplemented with each high level heavy metals. Samples were examined under scanning electron microscopy (SEM) equipped with energy-dispersive X-ray microanalysis (EDXA), High-performance liquid chromatography (HPLC) and X-ray powder diffraction (XRPD).

3.7.1 Purification of elemental crystals formation by wood-rotting fungi during growth on selected metal compounds (Gharieb and Gadd,1999)

Agar was taken from the crystallized zone that newly formed under and around the growing colonies of wood-rotting fungi. And then crystals were extracted from the agar by

gently homogenizing the agar with approximately 7 ml ddH_2O in a tested tube and then the tubes were placed in a water bath at 70-80 °C for 30 min until the agar dissolved. These elemental crystals were allowed to settle to the bottom of the test tube and the aqueous phase was removed. Repeated additions (approximately four) of ddH_2O, settling and removal of the aqueous phase yielded pure crystals for chemical analysis.

3.7.2 Preparation elemental crystal for scanning electron microscopy examination and elemental X-ray micro analysis (Gharieb and Gadd, 1999)

3.7.2.1 Scanning Electron Microscopy examination of elemental crystals

The elemental crystals formation by wood-rotting fungi which grown in the medium supplemented with copper sulfate, zinc sulfate, cadmium sulfate and lead nitrate were examined by Scanning Electron Microscope (SEM). Elemental crystals samples were prepared as described by Gharieb and Gadd (1999). Crystal samples were mounted on double-sided sticky tape on 1 cm dia. carbon stubs. Samples were dried in a vacuum desiccator at room temperature for at least 24 h. Samples were then sputter-coated with gold for 5 min by using a sputter coating machine (Balzer model SCD 040) and examined under JEOL Model JSM-6400 Scanning Electron Microscope at an accelerating voltage of 15 kV.

3.7.2.2 Elemental X-ray micro-analysis of elemental crystals

Specimens were prepared as described in the preparation in 3.7.2.1 except the specimens were not coated and elemental contents were analyzed by Energy Dispersive X-ray micro-Analysis (EDXA, Oxford Instrument (Link ISIS series 300)).

3.7.3 Determination type of organic acid in elemental crystals by HPLC (Sayer and Gadd, 1997)

One hundred milligram elemental crystals were dissolved in 1.0 ml concentrated orthophosphoric acid that was then diluted 100 times with 20 mM orthophosphoric acid. Analysis of free organic acids was carried out using a high-performance liquid chromatography (HPLC, Varian Prostar model 410). The mobile phase was 20 mM orthophosphoric acid at a flow rate of 1 ml min^{-1} and the column used was a Aquasil C-18 25 cm x 4.6 mm octadecyl bonded phase with a Mightysil 4.6/6mm guard pre-column. Both samples and the eluants were pre-filtered through membrane filters with pore size 0.22 µm and then 100 µl of samples were analyzed at a wavelength of 200 nm for 15 min at room temperature. A series of standard solutions of oxalic acid, citric acid, acetic acid, fumaric acid and succinic acid (sodium salts) were used and organic acids were identified by comparison to retention times of standards.

3.7.4 X-ray powder diffraction (XRPD) analysis

In order to confirm their homogeneity and identification as biogenic crystalline oxalate precipitates, the elemental crystals formed by wood-rotting fungi isolates MR40, KYO, WR3, WR4 and WR5 were examined using X-ray powder diffraction (XRPD) analysis. The finely samples were prepared as described by Fomina et al. (2005c) and mounted onto crystal silicon substrates and examined using a Bruker AXS Model D8 Discover X – ray diffractometer equipped with a VANTEC-1 detector. Samples were scanned from 2θ = 10° - 80°, using a monochromatic CuKα radiation, counting for 0.2 s per step by using a step size of 0.02°. Diffraction patterns were identified by reference to patterns in the International Centre for Diffraction Data (ICDD) powder diffraction file. The powder diffraction method gives the crystallographic structure of the studied sample. Moreover, the

diffraction pattern (position of the peaks and their relative intensities) may serve as a "fingerprint" of a given material. Thus, by comparison of the measured diffractogram with the ones reported in the literature it is possible to validate the presence of a certain crystalline material in the sample.

3.8 Analysis metal content of mycelia by atomic absorption spectrophotometry (AAS) (Jarosz-Wilkolazka and Gadd, 2003)

Zinc, copper, cadmium and lead accumulation in fungal biomass was determined using an atomic absorption spectrophotometer (AAS).

Fifty milligram fungal mycelia in 3.6.3 were digested in 3.0 ml of 6N HNO_3 by heating at 90 °C for 1 h. After cooling, the digest was diluted with an appropriate volume of double-distilled H_2O. The metal ion contents of the solutions were analyzed by using a PerkinElmer AAnalyst 200 atomic absorption spectrophotometer with comparisons to appropriate standard solutions in acidified double-distilled H_2O.

3.9 Identification of selected wood-rotting fungi

3.9.1 Identification by morphological characteristics

A taxonomic analysis of wood-rotting isolates MR40, KYO, WR3, WR4 and WR5 fungi were analyzed based on basidiocarp morphology which included size, color, shape, texture, pileus surface, gill/pore surface, stipe/stem, hyphae and basidiospores. Specimens for light microscopy were mounted in the water or KOH, Melzer's reagent, and lactophenol-cotton blue for observation of spores and other characteristics, and then identified. In this study, identification in genus level was using Wood-rotting Fungi of North America (Gilbertson, 1980) Genera of Polypores Nomenclature and Taxonomy (Ryvarden, 1991) European Polypores part 1 (Ryvarden and Gilbertson, 1993), European Polypores part 2

(Ryvarden and Gilbertson, 1994), and North American Mushrooms: A Field Guide to Edible and Inedible Fungi (Miller and Miller, 2006).

3.9.2 Identification by molecular methods

In this study, the sequence of the internal transcribed spacer (ITS) regions between the small, 5.8S, and large rRNA genes was determined. Cultures for genomic DNA preparation were grown on 2% malt extract agar and incubated at room temperature for 7 days. And then, mycelium was scraped into a 2.0-ml tube containing zirconia balls and transferred to a container with liquid nitrogen. After that, each sample was pulverized by using a homogenizer (Retsch Mixer Mill MM400, Haan, Germany) for 30 s and then 700 µl of 2X CTAB solution [2% cetyltrimethylammonium bromide, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 0.5% b-mercaptoethanol] was added to the tube and homogenized it again for 30 s. After incubation in a block heater at 65 °C for 1 h, 700 µl chloroform : isoamyl alcohol mixture (24 : 1) was added to the tube. After the tube had been vortexed and centrifuged (15,000 rpm, 8 min, room temperature), the supernatant was removed to another 1.5-ml tube. The DNA was precipitated by adding an equal volume of isopropyl alcohol and keeping the tube at -20 °C for 15 min. After centrifugation (8,000 rpm, 10 min, 4 °C), the DNA pellet was washed with 500 µl ethanol (70%) and dried. The DNA pellet was dissolved in 100 µl TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA), and stored at -20 °C until use.

The fungal internal transcribed spacer region was amplified by using primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990), and the expected product size was approximately 700 bp. PCRs were performed in duplicate with controls in 50-µl mixtures containing 5 µl of Taq buffer, 3 µl of $MgCl_2$, 5 µl of deoxynucleoside triphosphate, 1 µl of *Taq* polymerase, 2.5 µl of each primer at a concentration of 20 mM, 31 µl of distill water, and 4 µl of each template DNA. The optimal conditions for PCR amplification were as

follows: initial denaturation for 1 min at 94 °C, with subsequent denaturation for 1 min at 94 °C, primer annealing for 1 min at 51 °C, and extension for 1 min at 72 °C for a total of 35 cycles. A final extension for 5 min at 72 °C preceded a constant incubation at 4 °C until analysis by electrophoresis. PCR products were checked on a 1.5% agarose gel staining with ethidium bromide run at 100V for 45 min. All PCR products were purified and then sequenced by Pacific Science Co.,Ltd (Bangkok, Thailand) using the same primers as for amplification. The sequences obtained, were compared with the sequences of known species in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) using blast.

3.10 Statistics analysis

Statistics analysis was conducted with SPSS 13.0 for Windows. One-way ANOVA followed by Duncan's Multiple Range Test (DMRT) were use to compare the heavy metal concentrations and radial growth rate, biomass production and heavy metal accumulation with $P < 0.05$ as the level of significance.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of wood-rotting fungi from fruiting bodies, decayed-wood and fungal stock cultures

Totally sixty fungal mycelium isolates were used in this study originated from fifty three basidomes, three decayed-woods and four stock cultures (Table 4.1).

4.2 Determination of the type of wood-rotting fungi using the Bavendamm reaction

Sixty wood rotting fungi isolates were examined using the Bavendamm reaction, found that 55 isolates were white-rot fungi and only 5 isolates were brown-rot fungi (Table 4.1). By this reaction white-rot fungi distinguished from brown-rot fungi by use of certain substances added to the media. The most common of these were tannic and gallic acids. In Fig. 4.1, the darkening of these acids (positive oxidase reactions) by the white rots was stated to be due to the extracellular phenol oxidase enzyme, which was part of the lignin-degrading system of white-rot fungi. Brown rots did not cause darkening in tannic and gallic acid media. They gave negative oxidase reaction due to they did not produce this enzyme (Davidson et al., 1938 and Nobles, 1958). In this study found that total isolates of brown-rot fungi were less than white-rot fungi which agreed to the report from Gilberson (1981) found that of about 1,700 wood-degrading Basidiomycetes in North America, only 116 species (6%) caused brown rot and about 75 of these, or almost three-quarters of the total number, were in the family Polyporaceae.

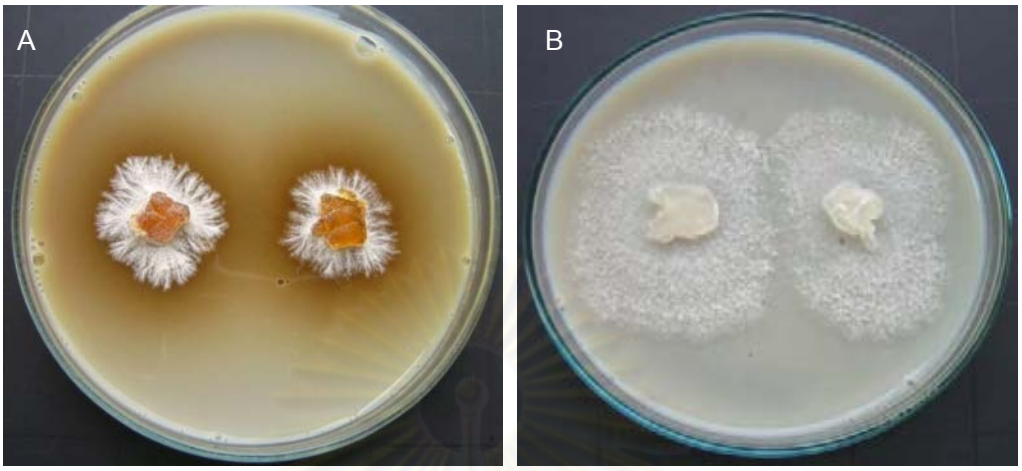

Fig. 4.1 The appearance of the Bavendamm reaction of the tested isolates of wood rotting fungi: (A) Positive Bavendamm reaction by the white-rot fungi and (B) Negative Bavendamm reaction by the brown-rot fungi when grown on agar medium containing tannic acid.

4.3 Screening of fungal tolerance to the heavy metal and detection of the crystals formation

Fifty five white-rot fungal isolates and five brown-rot fungal isolates were tested for their tolerance and their ability to produce the crystals formation in the presence of various concentrations of zinc sulfate ($ZnSO_4 \cdot 7H_2O$), copper sulfate ($CuSO_4 \cdot 5H_2O$), cadmium sulfate ($3CdSO_4 \cdot 8H_2O$) and lead nitrate ($Pb(NO_3)_2$), which were summarized in Table 4.1.

In zinc sulfate-amended plates, the most tolerant strains, which belonged to the isolate MR33, was able to grow at the highest concentrations tested 20000 ppm zinc, however no crystals were observed in this isolate. In contrast, the formations of numerous small crystals in the agar medium underneath the colonies were observed in brown-rot fungal isolate MR40, KYO and WR4 (Fig. 4.2), which were able to grow up to 3000, 2000 and 2000 ppm zinc, respectively.

In copper sulfate-amended plates, the most tolerant strains, which belonged to the white-rot fungal isolate MR17 and MR33, were able to grow at the highest concentrations

tested 20,000 ppm copper, however no crystals were observed. The elemental crystal formations were observed in the agar medium underneath the growing colonies and on the fungal mycelia of the brown-rot fungal isolate MR40, KYO and WR4 (Fig. 4.3), which were able to grow up to 2000, 5000 and 4000 ppm copper, respectively. During growth of three isolates in the presence of copper sulfate in the growth media, they were often observed green color elemental crystal which precipitated in the bottom of the petri dish, forming concentric ring patterns (Fig 4.3 A, C and E). These patterns were very similar to Liesegang patterns, which were formed by a complex interplay of diffusion, chemical reaction, and precipitation during reaction-diffusion processes (Henisch, 2002).

In cadmium sulfate-amended plates, brown-rot fungal isolate KYO showed the most tolerant species and was able to grow at the highest concentrations tested 13,000 ppm cadmium and formed the numerous crystals in the agar medium underneath the colonies. Similarly to brown-rot fungal isolate MR40 and WR4, and white-rot fungal isolate WR3 were detected the crystals formations in the agar medium underneath the colonies and grown up to 500, 500 and 8,000 ppm cadmium, respectively (Fig. 4.4). In the isolate KYO and WR4, stereo microscopy revealed that the crystals of these isolates over precipitated on the fungal mycelium (Fig. 4.4 I and J).

In lead nitrate-amended plates, brown-rot fungal isolate MR40 and KYO exhibited greater metal tolerance at the highest concentration tested 20,000 ppm lead and formed the numerous crystals in the agar medium under the colonies and on the fungal mycelia. Moreover, brown-rot fungal isolate WR4 and white-rot fungal isolate WR3 and WR5 which grown up to 6,000 13,000 and 5,000 ppm lead, respectively, were detected the crystals formations in the agar medium under and around the colonies (Fig. 4.5). In brown-rot fungal isolate MR40, KYO and WR4, stereo microscopy revealed that the crystals of three isolates over precipitated on the fungal mycelium (Fig. 4.5 K to M).

From this experiment found that, among sixty wood-rotting fungal isolates, there were only five isolates (brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate

WR3 and WR5) which were able to tolerance and produce the elemental crystals at various concentrations of heavy metals. Especially, brown-rot fungal isolate KYO was able to grow at concentration above 5000 ppm of copper sulfate, 2000 ppm of zinc sulfate, 13000 ppm of cadmium sulfate and 20000 ppm of lead nitrate-amended plates and produced numerous metal crystals. Brown-rot fungal isolate MR40, KYO and WR4 were capable to produce the crystalline metals in all types of heavy metals, in contrast white-rot fungal isolate WR3 and WR5 were able to produce the crystalline metals only in cadmium sulfate- and lead nitrate-amended plates. However, the five isolates were selected to determine the effect of heavy metal on the fungal growth.

The microscopic examination of the metal crystals formed by five isolates of wood-rotting fungi revealed the different characteristic crystalline structures for each of the metals tested. The crystals formation in the agar medium underneath the growing colonies and on the fungal mycelia of wood-rotting fungi may be related to the production of organic acids such as oxalic and citric acids, which were previously found to be effective in immobilizing (precipitation) soluble metal ions by forming insoluble metal oxalate (Gadd, 1997 and 1999).

Among 4 types of heavy metals tested, zinc and cadmium were the most toxic heavy metals for wood -rotting fungi, in the presence of zinc and cadmium at concentration of 3000 ppm in culture medium caused a complete inhibition of the growth in 50 isolates of these fungi. On the other hand, copper and lead were low toxic for these fungi in comparison with zinc and cadmium, since most of wood-rotting fungi were able to grow at higher concentrations of copper and lead. This was confirmed from previous studies which showed that zinc and cadmium were toxic for most of fungi such as Baldrian and Gabriel (1997) found the most potent inhibitor for all tested fungal strains was cadmium, which caused a decrease of growth rate in the presence of 1 mmol/L cadmium. Similarly Martha et al. (2002) reported that 10 ppm cadmium and 50 ppm zinc caused a growth inhibition in the white-rot fungal *Coriolopsis gallica*, 28% and 48%, respectively. Moreover, Baldrian and Gabriel (2002 and 2003) found that the addition of 1mM zinc sulfate and 2 mM cadmium

nitrate led to the decrease of laccase activity and Mn-peroxidase activity in *Pleurotus ostreatus*, respectively.

Table 4.1 Isolates of wood-rotting fungi tested for white rot/brown rot separation, heavy metal tolerance and crystal formation

Fungal isolates	Specimen of origin	Type of wood-rotting fungus	Zinc (II), Copper (II), Cadmium (II) and Lead (II) toxicity threshold (ppm)				Crystal formation
			Zinc (II)	Copper (II)	Cadmium (II)	Lead (II)	
MR4	Yasothorn	WRF	4000	2000	11000	9000	Not found
MR6	Yasothorn	WRF	500	500	300	1000	Not found
MR7	Kanjanaburi	WRF	500	500	200	3000	Not found
MR8	Kanjanaburi	WRF	500	500	50	1000	Not found
MR9	Kanjanaburi	WRF	500	1000	50	4000	Not found
MR12	Kanjanaburi	WRF	500	500	8000	4000	Not found
MR13	Kanjanaburi	WRF	1000	1000	3000	4000	Not found
MR14	Kanjanaburi	WRF	500	500	10000	5000	Not found
MR15	Kanjanaburi	WRF	18000	17000	200	8000	Not found
MR16	Kanjanaburi	WRF	16000	17000	8000	5000	Not found
MR17	Kanjanaburi	WRF	5000	20000	10000	5000	Not found
MR18	Kanjanaburi	WRF	500	500	400	2000	Not found
MR19	Nonthaburi	WRF	500	1000	8000	4000	Not found
MR23	Suphanburi	WRF	500	500	300	3000	Not found
MR24	Suphanburi	WRF	500	1000	1000	3000	Not found
MR26	Suphanburi	WRF	500	1000	1000	3000	Not found
MR28	Bangkok	WRF	500	2000	1000	4000	Not found

Table 4.1 (continued) Isolates of wood-rotting fungi tested for white rot/brown rot separation, heavy metal tolerance and crystal formation

Fungal isolates	Source	Type of wood-rotting fungus	Zinc (II), Copper (II), Cadmium (II) and Lead (II) toxicity threshold (ppm)				Crystal formation
			Zinc (II)	Copper (II)	Cadmium (II)	Lead (II)	
MR30	Chulalongkorn culture collection	WRF	500	500	2000	3000	Not found
MR32	Pathumthani	WRF	500	500	50	2000	Not found
MR33	Yasothorn	WRF	20000	20000	1000	3000	Not found
MR34	Kanjanaburi	WRF	500	500	200	4000	Not found
MR35	As above	WRF	4000	2000	900	8000	Not found
MR36	Bangkok	WRF	500	2000	50	4000	Not found
MR37	Nakornsrithammarat	WRF	1000	10000	7000	5000	Not found
MR38	Nakornsrithammarat	WRF	500	7000	3000	3000	Not found
MR40	Nonthaburi	BRF	3000	2000	500	20000	Found[a,b,c,d]
MR41	Nonthaburi	WRF	500	500	50	4000	Not found
MR42	Nonthaburi	WRF	500	2000	100	1000	Not found
MR43	Nan	WRF	500	500	50	2000	Not found
MR44	Nan	WRF	500	500	300	2000	Not found
MR48	Nan	WRF	2000	500	400	3000	Not found
MR50	Nan	WRF	500	4000	100	3000	Not found
MR51	Nan	WRF	2000	8000	400	2000	Not found
MR52	Nan	WRF	500	500	50	1000	Not found
MR54	Nan	WRF	500	500	200	2000	Not found

Table 4.1 (continued) Isolates of wood-rotting fungi tested for white rot/brown rot separation, heavy metal tolerance and crystal formation

Fungal isolates	Source	Type of wood-rotting fungus	Zinc (II), Copper (II), Cadmium (II) and Lead (II) toxicity threshold (ppm)				Crystal formation
			Zinc (II)	Copper (II)	Cadmium (II)	Lead (II)	
MR56	Nan	WRF	4000	500	100	3000	Not found
MR57	Nan	WRF	500	5000	50	2000	Not found
MR58	Nan	WRF	500	500	300	3000	Not found
MR60	Bangkok	WRF	500	500	50	2000	Not found
MR61	Bangkok	WRF	100	2000	100	3000	Not found
MR63	Bangkok	WRF	100	2000	300	3000	Not found
MR64	Bangkok	WRF	100	500	200	1000	Not found
MR66	Tak	BRF	5000	2000	50	1000	Not found
MR67	Tak	WRF	1000	4000	500	4000	Not found
MR69	Tak	BRF	500	9000	50	1000	Not found
MR72	Tak	WRF	100	1000	400	4000	Not found
MR73	Bangkok	WRF	100	1000	100	3000	Not found
MR75	Bangkok	WRF	5000	4000	50	4000	Not found
MR76	Kanjanaburi	WRF	100	500	50	1000	Not found
MR77	Kanjanaburi	WRF	100	500	100	1000	Not found
MR78	Nakornsrithammarat	WRF	500	1000	300	1000	Not found
MR79	Pathumthani	WRF	100	1000	50	1000	Not found
MR80	Pathumthani	WRF	500	1000	50	3000	Not found
MR81	Nakornpathom	WRF	500	500	100	1000	Not found

Table 4.1 (continued) Isolates of wood-rotting fungi tested for white rot/brown rot separation, heavy metal tolerance and crystal formation

Fungal isolates	Source	Type of wood-rotting fungus	Zinc (II), Copper (II), Cadmium (II) and Lead (II) toxicity threshold (ppm)				Crystal formation
			Zinc (II)	Copper (II)	Cadmium (II)	Lead (II)	
MR82	Nakornpathom	WRF	500	500	50	1000	Not found
MR83	Bangkok	WRF	100	100	50	500	Not found
KYO	Bangkok	BRF	2000	5000	13000	20000	Found[a,b,c,d]
WR3	Chulalongkorn culture collection	WRF	1000	2000	500	6000	Found[c,d]
WR4	Chulalongkorn culture collection	BRF	2000	4000	8000	13000	Found[a,b,c,d]
WR5	Chulalongkorn culture collection	WRF	500	500	800	5000	Found[d]

WRF: white rot fungi; BRF: brown rot fungi; [a] zinc sulfate-amended plate; [b] Copper sulfate-amended plate; [c] cadmium sulfate -amended plate; [d] lead nitrate–amended plate.

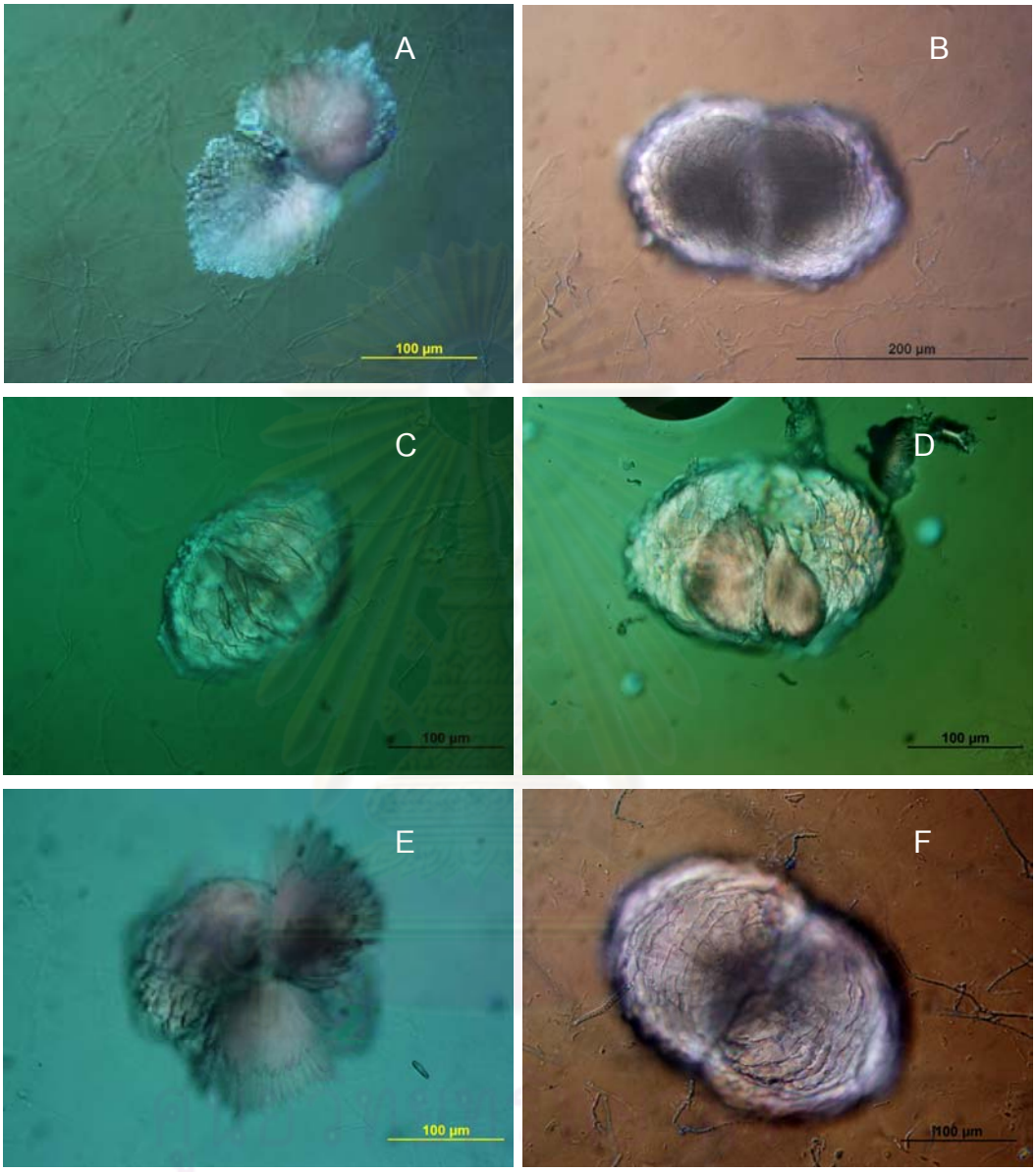

Fig. 4.2 Light microscopic images of crystals formation by brown-rot fungal isolate (A and B) MR40; (C and D) KYO and (E and F) WR4 after grown on 4% MEA media supplemented with zinc sulfate.

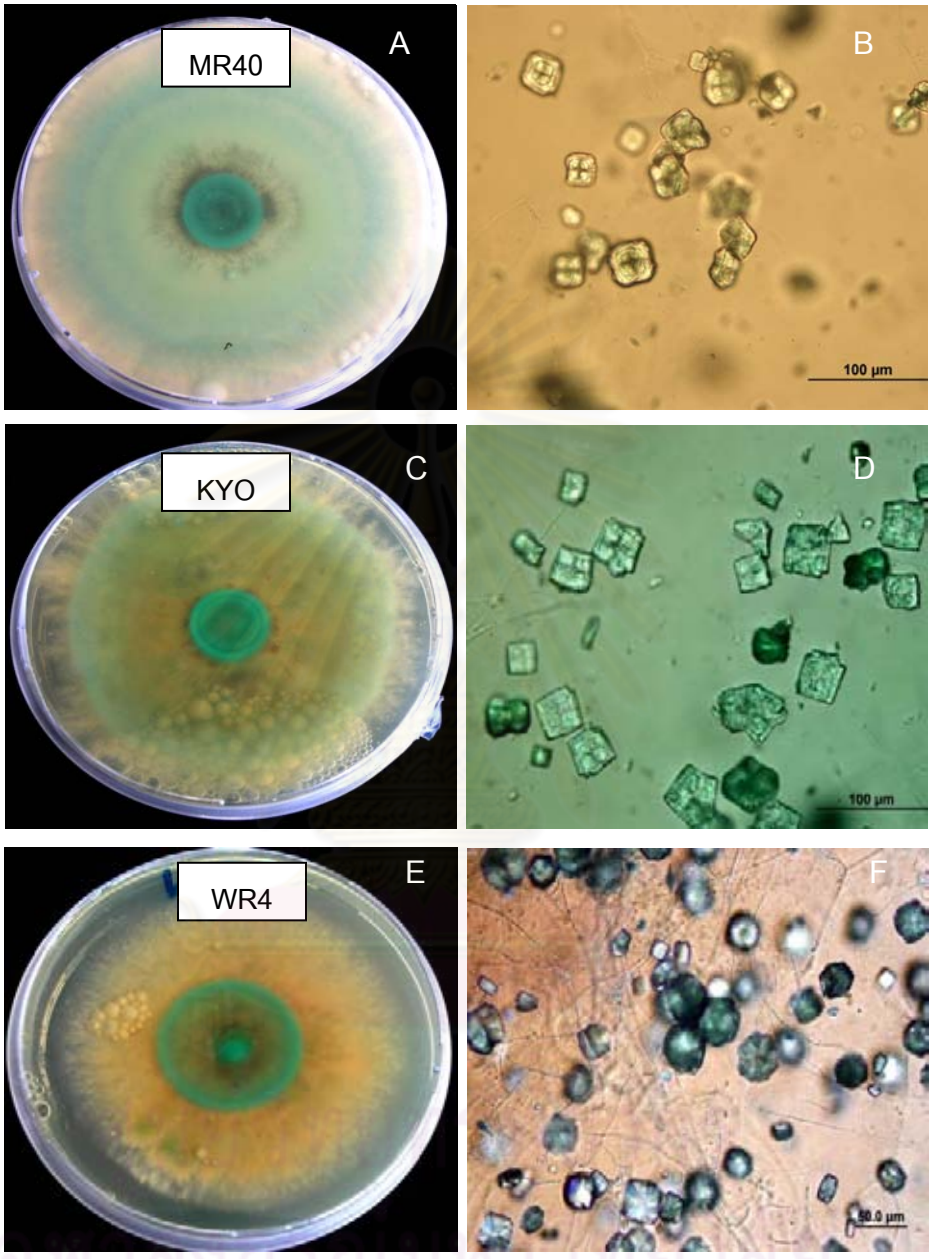

Fig. 4.3 (A, C and E) Photograph images of concentric ring patterns formation by brown-rot fungal isolate (A) MR40, (C) KYO and (E) WR4 after grown on 4% MEA media supplemented with copper sulfate. (B, D and F) Light microscopic images of the crystals formation by brown-rot fungal isolate (B) MR40, (D) KYO and (F) WR4 after grown on 4% MEA media supplemented with copper sulfate.

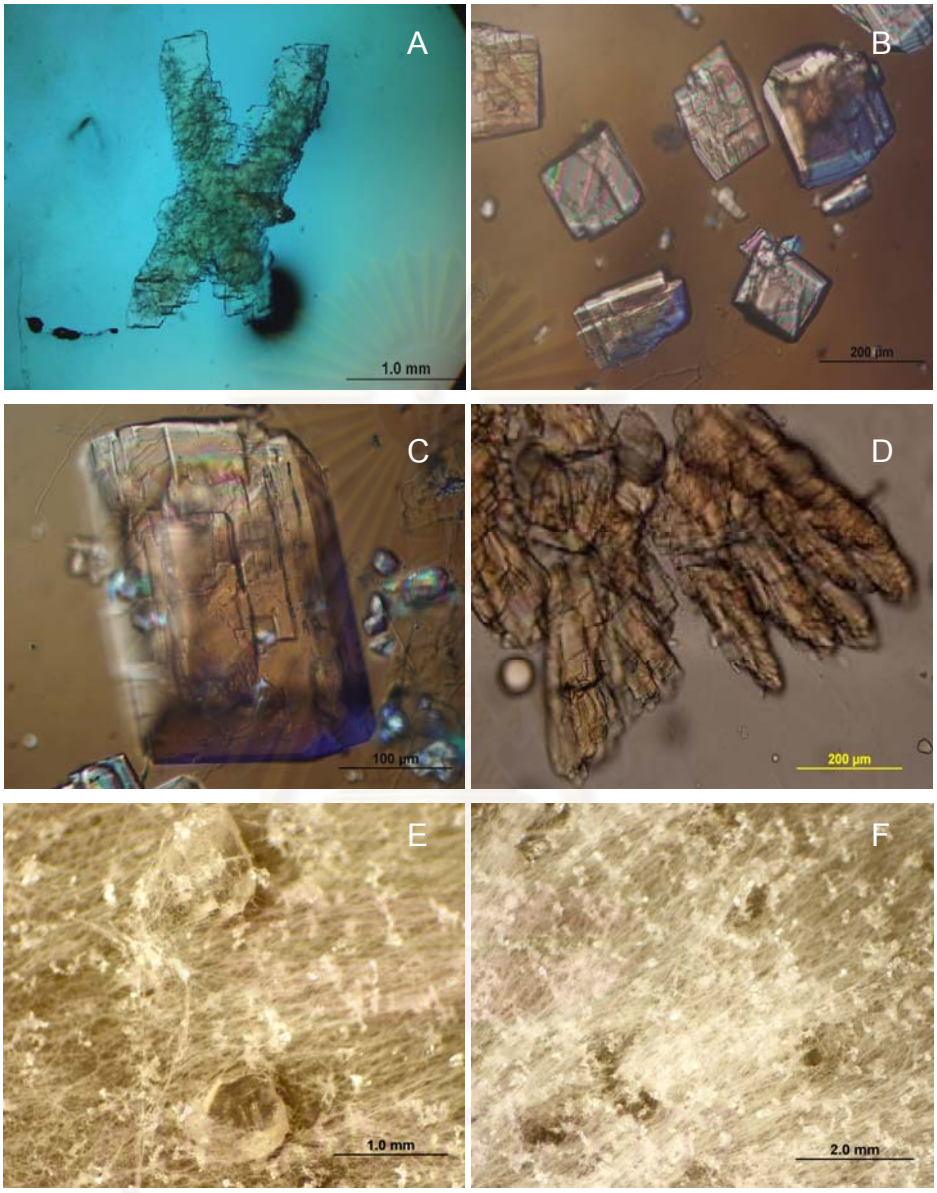

Fig. 4.4 (A to D) Light microscopic images of crystals formation by brown-rot fungal isolate (A) MR40; (B) KYO; (C) WR4 and white-rot fungal isolate (D) WR3 after grown on 4% MEA media supplemented with cadmium sulfate. (E and F) Stereo microscopic images of the crystals formation by brown-rot fungal isolate (E) KYO and (F) WR4 after grown on 4% MEA media supplemented with cadmium sulfate.

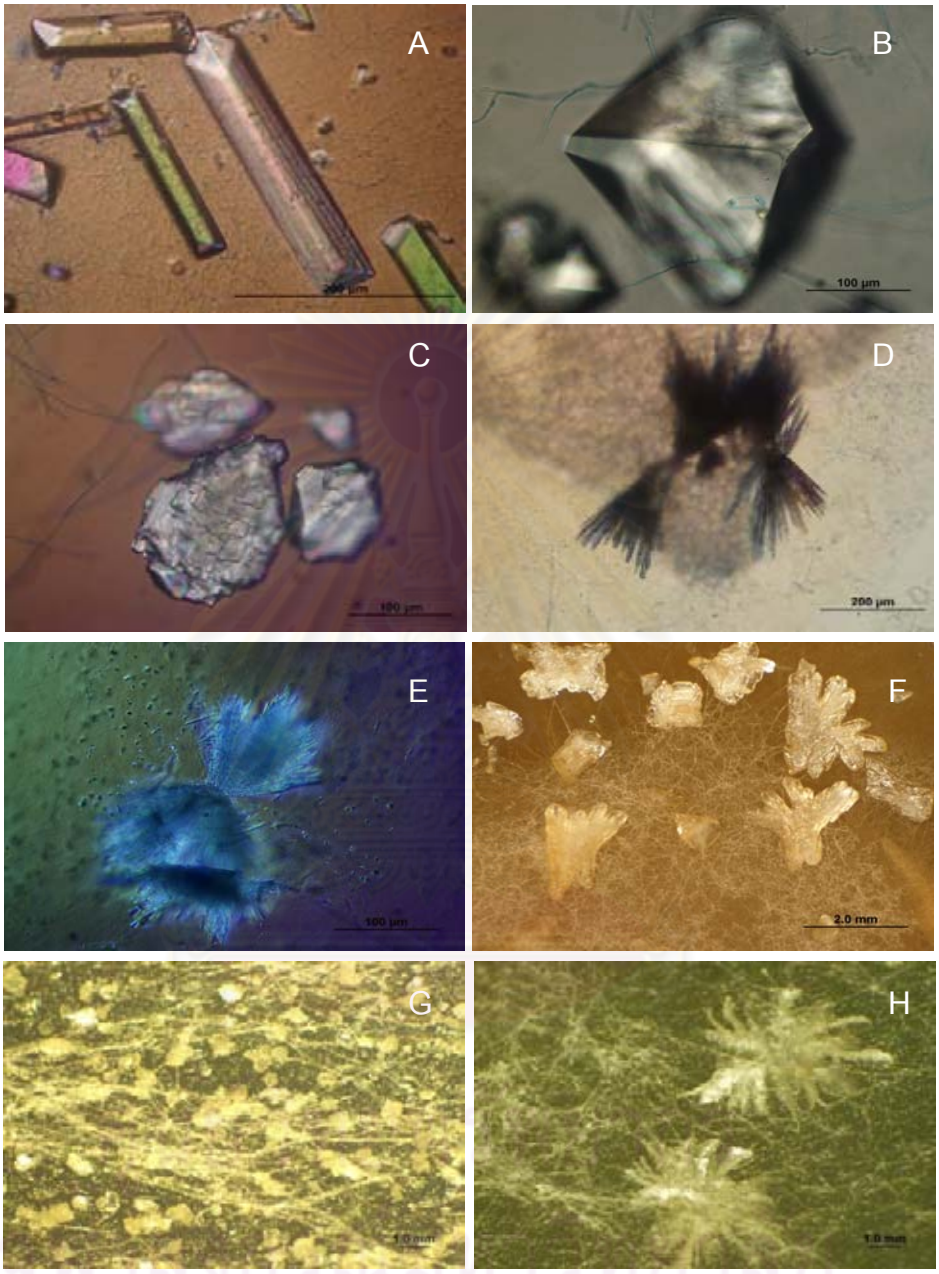

Fig. 4.5 (A to H) Light microscopic images of the crystals formation by brown-rot fungal isolate (A) MR40; (B) KYO; (C) WR4 and white-rot fungal isolate (D) WR3; (E) WR5 after grown on 4% MEA media supplemented with lead nitrate. (F to H) Stereo microscopic images of the crystals formation by brown-rot fungal isolate (F) MR40, (G) KYO and (H) WR4 after grown on 4% MEA media supplemented with lead nitrate.

4.4 Effects of heavy metals on the growth of wood-rotting fungi

4.4.1 Effect of heavy metals on the changes in the pH of medium

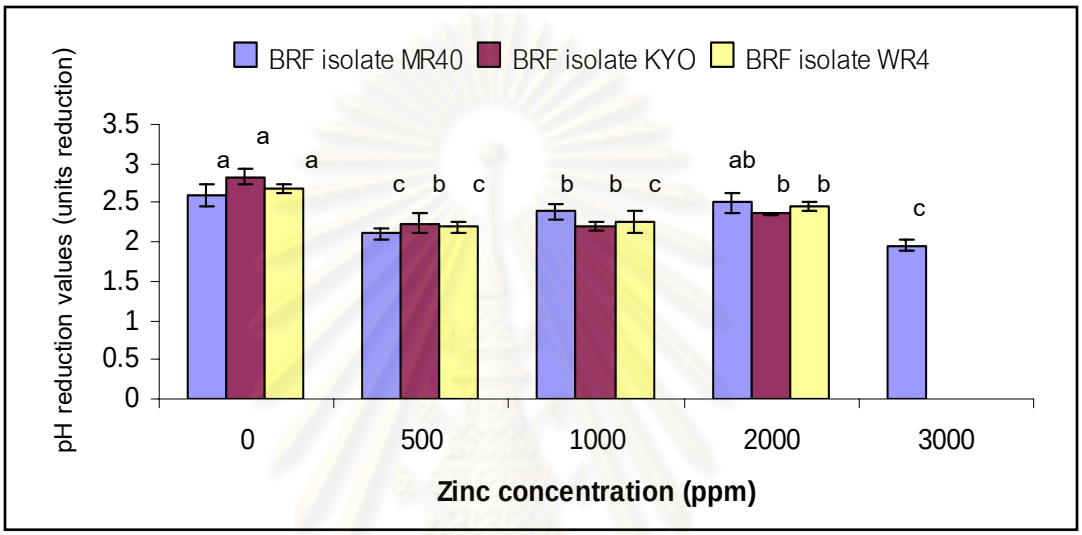

Fig. 4.6 Change in pH of MEA medium produced by brown-rot fungal isolates MR40, KYO and WR4 in the presence or absence of zinc sulfate. pH Reduction values were means and derived from at least three replicate determinations. For the same fungal isolates, the different letters (a–c) above columns indicated statistically significant differences for pH reduction values in the presence or absence of zinc sulfate at $p<0.05$ using Duncan test (one-way ANOVA).

In zinc sulfate-amended plates, the initial pH values were 4.70, 4.60, 4.52 and 4.46 for 500, 1000, 2000 and 3000 ppm zinc, respectively (Appendix B (Table B1 and B1 to B3)). When the tested fungal isolates grew on these plates, a tendency to increase acidification as the metal concentration increased was observed in all fungal cultures. At 500 ppm of zinc the greatest reduction in pH with respect to the initial pH value was obtained with brown-rot fungal isolates KYO and WR4 (Fig. 4.6) (2.2 units reduction), whereas at 1000, 2000, and 3000 ppm zinc the greatest pH reduction was belonging to brown-rot fungal

isolate MR40 (2.4, 2.5 and 2.0 units reduction, respectively). Although three brown-rot fungal isolates caused the greatest reduction in the zinc sulfate-amended plates, however the pH reductions in these cultures were slightly lower than the control cultures.

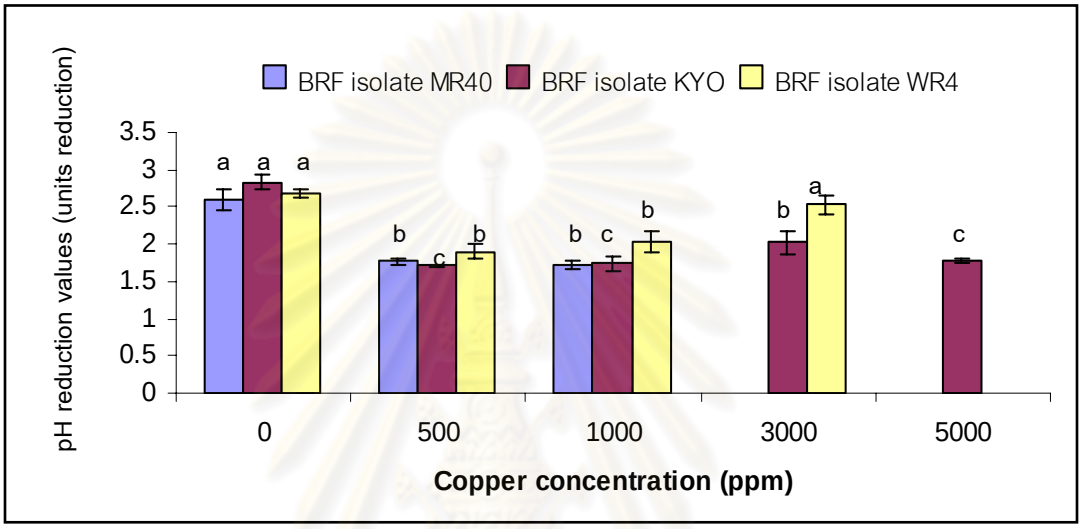

Fig. 4.7 Change in pH of MEA medium produced by brown-rot fungal isolates MR40, KYO and WR4 in the presence or absence of copper sulfate. pH Reduction values were means and derived from at least three replicate determinations. For the same fungal isolates, the different letters (a–c) above columns indicated statistically significant differences for pH reduction values in the presence or absence of copper sulfate at $p<0.05$ using Duncan test (one-way ANOVA).

In copper sulfate-amended plates, the initial pH values were 3.73, 3.46, 3.2 and 3.15 for 500, 1000, 3000 and 5000 ppm copper, respectively. After growth of fungi for 30 days, final pH values were significantly reduced, especially for brown-rot fungal isolate WR4 was the greatest pH reduction with respect to the initial pH values (Appendix B (Table B2 and B4 to B6)). At 500, 1000 and 3000 ppm copper, it could reduce the pH of the medium 1.9, 2.0, and 2.5 units reduction, respectively (Fig. 4.7). However, at the highest concentration of copper (5000 ppm), only brown-rot fungal isolate KYO could reduce the

pH of the medium (1.8 units reduction). Although brown-rot fungal isolates WR4 and KYO represented the greatest pH reduction in copper sulfate-amended plates, however they showed the lower pH reduction values when compared with the control cultures.

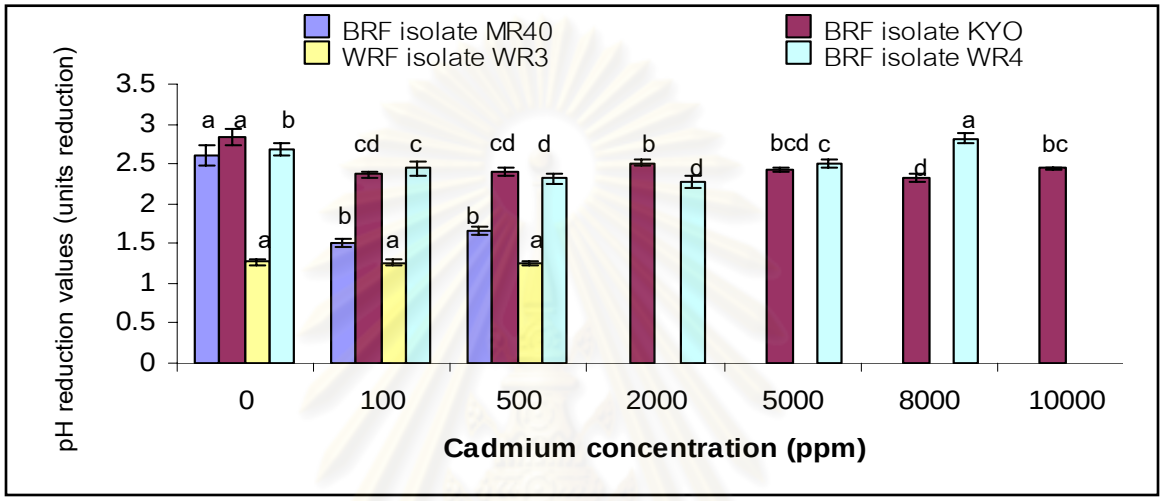

Fig. 4.8 Change in pH of MEA medium produced by brown-rot fungal isolates MR40, KYO and WR4, and white-rot fungal isolate WR3 in the presence or absence of cadmium sulfate. pH Reduction values were means and derived from at least three replicate determinations. For the same fungal isolates, the different letters (a–d) above columns indicated statistically significant differences for pH reduction values in the presence or absence of cadmium sulfate at $p<0.05$ using Duncan test (one-way ANOVA).

Initial pH values of media were 5.1, 4.76, 4.39, 4.3, 4.23 and 4.15 with supplemented with cadmium 100, 500, 2000, 5000, 8000 and 10000 ppm, respectively (Appendix B (Table B3 and B7 to B10)). The effect of adding cadmium in growth media might cause pH reduction in most cultures, especially brown-rot fungal isolates KYO and WR4 (Fig. 4.8). At 100, 5000 and 8000 ppm cadmium, brown-rot fungal isolate WR4 showed the greatest pH reduction 2.4, 2.5 and 2.8 units reduction, respectively with respect to the initial pH values. While, at 500, 2000 and 10000 ppm cadmium, brown-rot fungal isolate

KYO showed a tendency to increase acidification (2.4, 2.5 and 2.4 units reduction, respectively), with respect to the initial pH values. Although brown-rot fungal isolate WR4 and KYO caused a high pH reduction with respect to the initial pH values, however pH reduction patterns were similar to with slightly lower than in control cultures.

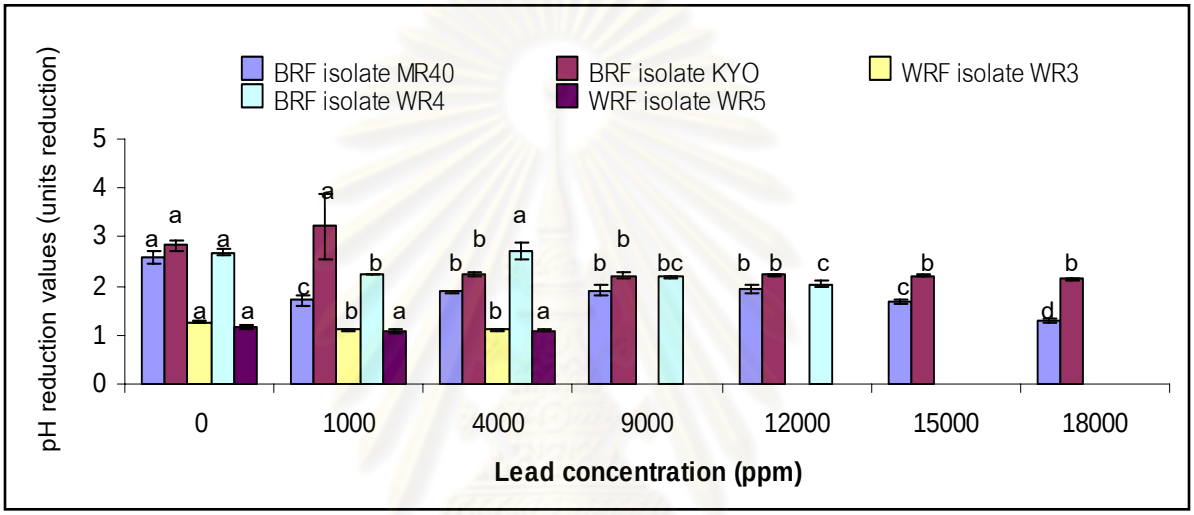

Fig. 4.9 Change in pH of MEA medium produced by brown-rot fungal isolates MR40, KYO and WR4, and white-rot fungal isolates WR3 and WR5 in the presence or absence of lead nitrate. pH Reduction values were means and derived from at least three replicate determinations. For the same fungal isolates, the different letters (a–d) above columns indicated statistically significant differences for pH reduction values in the presence or absence of lead nitrate at $p<0.05$ using Duncan test (one-way ANOVA).

Initial pH values of media were 4.5, 3.73, 3.6, 3.53, 3.48 and 3.43 with supplemented with lead 1000, 4000, 9000, 12000, 15000 and 18000 ppm, respectively. (Appendix B (Table B4 and B11 to B15)). When lead sulfate was added to MEA medium, the pH values obtained with the brown-rot fungal isolates KYO and WR4 were significantly lower than those of white-rot fungi, with respect to the initial pH values (Fig. 4.9). At 1000, 9000, 12000, 15000 and 18000 ppm lead, brown-rot fungal Isolate KYO showed the

greatest pH reduction with respect to the initial pH values (3.1, 2.2, 2.2, 2.2 and 2.1 units reduction, respectively). At 4000 ppm of lead, brown-rot fungal isolate WR4 presented the greatest pH reduction, which reduced pH medium 2.7 units reduction. Despite the fact that brown-rot fungal isolates KYO and WR4 caused the greatest pH reduction in lead nitrate-amended agar, however the pH reduction values of these cultures were similar and slightly lower than the control cultures.

From these results found that the similar patterns of pH reduction were seen in both control and metal-amended agar under the growing colonies of wood-rotting fungi, which indicated that the presences of the metal compounds did not stimulate pH reduction and/or acid production. This evidence supported research work of Burgstaller and Schinner (1993) in which the presences of the metal compound did not necessary in stimulation organic acid production by *Aspergillus niger*, whereas oxalic, citric and gluconic acids could be produced whether a metal compound was present or not. Similarly Sayer and Gadd (1997) found that the drop in pH of the agar under the growing colonies of *Aspergillus niger* was similar in the absence or presence of the metal compounds tested.

The pH reduction observed in both control and treatment which might be relate to organic acid productions such as oxalic and/or citric acids. In generally, most of brown-rot and some white-rot fungi produced significant quantities of oxalic acid in synthetic medium, although there were differences between the two fungal groups since the brown-rot fungi accumulate this acid in the medium, provoking a strong drop in the pH of the cultures. By contrast, white-rot fungi might use this acid in other metabolic pathways, impeding its accumulation as well as the marked decrease in pH (Jellison et al., 1997, Machuca et al., 2001 and Milagres et al., 2002). Other studies found that oxalic acid secreted by wood-rotting fungi has several roles in cellulose and lignin degradation, e.g. chelation and stabilization of Mn(III), providing H_2O_2 and buffering of the environment, all of which were important factors for the performance of lignin-degrading peroxidases (Perez and Jeffries, 1993; Hyde and Wood, 1997; Gadd, 1999).

4.4.2 Effect of heavy metals on the changes in the radial growth rate and biomass production

4.4.2.1 Effect of zinc sulfate on the changes in the radial growth rate and biomass production

Consequently, increasing zinc concentrations in growth medium caused a significant decrease in the radial growth of three brown-rot fungal isolates (Fig. 4.10) (Appendix B (B16 to B18)). At the zinc concentrations 500 and 1000 ppm, the radial growth of brown-rot fungal isolates MR40, KYO and WR4 were partially inhibited, whereas at higher concentration (2000 ppm of zinc) the radial growth were significantly reduced. At the highest concentration (3000 ppm of zinc), complete growth inhibition were observed in brown-rot fungal isolates KYO and WR4, and only brown-rot fungal isolate MR40 was able to grow although a significant drop in the radial growth was observed.

In contrast to the growth rate results, the presence of zinc in the MEA medium led to a significant increase of the fungal biomass in brown-rot fungal isolates KYO and WR4, which did not correlate with a marked reduction in the growth rate (Fig. 4.11). Except for brown-rot fungal isolate MR40 presented a reduction in biomass when zinc concentration was increased.

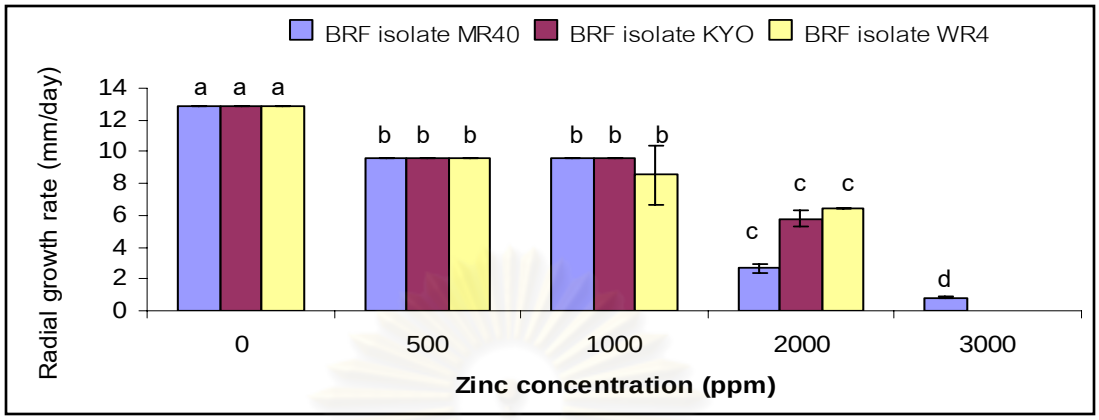

Fig. 4.10 Radial growth rate (mm/day) of brown-rot fungal isolates MR40, KYO and WR4 growing in MEA medium in the presence or absence of zinc sulfate. Means of three replicates were shown. For the same fungal isolates, the different letters (a–d) above columns indicated statistically significant differences for fungal radial growth rate (mm/day) in the presence or absence of zinc sulfate at $p<0.05$ using Duncan test (one-way ANOVA).

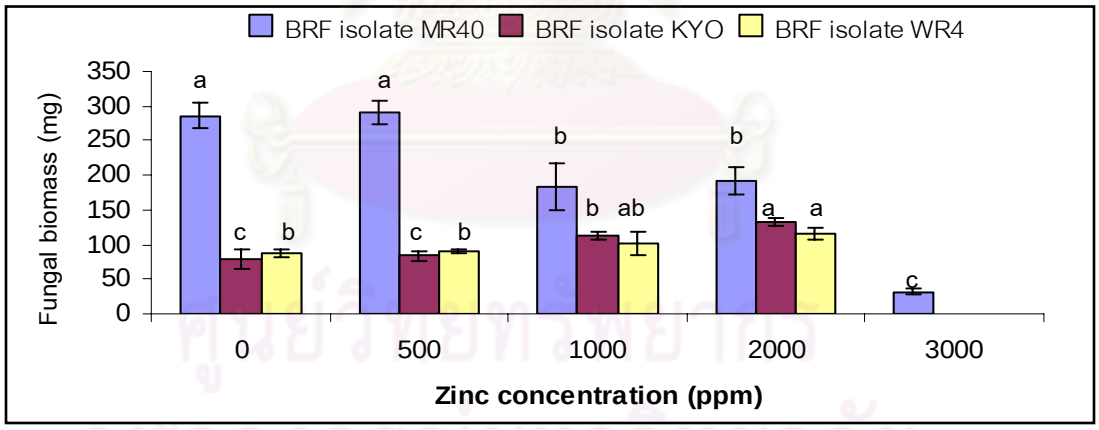

Fig. 4.11 Biomass production (mg) by brown-rot fungal isolates MR40, KYO and WR4 growing in MEA medium in the presence or absence of zinc sulfate. Means of three replicates were shown. For the same fungal isolates, the different letters (a–c) above columns indicated statistically significant differences for fungal biomass (mg) in the presence or absence of zinc sulfate at $p<0.05$ using Duncan test (one-way ANOVA).

4.4.2.2 Effect of copper sulfate on the changes in the radial growth rate and biomass production

The presence of various concentrations of copper sulfate in growth media caused the reduction of radial growth rate in all fungi (Fig. 4.12) (Appendix B (B19 to B21)). The lowest copper concentration (500 ppm copper) did not affect the growth of brown-rot fungal isolate KYO and WR4, whereas brown-rot fungal isolate MR40 was partially inhibited. Increasing copper concentration to 1000 ppm copper, brown-rot fungal isolate KYO, WR4 and MR40 showed a significant reduction in the radial growth. Complete growth inhibition in brown-rot fungal isolate MR40 was observed at 3000 ppm copper, while brown-rot fungal isolate KYO and WR4 showed a significant drop in the growth rate. At 5000 ppm copper, only brown-rot fungal isolate KYO was able to grow, however the rate of growth was decreased.

In contrast to the growth rate results, the presence of copper in the MEA medium led to a significant increase of the fungal biomass in three brown-rot fungi compared to their control culture (Fig. 4.13), which did not correlate with a strong reduction in the growth rate. Especially for brown-rot fungal isolate MR40, the biomass values obtained with this fungus in the absence or presence of copper was significantly greater than brown-rot fungal isolate KYO and WR4.

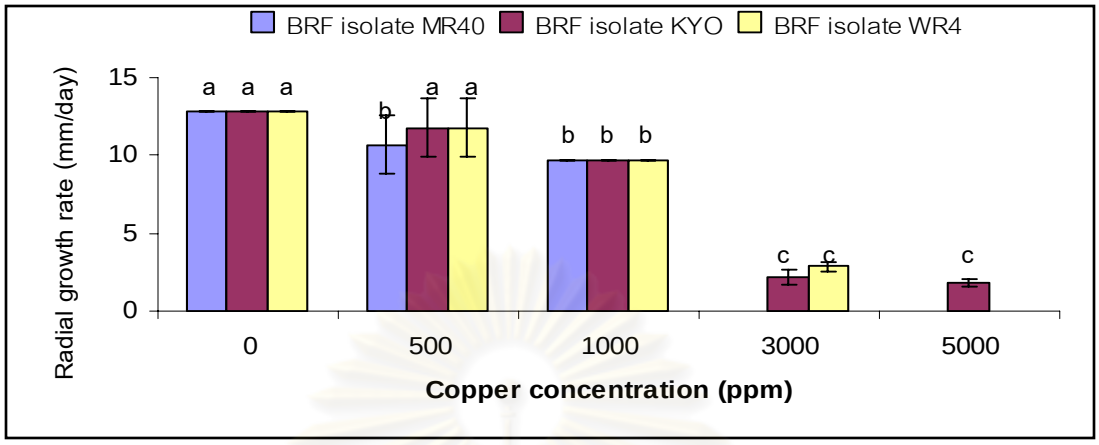

Fig. 4.12 Radial growth rate (mm/day) of brown-rot fungal isolates MR40, KYO and WR4 growing in MEA medium in the presence or absence of copper sulfate. Means of three replicates were shown. For the same fungal isolates, the different letters (a–c) above columns indicated statistically significant differences for fungal radial growth rate (mm/day) in the presence or absence of copper sulfate at $p<0.05$ using Duncan test (one-way ANOVA).

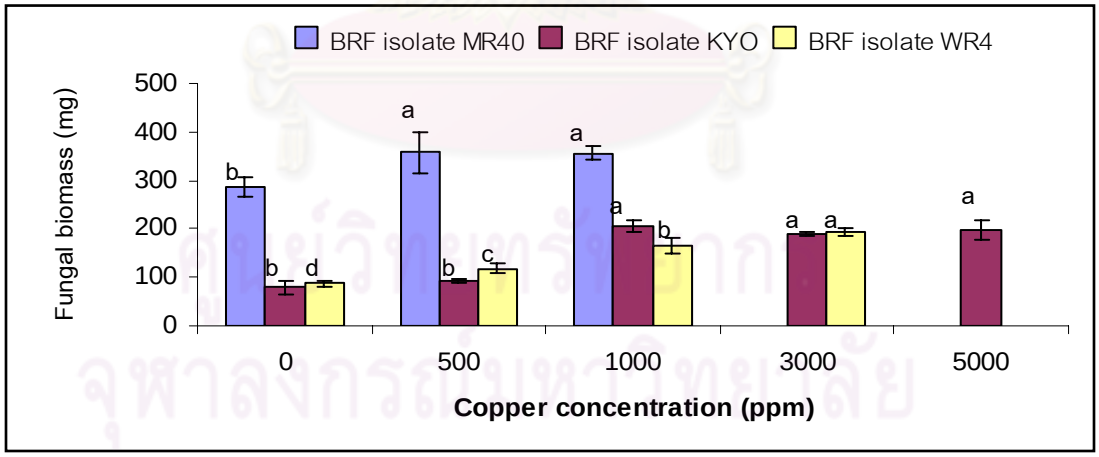

Fig. 4.13 Biomass production (mg) by brown-rot fungal isolates MR40, KYO and WR4 growing in MEA medium in the presence or absence of copper sulfate. Means of three replicates were shown. For the same fungal isolates, the different letters (a–d) above columns indicated statistically significant differences for fungal biomass (mg) in the presence or absence of copper sulfate at $p<0.05$ using Duncan test (one-way ANOVA).

4.4.2.3 Effect of cadmium sulfate on the changes in the radial growth rate and biomass production

When cadmium salt was added to the medium, the radial growth of all fungi decreased significantly with increasing cadmium concentration (Fig. 4.14) (Appendix B (B22 to B25)). The 100 ppm cadmium concentration effected in a significant decrease the radial growth of brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3. Especially brown-rot fungal isolate MR40 and white-rot fungal isolate WR3 showed a low radial growth in the presence of cadmium 100 ppm. When the cadmium was increased to 2000 ppm cadmium, complete growth inhibitions were observed in brown-rot fungal isolate MR40 and white-rot fungal WR3, while brown-rot fungal isolate KYO and WR4 were able to grow although the significant drop in the growth were observed. At 10000 ppm cadmium, only brown-rot fungal isolate KYO was able to grow, however the rate of growth was significantly reduced.

The increasing cadmium concentrations in the culture medium effected in a progressive decrease in the fungal biomass of brown-rot fungal isolate MR40 and white-rot fungal isolate WR3 compared to their control cultures, which correlated with a marked reduction in the growth rate at 500 ppm cadmium (Fig. 4.15). Brown-rot fungal isolate KYO demonstrated greater tolerance to the presence of cadmium in the MEA medium, was observed a significant stimulation of the biomass which did not correlate with a significant reduction in the radial growth. Similarly the fast growth brown-rot fungal isolate WR4 produced the high biomass in the presence of cadmium at higher concentration.

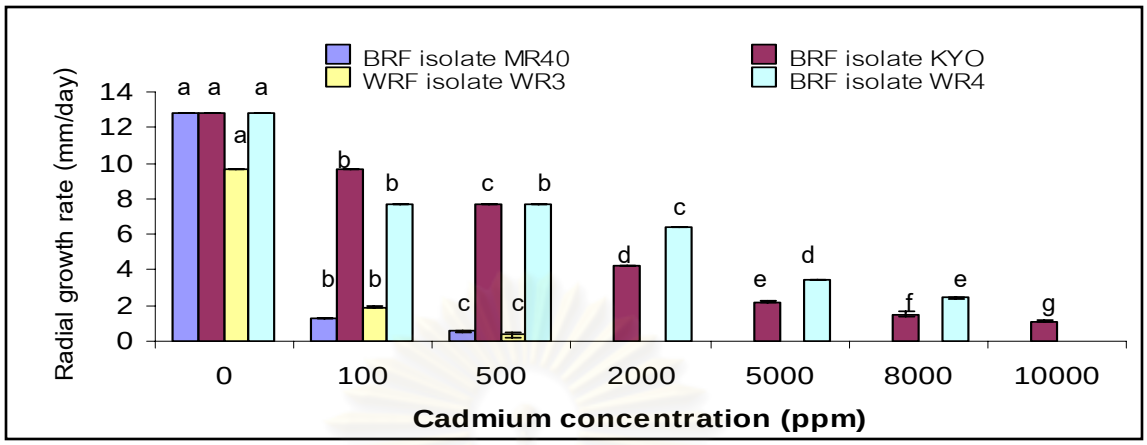

Fig. 4.14 Radial growth rate (mm/day) of brown-rot fungal isolates MR40, KYO and WR4, and white-rot fungal isolate WR3 growing in MEA medium in the presence or absence of cadmium sulfate. Means of three replicates were shown. For the same fungal isolates, the different letters (a–f) above columns indicated statistically significant differences for fungal radial growth rate (mm/day) in the presence or absence of cadmium sulfate at $p<0.05$ using Duncan test (one-way ANOVA).

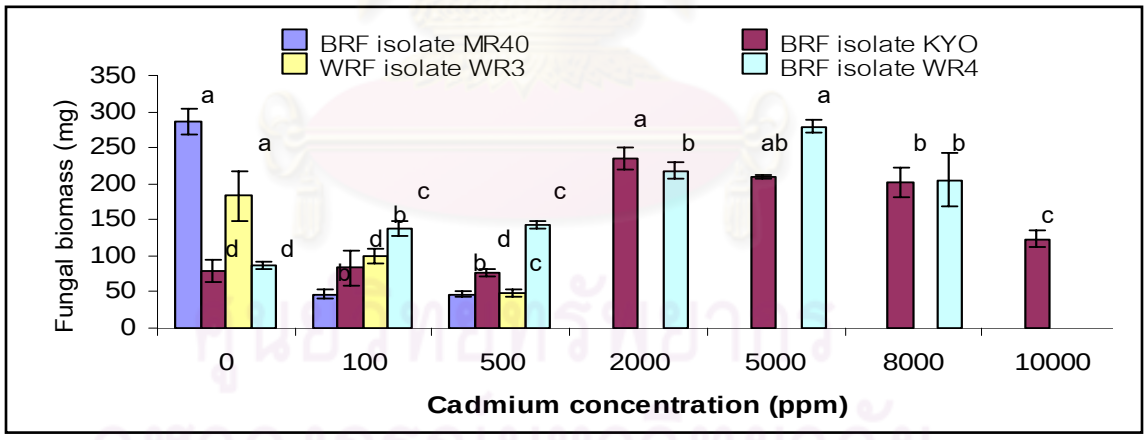

Fig. 4.15 Biomass production (mg) by brown-rot fungal isolates MR40, KYO and WR4, and white-rot fungal isolate WR3 growing in MEA medium in the presence or absence of cadmium sulfate. Means of three replicates were shown. For the same fungal isolates, the different letters (a–d) above columns indicated statistically significant differences for fungal biomass (mg) in the presence or absence of cadmium sulfate at $p<0.05$ using Duncan test (one-way ANOVA).

4.4.2.4 Effect of lead nitrate on the changes in the radial growth rate and biomass production

Increasing lead nitrate concentrations in the MEA medium effected in a progressive and significantly decrease in the radial growth of brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3, except to white-rot fungal isolate WR5 which was observed a significant stimulation of the radial growth at 1000 ppm lead (Fig. 4.16) (Appendix B (B26 to B30)). However at 4000 ppm lead, the radial growth reduction was observed in this isolate. Complete growth inhibition in white-rot fungal isolate WR3 and WR5 was observed at 9000 ppm of lead, while the growth rate of brown-rot fungal isolate MR40, KYO and WR4 were significantly reduced. At 15000 and 18000 ppm lead, only brown-rot fungal isolate MR40 and KYO showed the ability to grow, however the radial growth of these isolates were significantly reduced at the highest lead concentration. It was notice that, in the presence and absence of lead, the radial growth obtain with the brown-rot fungi were higher than those of white-rot fungi. Especially the growth rates attained by brown-rot fungal isolate KYO, was always greater than those obtained with the other species, thus proving it to be a fast-growth species with an elevated lead tolerance.

When analyzing the effect of lead on biomass production, the different fungal species presented different behavior (Fig. 4.17). In contrast to the growth rate results, the biomass values obtained with the white-rot fungi in the absence or presence of lead were significantly greater than those of the brown-rot fungi. Except for brown-rot fungal isolate MR40 showed the highest biomass in lead absence or presence. However the radial growths of white-rot fungi were significantly reduced at the higher lead concentration. A stimulatory effect on the biomass was observed in white-rot fungal isolate WR5 at 1000 ppm lead, which correlated with a significant stimulation in the growth rate at this concentration. The presence of higher lead concentration in the growth media effectted a significant

increase in the biomass production of brown-rot fungal isolate KYO and WR4, which did not correlate with a significant reduction in the growth rate.

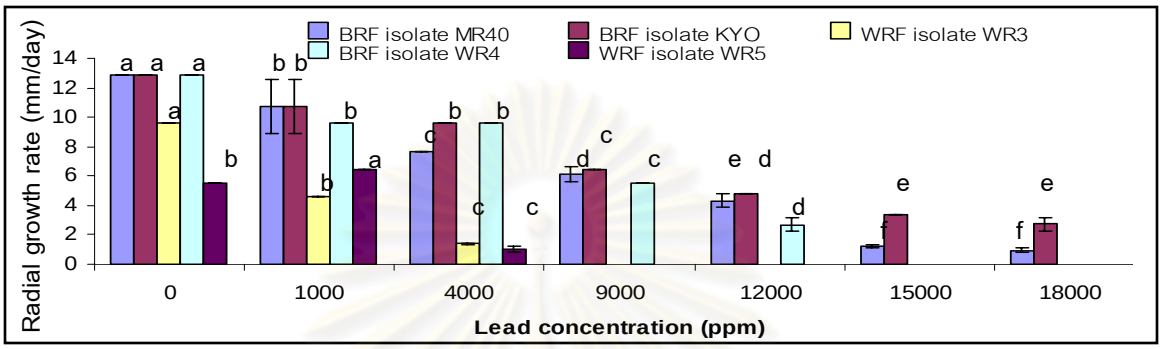

Fig. 4.16 Radial growth rate (mm/day) of brown-rot fungal isolates MR40, KYO and WR4, and white-rot fungal isolates WR3 and WR5 growing in MEA medium in the presence or absence of lead nitrate. Means of three replicates were shown. For the same fungal isolates, the different letters (a–f) above columns indicated statistically significant differences for fungal radial growth rate (mm/day) in the presence or absence of lead nitrate at $p<0.05$ using Duncan test (one-way ANOVA).

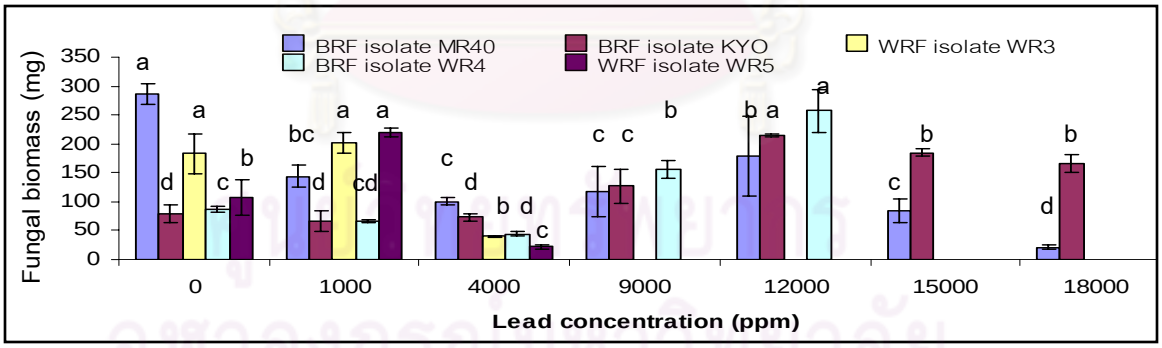

Fig. 4.17 Biomass production (mg) by brown-rot fungal isolates MR40, KYO and WR4, and white-rot fungal isolates WR3 and WR5 growing in MEA medium in the presence or absence of lead nitrate. Means of three replicates were shown. For the same fungal isolates, the different letters (a–d) above columns indicated statistically significant differences for fungal biomass (mg) in the presence or absence of lead nitrate at $p<0.05$ using Duncan test (one-way ANOVA).

From this result, it was evident that increasing concentrations of heavy metal compounds in the culture medium effected in a significant decrease in the radial growth of brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3 and WR5. In contrast, the presence of heavy metals in the culture medium led to a significant increase of the fungal biomass in most of the cultures. If we compared the radial growth rate and the biomass production in heavy metals presence, it was apparent that the radial growth rate was much more sensitive to the heavy metal compounds than the biomass production. The great biomass production was observed in the heavy metal presence might be correlated with the fungal mycelia grown at higher heavy metal concentrations on agar plates were more dense, which could probably be attributed to changes in hyphal branching. This evidence supported the research of Darlington and Rauser (1988), we found that addition of cadmium led to the increase in hyphal density of *Paxillus involutus*, which caused by increased number of laterals per branch point and a decrease of the distance between branch points. In another study by Gabriel et al. (1996) found that cadmium increases the density of mycelium by increased branching. Similarly to Baldrian and Gabriel (2002) reported that addition of cadmium to nutrient media resulted in reduction of relative growth rate of *Piptoporus betulinus*, while the biomass production was not affected. Moreover, in liquid culture, where *P. betulinus* forms spherical pellets, pellets in absence of metals were ‘‘hairy’’ with loose, longer hyphae, whereas in the presence of cadmium the surface of a pellet was smooth, formed by a dense layer of hyphal tips.

The unexpected great increasing in biomass might be related to the adsorption of metal ions onto the fungal mycelium. Several metals had been found in the mycelium in high concentrations when the fungal species grow in the presence of metal ions in solid medium or in preserved wood; in such case, the adsorption onto the cell wall was species-specific (Falih, 1997; De Groot and Woodward, 1999; Gabriel et al., 2001; Baldrian, 2003). Additionally the fungal species in this study, which could produce high amounts of biomass in the presence of zinc, copper, cadmium and lead should be used to treat heavy-metal-

contaminated industrial and municipal effluents through biosorption. Basidiomycete fungi that colonize wood were promissing organisms for metal biosorption, since they were easily cultivated on varieties of substrates, synthetic or lignocellulosics and show high biomass production (Baldrian, 2003).

4.5 Analysis of heavy metal transformation by brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3 and WR5

4.5.1. Elemental composition and morphology of crystals produced by brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3 and WR5 during growth on zinc sulfate, copper sulfate, cadmium sulfate and lead nitrate-amend medium

The numerous crystals formed by brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3 and WR5 when grown on zinc sulfate, copper sulfate, cadmium sulfate and lead nitrate-amended plates were examined the elemental composition using Energy Dispersive X-ray micro-Analysis (EDXA) and observed the crystals structure by using Scanning Electron Microscope (SEM). The results showed that the X-ray microanalysis of individual crystals showed a peak for zinc in the crystals from zinc sulfate-amended plates (Fig. 4.18A), a peak for copper in the crystals from copper–amended plates (Fig. 4.18B), a peak for cadmium in the crystals from cadmium sulfate-amended plates (Fig. 4.18C) and a peak for lead in the crystals from lead nitrate-amended plates (Fig. 4.18D).

Fig. 4.19-4.22 showed scanning electron micrographs of the crystals formed by brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3 and WR5 and the original zinc sulfate, copper sulfate, cadmium sulfate and lead nitrate. From the experiment found that the growing of brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3 and WR5 on the zinc sulfate, copper sulfate, cadmium sulfate and lead nitrate-amended medium led to the different form of crystals when compared to the original crystals of zinc sulfate, copper sulfate, cadmium sulfate and lead nitrate.

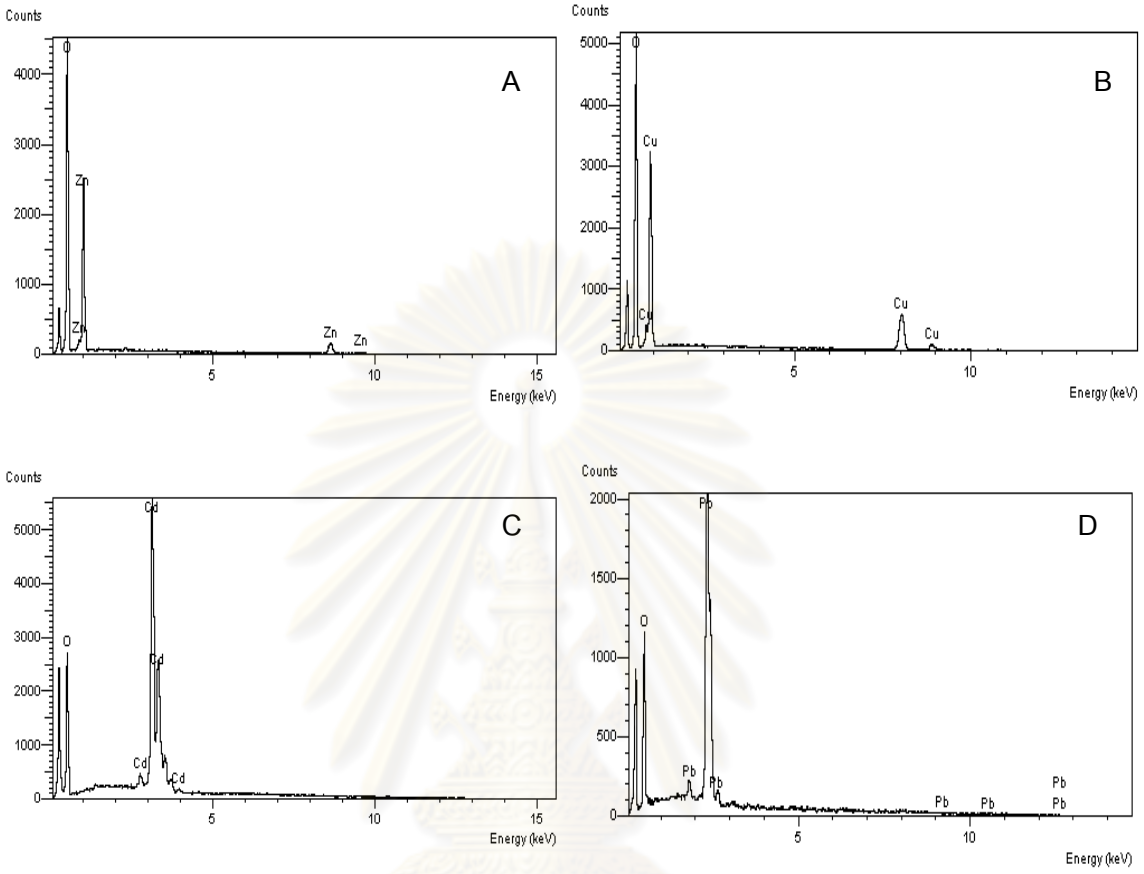

Fig. 4.18 Elemental analysis of crystals by Energy-Dispersive X-ray micro-Analysis (EDXA) showed X-ray spectra pattern. (A) Zinc spectra pattern of crystals produced by brown-rot fungal isolate MR40 grown in MEA media supplemented with zinc sulfate; (B) copper spectra pattern of crystals produced by brown-rot fungal isolate MR40 grown in MEA media supplemented with copper sulfate; (C) cadmium spectra pattern of crystals produced by brown-rot fungal isolate MR40 grown in MEA media supplemented with cadmium sulfate, and (D) lead spectra pattern of crystals produced by brown-rot fungal isolate MR40 grown in MEA media supplemented with lead nitrate. Identical peaks were also obtained for purified crystals from brown-rot fungal isolate KYO and WR4 and white-rot fungal isolate WR3 and WR5 (Appendix C).

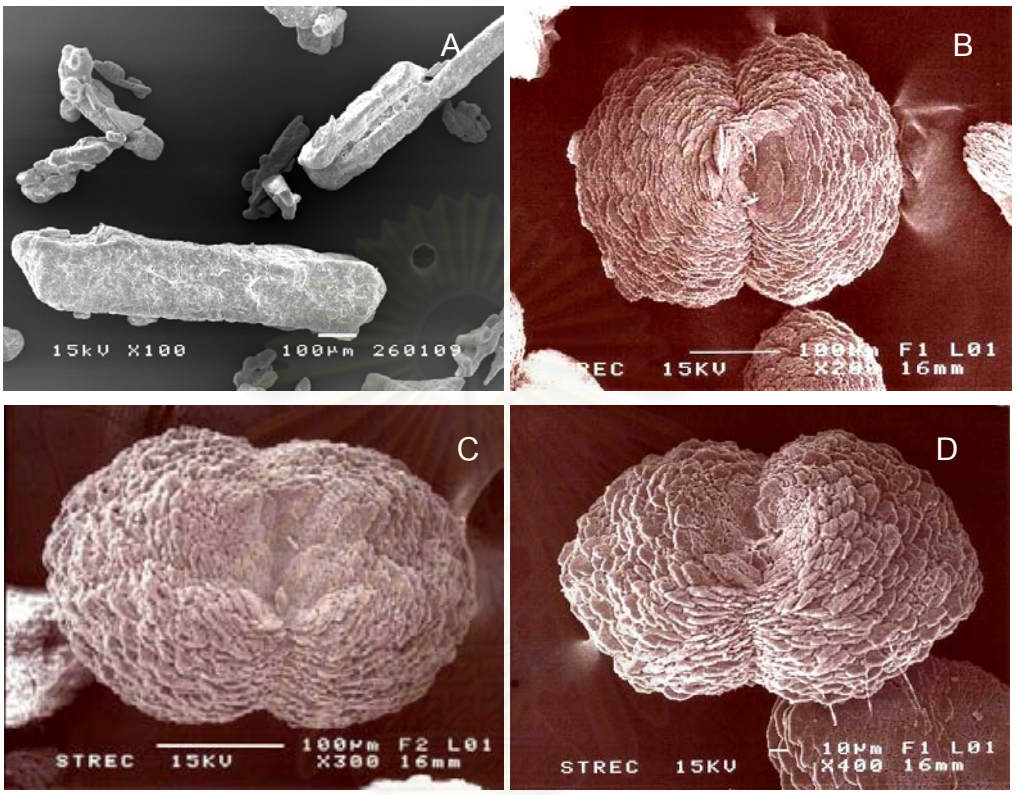

Fig. 4.19 (A) Scanning electron micrograph of zinc sulfate salt; and zinc crystals formed by brown-rot fungal (B) isolate MR40; (C) isolate KYO; and (D) isolate WR4.

In zinc sulfate-amended plates, brown-rot fungal isolate MR40, KYO and WR4 turned zinc sulfates into twinned bulbous crystals of zinc (Fig. 4.19B to D), which differed from zinc sulfate salt (Fig. 4.19A). The crystals produced by three brown-rot fungal isolates were white and the sizes of the crystals were approximately 50 mm x 70 mm (isolate MR40), 60 mm x 100 mm (isolate KYO) and 40 mm x 80 mm (isolate WR4).

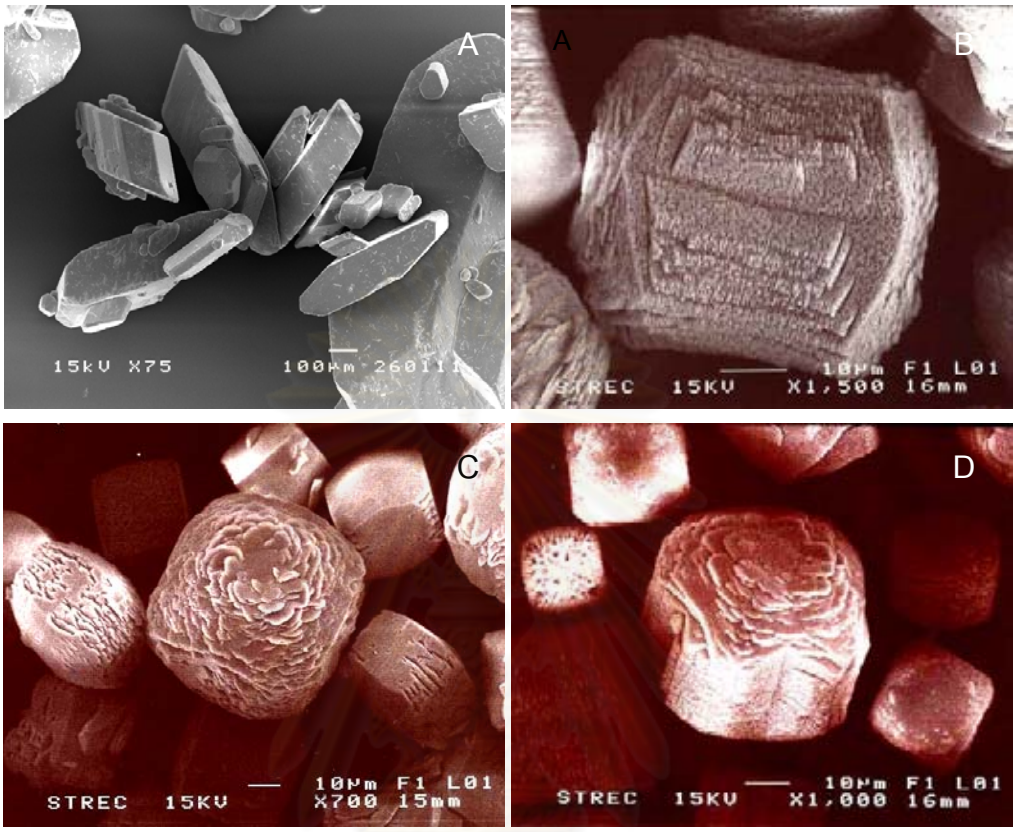

Fig. 4.20 (A) Scanning electron micrograph of copper sulfate salt; and copper crystals formed by brown-rot fungal (B) isolate MR40; (C) isolate KYO; and (D) isolate WR4.

In copper sulfate-amended plates, brown-rot fungal isolate MR40, KYO and WR4, turned copper sulfate into tetragonal crystals of copper (Fig. 4.20B to D), which were green and differed from copper sulfate salt (Fig. 4.20A). The sizes of copper crystals produced from these isolates were approximately 65 mm x 80 mm (isolate MR40), 40 mm x 50 mm (isolate KYO) and 30 mm x 40 mm (isolate WR4).

In cadmium sulfate-amended plates, brown-rot fungal isolate MR40 turned cadmium sulfate into the star-shaped crystals with four arms (Fig. 4.21B), which were white. Each arm was approximately 20 mm in wide and 50 mm in length. Brown-rot fungal isolate KYO and WR4 turned cadmium sulfate into tabular crystals of cadmium (Fig. 4.21C and D). In contrast, cadmium crystals formed by white-rot fungal isolate WR3 were light brown with two arms, the size of each arm was approximately 20 mm in wide and 90 mm in length (Fig. 4.21E). These crystals were white and the sizes of crystals were approximately 40 mm x 50 mm (isolate KYO) and 45 mm x 55 mm (isolate WR4). The crystal formation from all isolates differed from the original crystal of cadmium sulfate (Fig. 4.21A).

In lead nitrate-amended plates, brown-rot fungal isolate MR40 and white-rot fungal isolate WR3 and WR5 turned lead nitrate into aggregated-prismatic bar crystals of lead (Fig. 4.22B, E and F, respectively). Lead crystals formed by brown-rot fungal isolate MR40 were dark brown and were too large approximately 20 mm in wide and 90 mm in length. In white-rot fungal isolate WR3 and WR5, lead crystals were light brown and the size were approximately 25 mm x 90 mm and 2 mm x 12 mm, respectively. Brown-rot fungal isolate KYO turned lead nitrate into single prismatic bar crystals of lead (Fig. 4.22C). These crystals were yellow-brown and the size was approximately 10 mm in wide and 40 mm in length. Brown-rot fungal isolate WR4 turned lead nitrate into lead crystals, which were cream-white and had four arms. Each arm was approximately 20 mm in wide and 55 mm in length. (Fig. 4.22D). Lead crystals produced by all fungal isolates differed from the original crystal of lead nitrate (Fig. 4.22A).

From these crystallline images were found that different metals resulted in different and characteristic crystal morphologies.

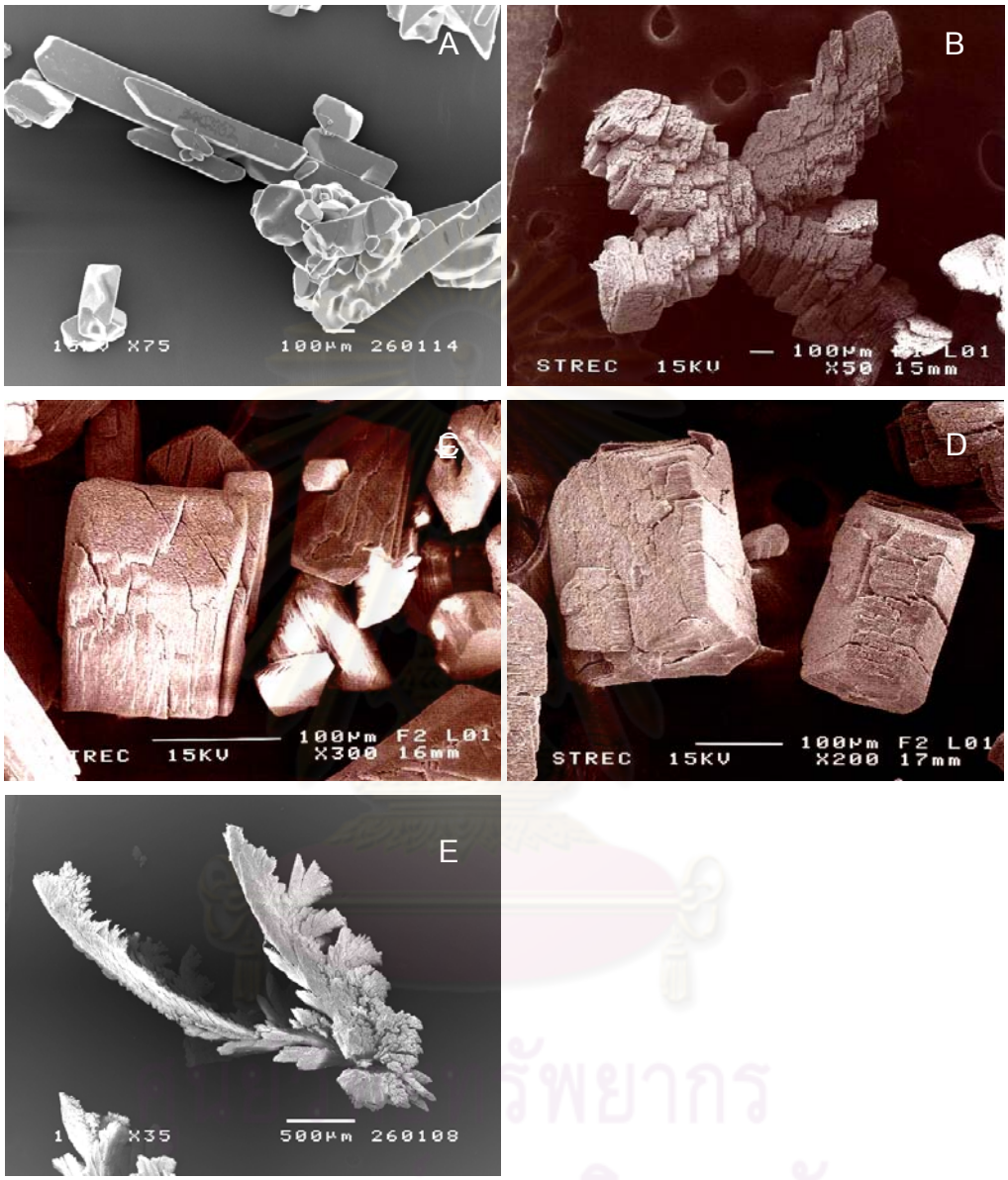

Fig. 4.21 (A) Scanning electron micrograph of cadmium sulfate salt; and cadmium crystals formed by brown-rot fungal (B) isolate MR40; (C) isolate KYO; (D) isolate WR4; and (E) white-rot fungal isolate WR3.

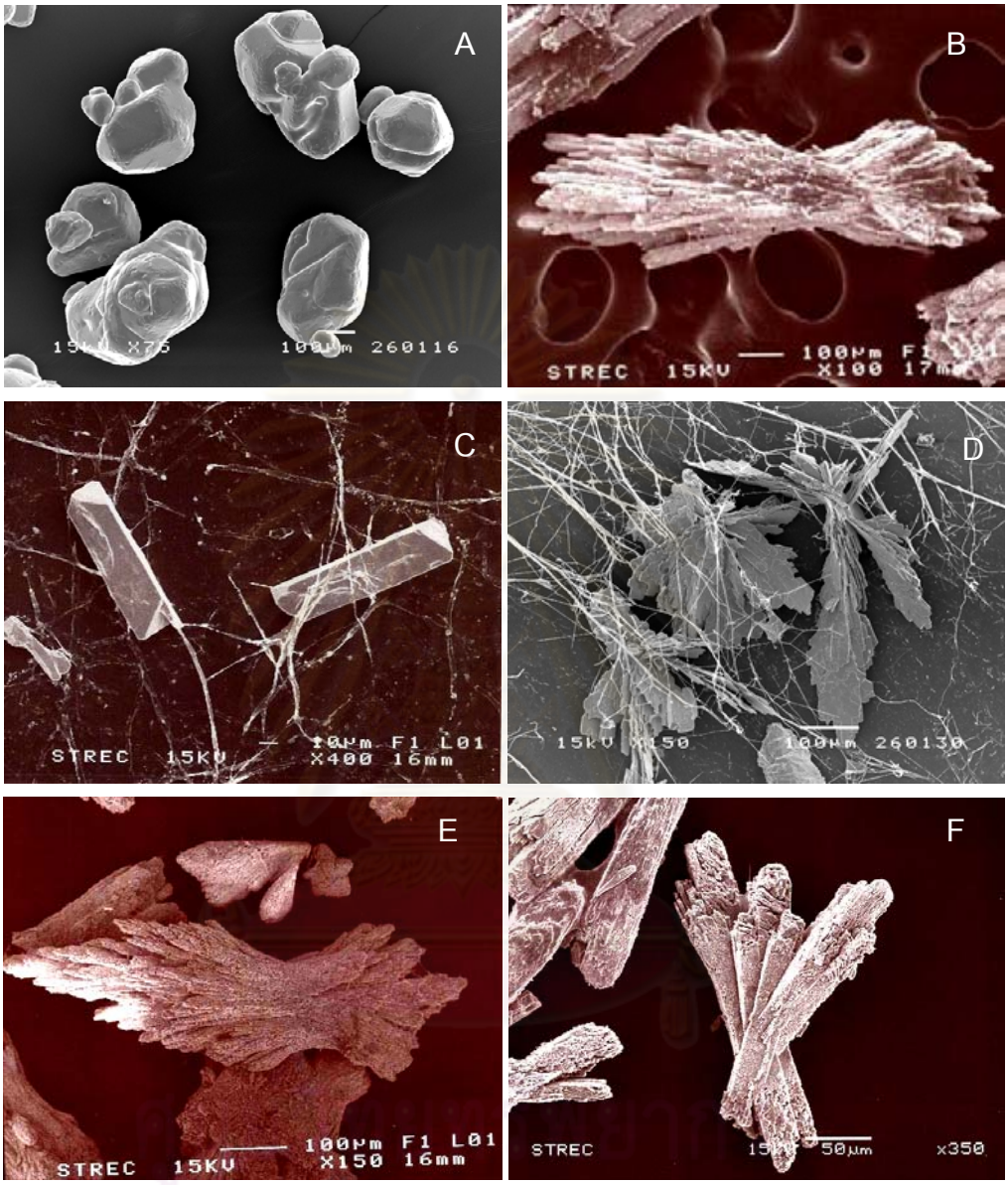

Fig. 4.22 (A) Scanning electron micrograph of lead nitrate salt; and lead crystals formed by brown-rot fungal (B) isolate MR40; (C) isolate KYO; (D) isolate WR4; and white-rot fungal (E) isolate WR3 and (F) isolate WR5.

The metal crystals were observed not only in the agar but also on the fungal mycelium above the dialysis membrane (Fig. 4.23-4.26). Scanning electron microscopy revealed that zinc crystals formed by brown-rot fungal isolate WR4 were encrusted with a net of fungal mycelium (Fig. 4.23A and B). Copper crystals formed by brown-rot fungal isolate MR40, KYO and WR4 were entrapped with fungal hyphae (Fig. 4.24A to F). Especially brown-rot fungal isolate KYO and WR4 when grown on copper sulfate-amend plates, the crystals formed by these isolates were embedded with the mycelial cords. Cadmium crystals were embedded with fungal hyphae of brown-rot fungal isolate KYO (Fig. 4.25A to C) and WR4 (Fig. 4.25D to F). Similarly lead crystals were usually entrapped with the fungal hyphae of brown-rot fungal isolate MR40, KYO and WR4 (Fig.4.26A to F).

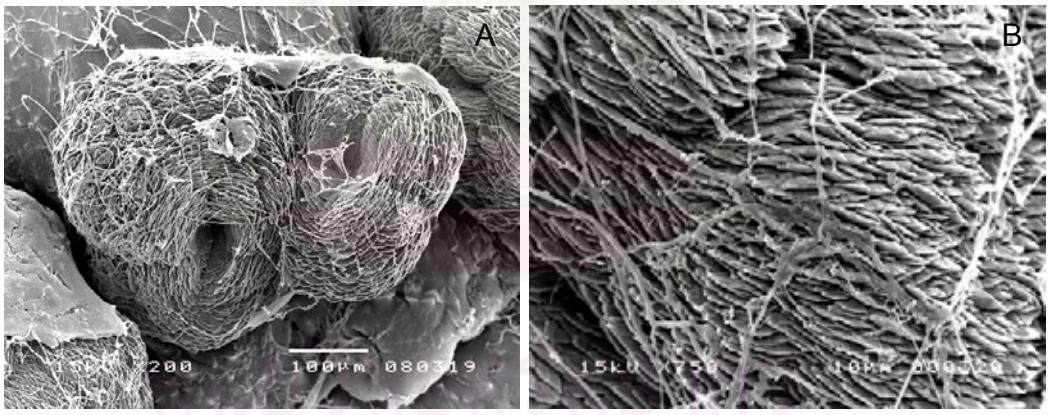

Fig. 4.23 (A) Scanning electron micrograph of zinc crystals associated with the fungal mycelium of brown-rot fungal isolate WR4. (B) High magnification scanning electron micrograph of zinc crystals with the fungal mycelium.

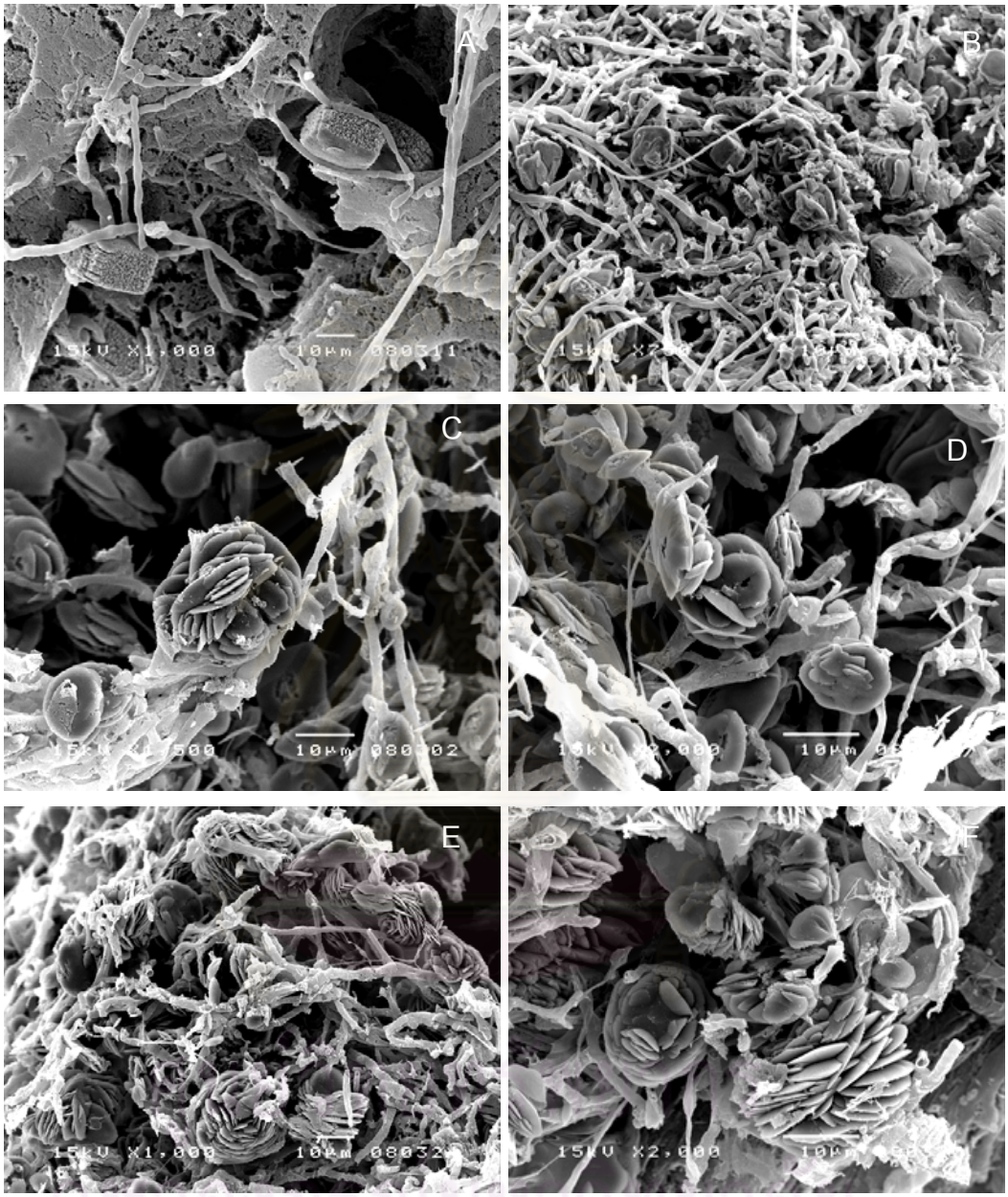

Fig. 4.24 Scanning electron micrograph of copper crystals formed in the culture of brown-rot fungal (A and B) isolate MR40, (C and D) isolate KYO and (E and F) isolate WR4.

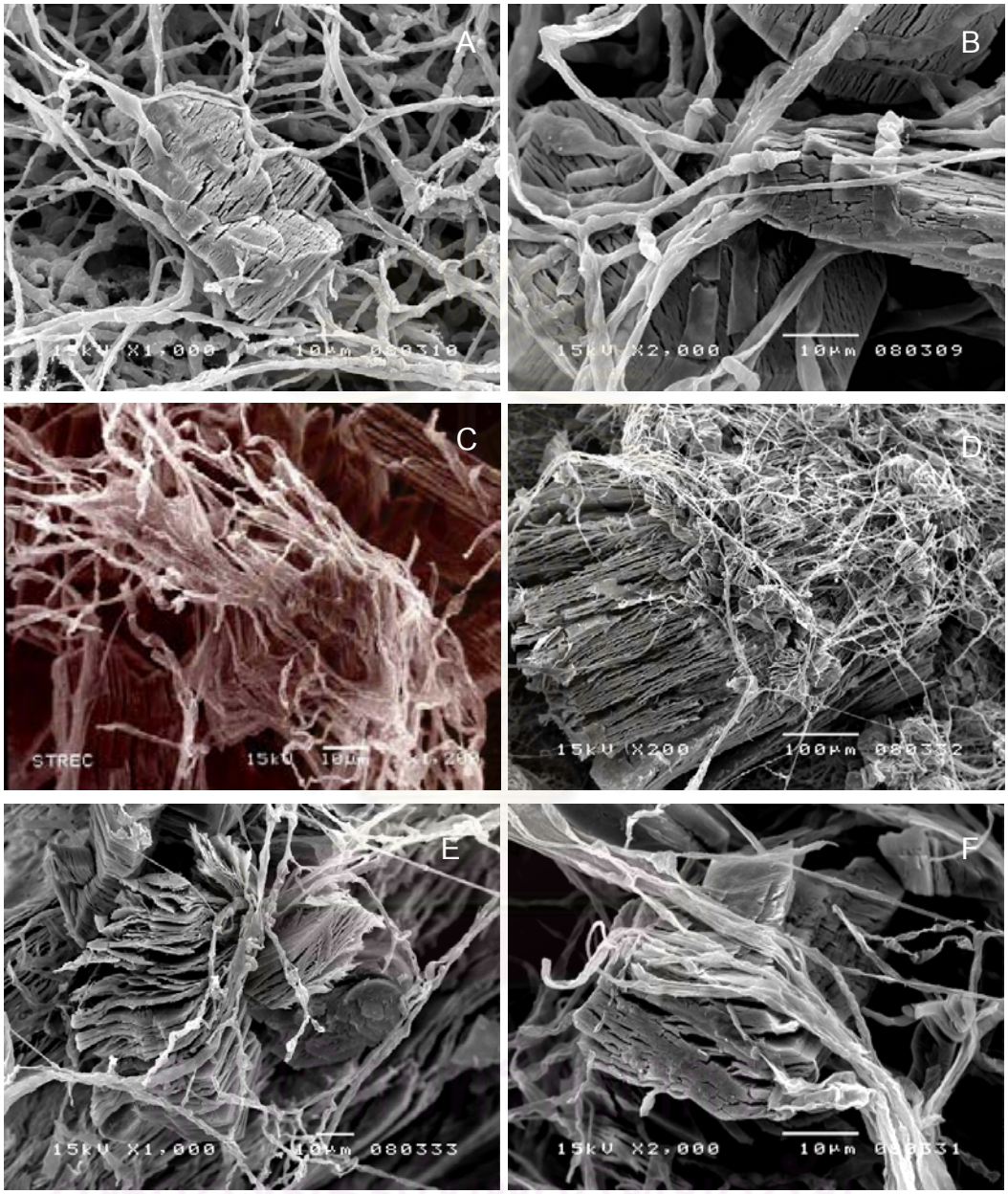

Fig. 4.25 Scanning electron micrograph of cadmium crystals associated with the fungal mycelium of brown-rot fungal (A to C) isolate KYO and (D to F) isolate WR4.

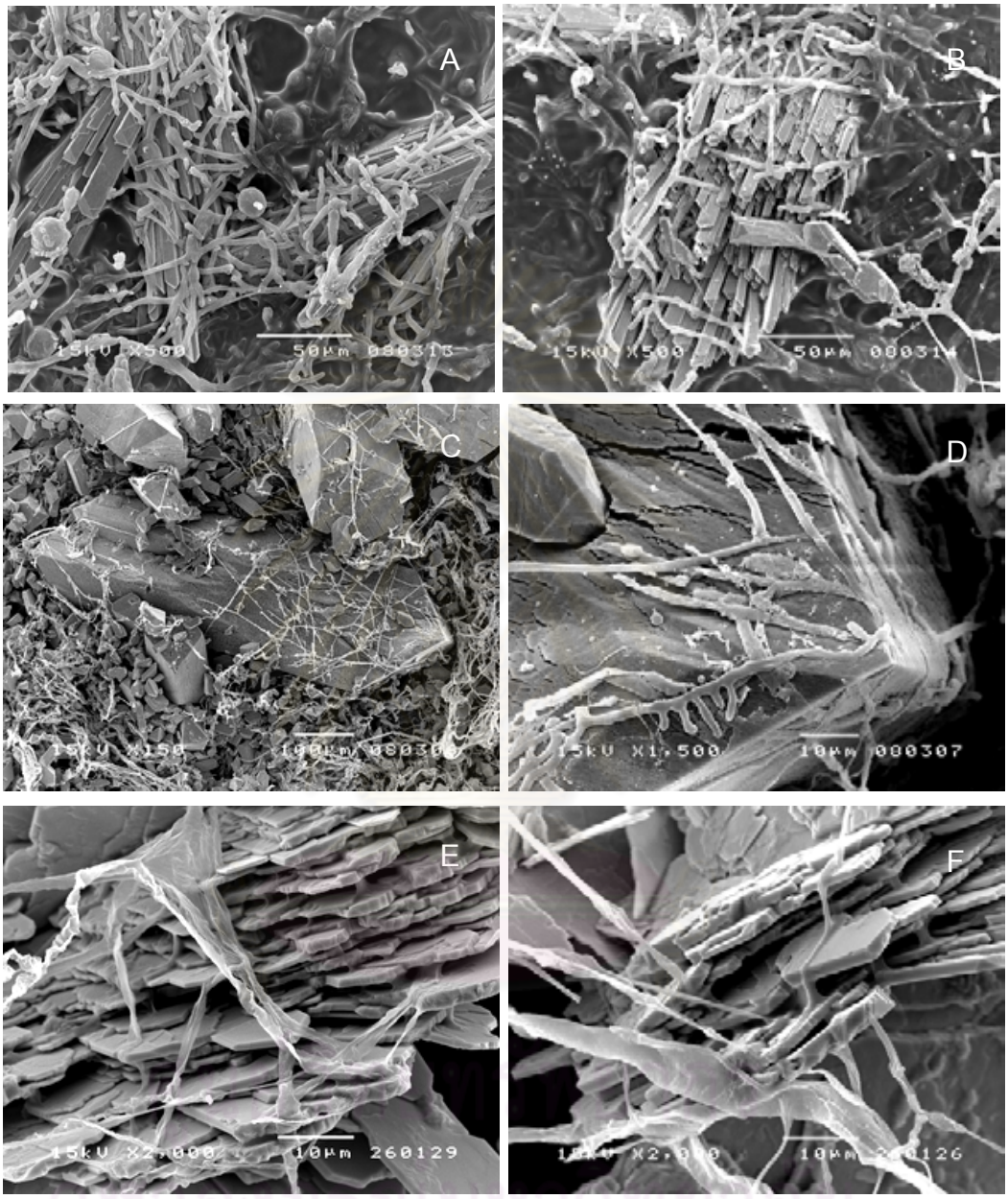

Fig. 4.26 Scanning electron micrograph of lead crystals associated with the fungal mycelium of brown-rot fungal (A and B) isolate MR40, (C and D) isolate KYO and (E and F) isolate WR4.

4.5.2 Determination of organic acids by HPLC

After dissolution of samples of these metal crystals, typical HPLC chromatograms of these crystals spiked with aliquots of oxalic acid. It could be seen that the peak for oxalic acid (occurring at 3.33 min) (Fig. 4.27A) was an exact match with the peak for the zinc crystals (Fig. 4.27F), copper crystals (Fig. 4.27G), cadmium crystals (Fig. 4.27H) and lead crystals (Fig. 4.27I). In contrast, citric acid (peak occurring at 6.6 min) (Fig. 4.27B), acetic acid (peak occurring at 5.21 min) (Fig. 4.27C), fumalic acid (peak occurring at 9.57 min) (Fig. 4.27D) and succinic acid (peak occurring at 6.9 min) (Fig. 4.27E) did not match any peaks for these crystals. (In the present study, white rot fungal isolates WR3 and WR5 did not study in HPLC analysis because of they produced little quantity of metal oxalate crystals).

These results suggest that the crystals consist mainly of oxalic acid which indicated that oxalic acid as an important metabolite elaborated in the response of wood-rotting fungi to toxic metal stress. This phenomenon support the report by Rabanus (1933) and Shimazono and Takubo (1952), which suggested that metal tolerance of brown-rot fungi was linked to oxalic acid production, which presumably precipitated copper into the insoluble form of copper oxalate, rendering the metal metabolite inert. Similarly study of Sutter et al. (1983) which reported that the wood rotting fungi *Poria placenta* and *Poria vaillantii* could immobilize copper in wood treated with copper sulfate as a preservative. The copper was removed almost completely from treated wood by the production of oxalic acid and appeared on the surface of the wood and around the hyphae as copper oxalate, reported to be non-toxic to the fungi because of its insolubility. Sayer and Gadd (1997) and Gadd (1999) suggested that the production of oxalic acid by fungi provided a means of immobilizing soluble metal ions, or complexes, as insoluble oxalates, which decreasing bioavailability and increasing tolerance to the toxic metals. In another study Green and Clausen (2003) showed that oxalic acid was a key component in the successful colonization and degradation of copper citrate-treated wood by brown-rot fungi.

In treated wood, oxalic acid reacts with copper in the wood to form an insoluble copper oxalate, which was precipitated. This mechanism was considered to contribute to the detoxification of copper in copper-treated wood and this enables fungi to tolerate environments containing high concentrations of copper and other toxic metals. Since copper oxalate was insoluble, copper in this form has less inhibitory effect on fungal growth (Richardson, 1997; Humar et al., 2002).

In this study white rot fungal isolate WR3 and WR5 produced less quantity of metal oxalate crystals than the brown rot fungal isolate MR40, KYO, WR4, which might be involved the production of oxalate decarboxylase enzyme by white-rot fungi. White rot fungi were known to excrete oxalic acid into their surroundings at lower concentrations than brown rot fungi (Akamatsu et al., 1994). This difference was thought to be due to the expression of oxalate decarboxylase in white rot fungi, which decomposes oxalic acid to formic acid and carbon dioxide, and was considered to have a key role in regulating the level of oxalate inside the fungal cells.

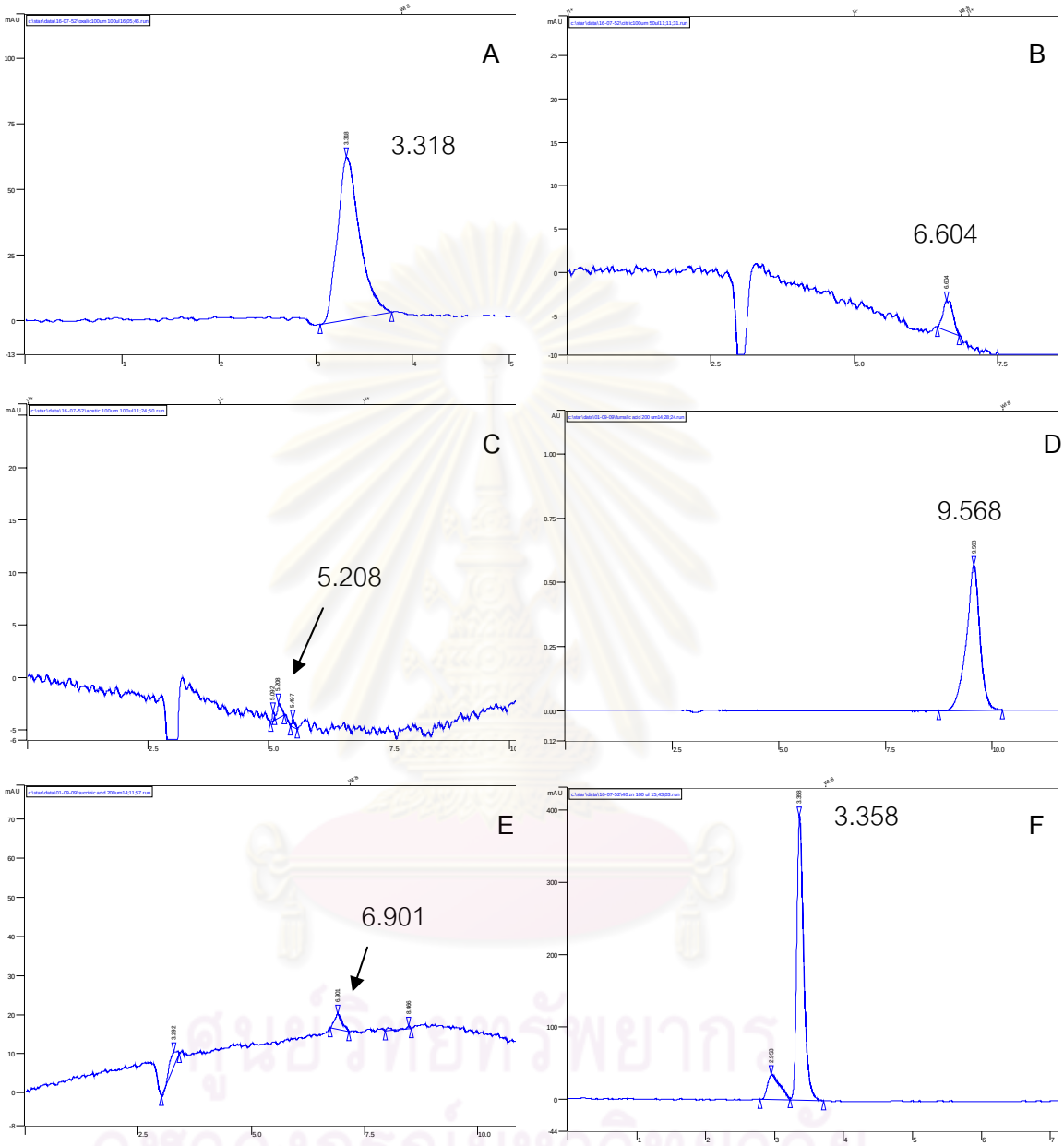

Fig. 4.27 HPLC chromatogram showing the organic acid peaks of standard oxalic (A); citric (B); acetic (C); fumaric (D), and succinic acids (E); and extract of metal crystals formed by brown-rot fungal isolate MR40 after growth on zinc sulfate-amended agar (F); copper sulfate-amended agar (G); cadmium sulfate-amended agar (H), and lead nitrate-amended agar (I). A similar pattern of peaks were also observed for the crystals produced by brown-rot fungal isolate KYO and WR4 (Appendix D). X-axis—time (min), Y -axis—mAU.

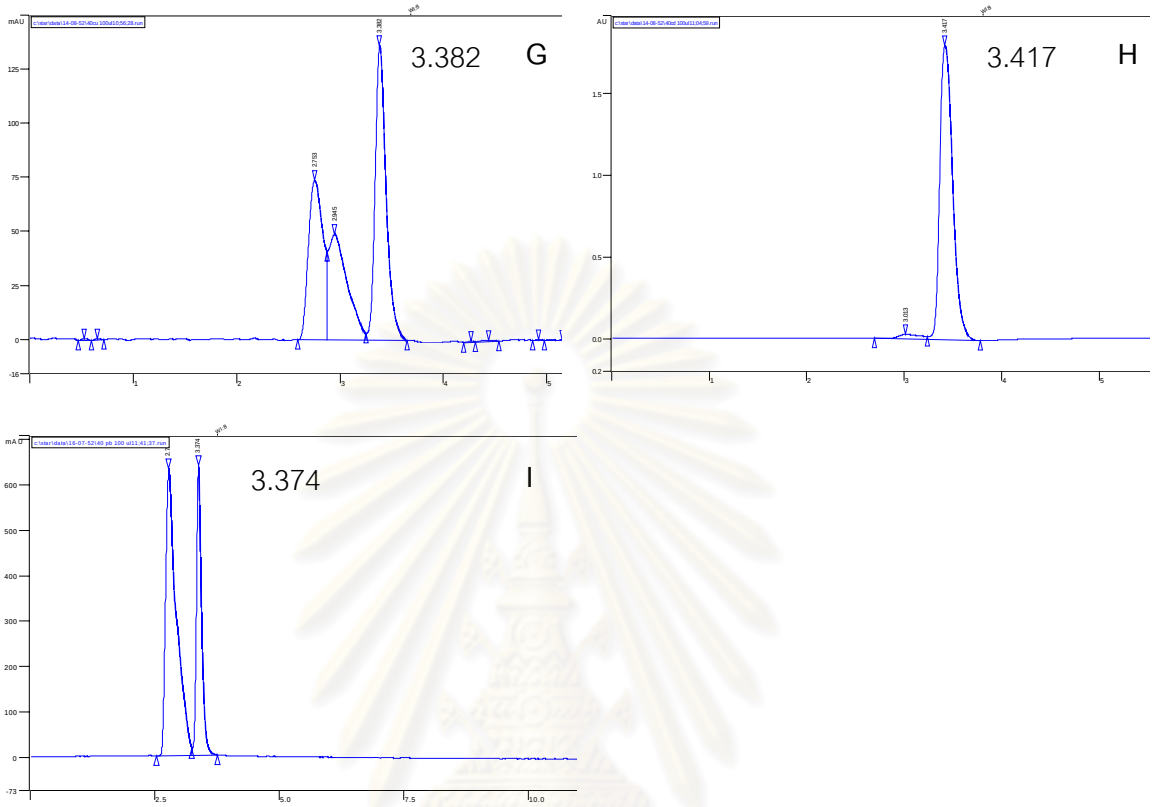

Fig. 4.27 (continued)

4.5.3 Confirmation of their homogeneity and identification as biogenic crystalline precipitates

X-ray powder diffraction (XRPD) analysis of these metal crystals produced by wood rotting fungi (Fig. 4.28A to D) showed the presence of a well-crystallized compound with an excellent match to reference patterns for zinc oxalate hydrate (PDF-14-0740) (Fig. 4.28A), copper oxalate hydrate (moolooite) (PDF-21-0297) (Fig. 4.28B), cadmium oxalate hydrate (PDF-54-0317 and -53-0085) (Fig. 4.28C) and lead oxalate (PDF-11-0723) (Fig. 4.28D). The formulae for zinc oxalate hydrate, copper oxalate hydrate (moolooite), cadmium oxalate hydrate and lead oxalate were $C_2O_4Zn \cdot 2H_2O$, $CuC_2O_4 \cdot xH_2O$, $C_2CdO_4 \cdot 2.5H_2O$ and $C_2CdO_4 \cdot 3H_2O$, and PbC_2O_4 respectively. From these results found that overexcretion of oxalic acid by

brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3 and WR5 led to the transformation of soluble metal minerals into insoluble metal oxalates (Fig. 4.19 to 4.28). Brown-rot fungal isolate MR40, KYO and WR4 turned zinc sulfates ($ZnSO_4 \cdot 7H_2O$) into twinned bulbous crystals of zinc oxalate hydrate ($C_2O_4Zn \cdot 2H_2O$) (Fig. 4.19 and 4.28A) and turned copper sulfate ($CuSO_4 \cdot 5H_2O$) into tetragonal crystals of copper oxalate hydrate (moolooite) ($CuC_2O_4 \cdot xH_2O$) (Fig. 4.20 and 4.28B). Brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3 turned cadmium sulfate ($3CdSO_4 \cdot 8H_2O$) into the crystals of cadmium oxalate hydrate ($C_2CdO_4 \cdot 2.5H_2O$ and $C_2CdO_4 \cdot 3\ H_2O$) (Fig. 4.21 and 4.28C). And the brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3 and WR5 turned lead nitrate ($Pb(NO_3)_2$) into the crystals of lead oxalate (PbC_2O_4) (Fig. 4.22 and 4.28D). This was agreement with the report from Sayer and Gadd (1997) found that *Aspergillus niger*, a fungus capable of oxalic acid production, was therefore capable of transforming inorganic insoluble metal compounds (ZnO, $Zn_3(PO_4)_2$ and $CO_3(PO_4)_2$) into organic insoluble metal compounds. Jarosz-Wilkolazka and Gadd (2003) reported that oxalic acid as an important metabolite elaborated in the transforming of $CaCO_3$, $Co_3(PO_4)_2$ or $Zn_3(PO_4)_2$- into calcium, cobalt and zinc oxalate crystals in the white-rot fungi (*Bjerkandera fumosa*, *Phlebia radiata*, and *Trametes versicolor*) and the brown-rot fungus *Fomitopsis pinicola*. Similarly Gharieb et al. (2004) found that in copper oxychloride-amended medium, *Aspergillus niger* would excrete oxalic acid and transformed the inorganic metal-containing compound (copper oxychloride) into insoluble organic compound (copper oxalate). In a further study Fomina et al. (2005c) suggested that the amount of oxalic acids excreted by *Beauveria caledonica* was the main mineral transforming agent, which transformed cadmium, copper, lead, and zinc minerals, converting them into oxalates.

Most metal oxalates were immobile and resistant to further solubilization, with only a few species of bacteria and fungi able to degrade them readily (Morris and Allen, 1994). Gadd (1993) suggested that metal toxicity might be reduced if the mobilized toxic metal

forms complexes with organic ligands excreted by the fungus and especially if toxic metals were precipitated as highly insoluble oxalates.

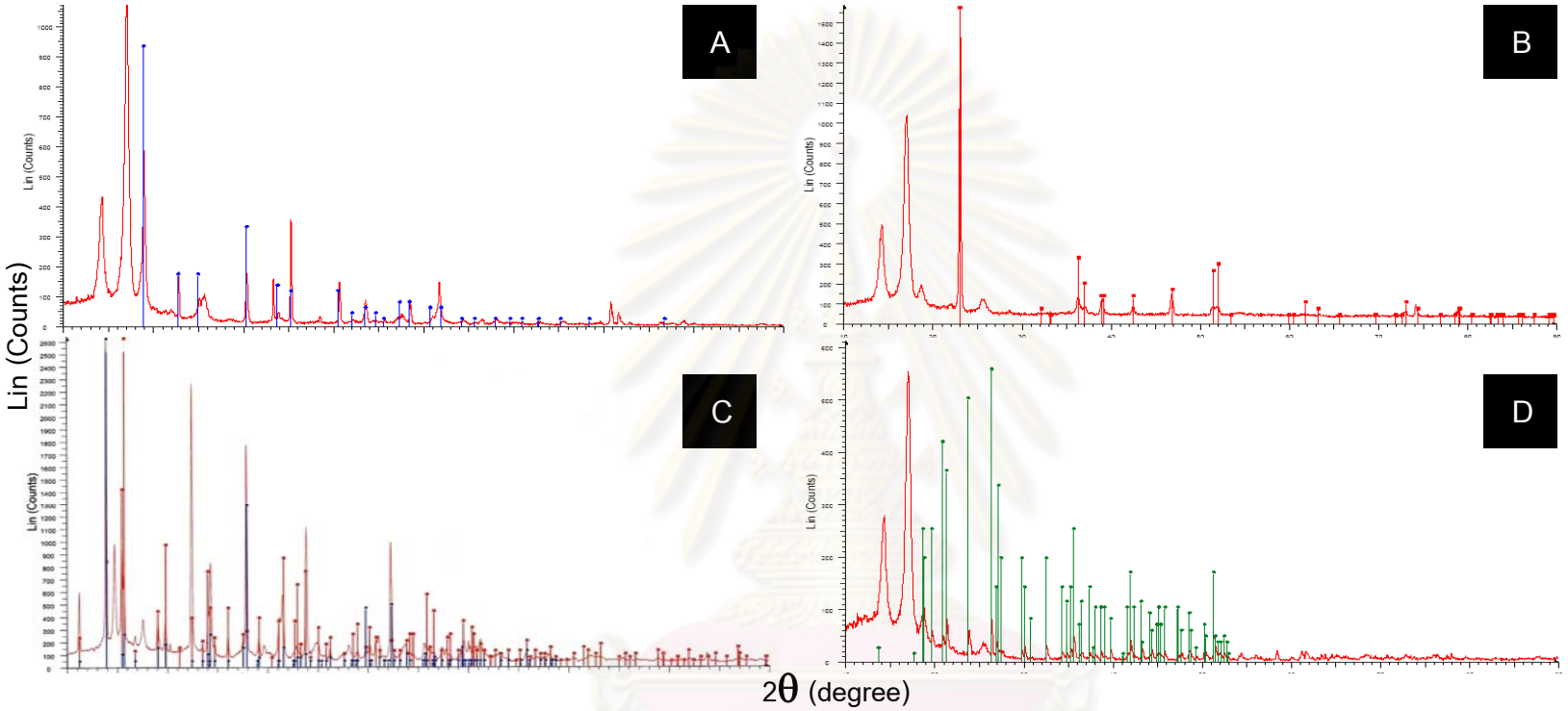

Fig. 4.28 Typical XRPD patterns for metal oxalate crystals precipitated by brown-rot fungal isolate MR40 after grown on toxic metal medium. (A) zinc oxalate hydrate formed on medium containing zinc sulfate; (B) copper oxalate hydrate (moolooite) formed on medium containing copper sulfate; (C) cadmium oxalate hydrate formed on medium containing cadmium sulfate; (D) lead oxalate formed on medium containing lead nitrate. The vertical bars indicate the peak positions and relative intensities for standard XRPD patterns of metal oxalates as given in the International Centre for Diffraction Data powder diffraction file. The standards used were zinc oxalate hydrate (PDF-14-0740), copper oxalate hydrate (PDF-21-0297), cadmium oxalate hydrate (PDF-54-0317 and PDF-53-0085), and lead oxalate (PDF-11-0723). A similar pattern of peaks were also observed for the crystals produced by isolate KYO, WR3, WR4 and WR5 (Appendix E).

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4.6 Heavy metal accumulation by wood-rotting fungi

Metals immobilized by wood-rotting fungi were accumulated not only within extracellular crystalline oxalate precipitates but also within the fungal biomass. Metal accumulation by fungal biomass was expressed in terms of specific metal uptake ($mg.g^{-1}$dry weight) biomass (Fig. 4.29A to D) (Appendix B (B31 to B34)).

The AAS analysis was carried out on mycelia cultivated on media without of heavy metals added and on media supplemented with various concentrations of heavy metals. No zinc, copper, cadmium and lead were detected in mycelia from control cultures. Since, the machine could not detect a very low metal concentration in the control culture.

There were no significant difference between zinc uptake data for brown-rot fungal isolate MR40, KYO, and WR4 in the medium supplemented with zinc sulfate 500 and 1000 ppm (Fig. 4.29A). In contrast to the low zinc conditions, high zinc uptake values were found for brown-rot fungal isolate MR40 at 2000 and 3000 ppm of zinc with dry weight 610 and 2500 mg/g, respectively.

High copper accumulations were found in brown-rot fungal isolate KYO and WR4 in the medium supplemented with copper sulfate (Fig. 4.29B). However at 1000 ppm of copper, the copper uptake value in brown-rot fungal isolate KYO was significantly lower than the brown-rot fungal isolate WR4. In contrast to under low-copper conditions (500 and 1000 ppm of copper), the highest copper accumulation was observed in brown-rot fungal isolate KYO at the highest concentration tested 5000 ppm of copper with dry weight 1000 mg/g.

High cadmium content was found for the white-rot fungal isolate WR3 and the low cadmium accumulation was observed in the brown-rot fungal isolate KYO in the medium supplemented with cadmium sulfate 100 and 500 ppm (Fig. 4.29C). At high concentration of cadmium, only brown-rot fungal isolate KYO and WR4 presented high cadmium uptake values. Brown-rot fungal isolate WR4 grown under high cadmium condition accumulated

considerably more cadmium than that grown under low cadmium condition. Similarly brown-rot fungal isolate KYO showed the highest cadmium uptake value at 8000 ppm of cadmium with dry weight 5000 mg/g.

In presence of lead nitrate concentration 1000 and 4000 ppm, high lead uptake values were observed in brown-rot fungal isolate WR4 and white-rot fungal WR3 and WR5 (Fig. 4.29D). Except at 4000 ppm of lead, brown-rot isolate WR4 presented the lower level of lead accumulation than the other isolates. In contrast, under low-lead condition, higher lead accumulations were observed in brown-rot fungal isolate MR40, KYO and WR4 at higher lead condition. The highest lead accumulation was found in brown-rot fungal isolate WR4 at 9000 ppm of lead with dry weight 8300 mg/g and at the highest lead concentration tested only brown-rot fungal isolate KYO presented high lead uptake values with dry weight 6300 mg/g.

From this experiment, the accumulated heavy metals significantly increased by increasing the concentration of heavy metals and the ability of wood-rotting fungi to withstand stress induced by heavy metals might be connected with their ability to immobilize and bind toxic metals with biomass. Metal ion could be bound to the biomass since the fungal biomass might provide a variety of ligands that form complexes with heavy metals (Gadd, 2007a). Additionally the increasing in fungal biomass should be related to the accumulation of metal ions into the fungal mycelium. Because of the increasing concentrations of heavy metal in the MEA medium led to a significant increase of fungal biomass in the wood-rotting fungi, which correlated with a significant accumulation of heavy metals were significantly increased by increasing the concentrations of heavy metal.

In this study, amounts of metals accumulated by the biomass were high in comparison with published values for a range of fungi grown in predominantly liquid media (Table 4.2).

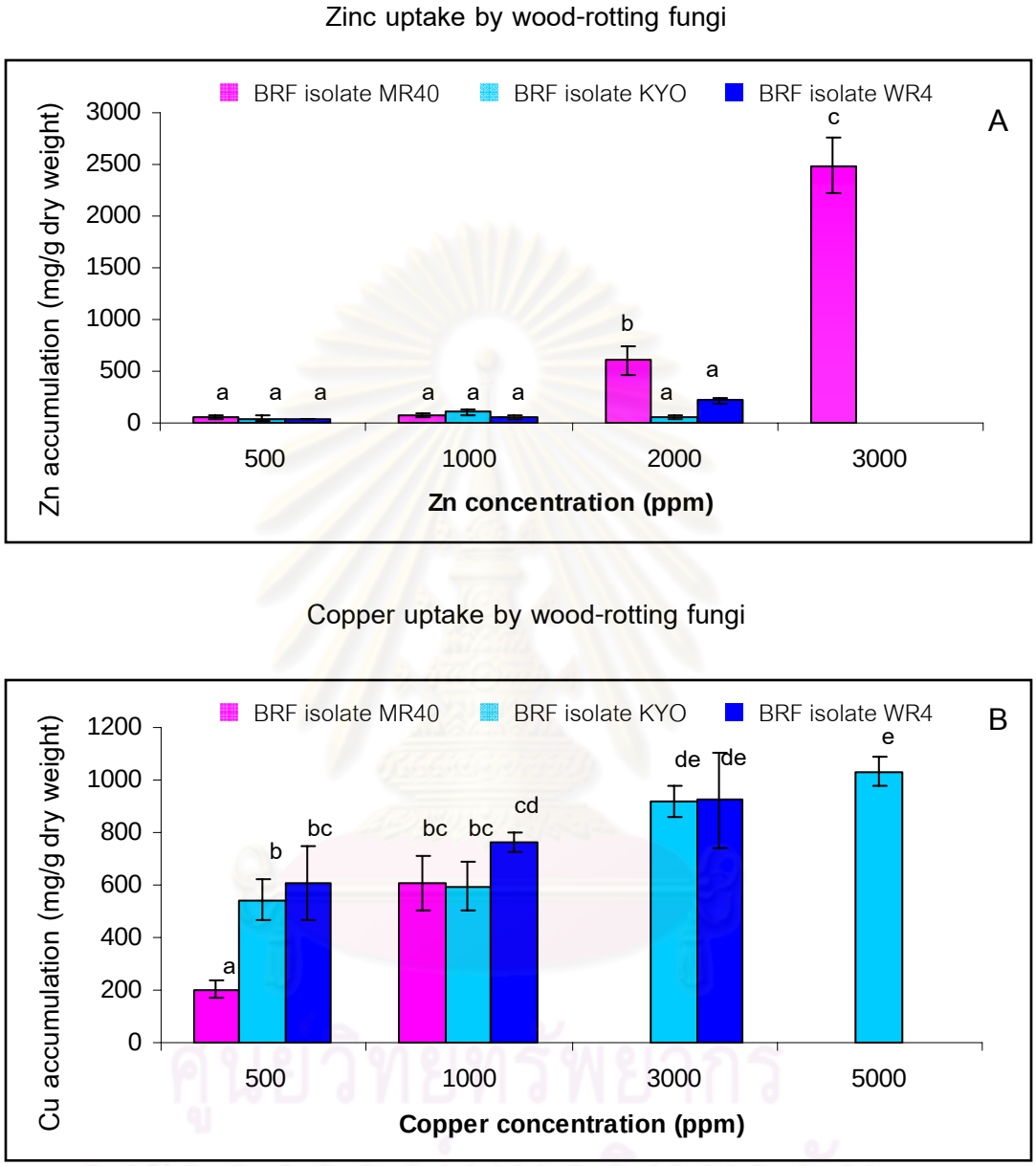
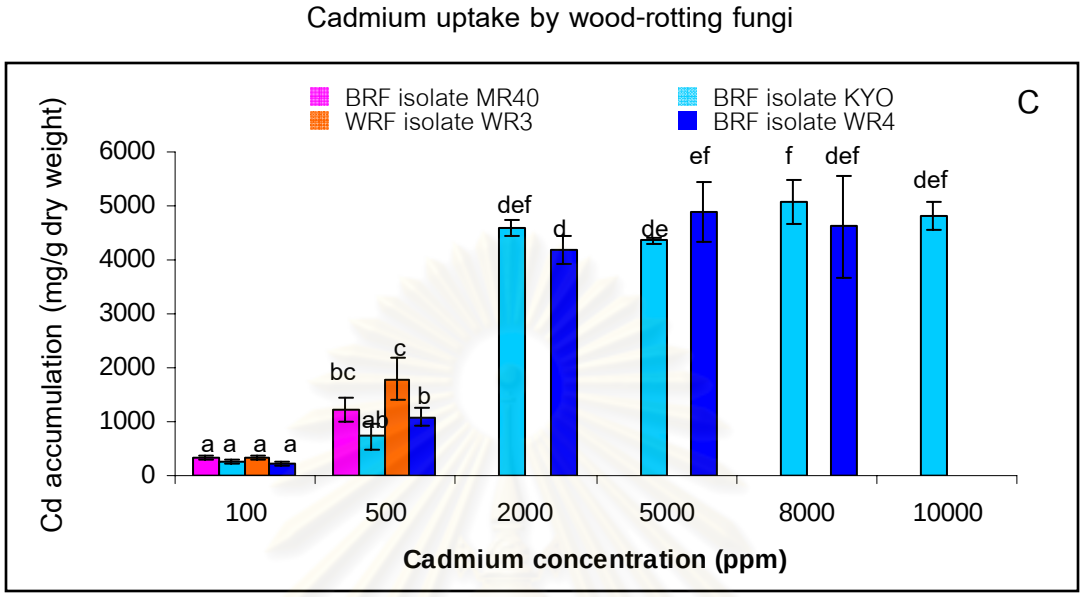


Fig. 4.29 Elemental accumulation (A) zinc; (B) copper; (C) cadmium; and (D) lead in the mycelia of wood-rotting fungi grown on media amended with various concentrations of zinc sulfate, copper sulfate, cadmium sulfate and lead nitrate, respectively. Values were means and derived from at least three replicate determinations. The different letters (a–l) above columns indicate a statistically significant differences for zinc, copper, cadmium and lead accumulation at $p < 0.05$ using Duncan test (one-way ANOVA).



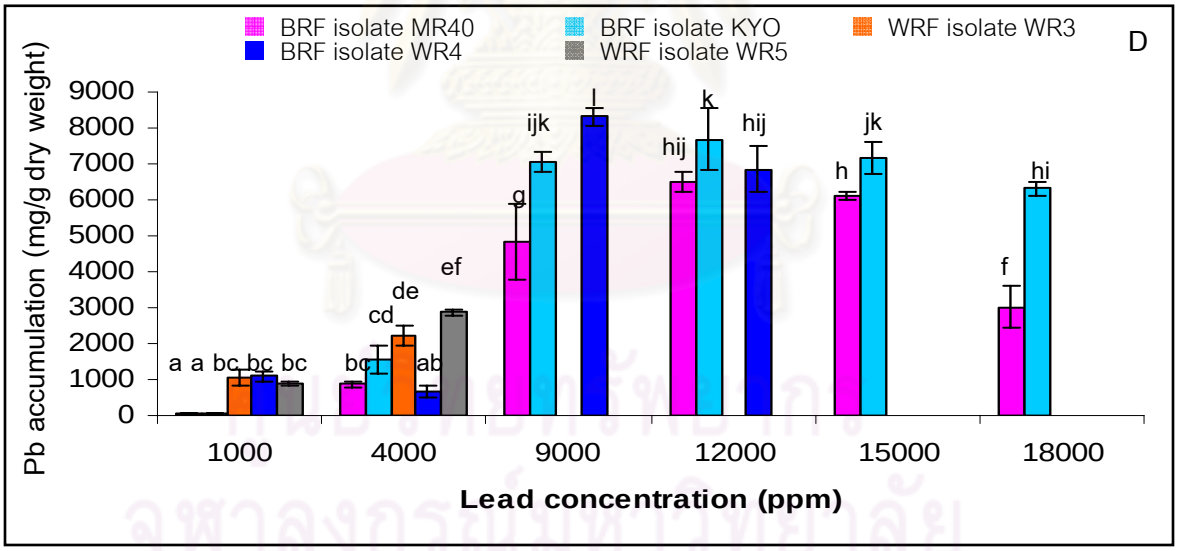


Fig. 4.29 (continued)

Table 4.2 Comparison of zinc, copper, cadmium and lead maximum uptake capacity between results from this study and other studies.

Metal type	Biosorptive capacity mg/g dry weight	Biosorbent type	References
Zinc	106.383	*Rhizopus arrhizus*	Yin et al., 1999
	53.85	*Mucor rouxii*	Yan and Viraraghavan, 2003
	109.3	*Trametes versicolor*	Bayramoglu et al., 2003
	50.9	*Phanerochaete chrysosporium*	Iqbal and Edyvean, 2004
	18.8	*Cunninghamella echinulata*	El-Morsy, 2004
	57.7	*Lentinus edodes*	Bayramoglu et al., 2008
	2,500	Isolate MR40	This study
	100	Isolate KYO	This study
	220	Isolate WR4	This study
Copper	5.66	*Aspergillus niger* 405	Filipovic-Kovacevic et al.,2000
	160–180	*Aspergillus terreus*	Gulati et al., 2002
	26.55	*Phanerochaete chryosporium*	Say et al., 2001
	117.2	*Trametes versicolor*	Bayramoglu et al., 2003
	102.8	*Phanerochaete chrysosporium*	Iqbal and Edyvean,2004
	20	*Cunninghamella echinulata*	El-Morsy, 2004
	28.70	*Aspergillus niger*	Dursun, 2006
	25.32	*Penicillium brevicompactum*	Tsekova et al., 2007
	2.76	*Pycnoporus sanguineus*	Yahaya et al.,2009
	34.13	*Aspergillus niger*	Tsekova et al., 2010
	600	Isolate MR40	This study
	1000	Isolate KYO	This study
	920	Isolate WR4	This study

Table 4.2 (continued) Comparison of zinc, copper, cadmium and lead maximum uptake capacity between results from this study and other studies.

Metal type	Biosorptive capacity mg/g dry weight	Biosorbent type	References
Cadmium	6.94	*Mucor rouxii*	Yan and Viraraghavan, 2000
	27.79	*Phanerochaete chryosporium*	Say et al., 2001
	120.6	*Trametes versicolor*	Arica et al., 2001
	153	*Trametes versicolor*	Yalcinkaya et al., 2002
	3.0	*Trametes versicolor*	Jarosz-Wilkolazka, et al., 2002
	123.5	*Lentinus sajor-caju*	Bayramoglua et al., 2002
	110.4	*Penicillium purpurogenum*	Say et al., 2003
	20.31	*Mucor rouxii*	Yan and Viraraghavan,2003
	54.0	*Pseudomonas veronii 2E*	Vullo et al., 2008
	8.46	*Mucor rouxii*	Yan and Viraraghavan, 2003
	2.72	*Rhizopus* sp.	Zafar et al., 2007
	2.91	*Aspergillus* sp.	Zafar et al., 2007
	3.18	*Pycnoporus sanguineus*	Mashitah et al., 2008
	274.3	*Lentinus edodes*	Bayramoglu et al., 2008
	60.24	*Aspergillus niger*	Tsekova et al., 2010
	1230	Isolate MR40	This study
	5000	Isolate KYO	This study
	1800	Isolate WR3	This study
	4900	Isolate WR4	This study

Table 4.2 (continued) Comparison of zinc, copper, cadmium and lead maximum uptake capacity between results from this study and other studies.

Metal type	Biosorptive capacity mg/g dry weight	Biosorbent type	References
Lead	53.6	*Phanerochaete chrysosporium,*	Çeribasi and Yetis, 2000
	53.6	*Phanerochaete chrysosporium*	Çeribasi and Yetis, 2000
	80	*Phanerochaete chrysogenum*	Yetis et al., 2000
	85.86	*Phanerochaete chryosporium*	Say et al., 2001
	47	*Rhizopus nigricans*	Bai and Abraham, 2001
	252.8	*Penicillium purpurogenum*	Say et al., 2003
	227.8	*Trametes versicolor*	Bayramoglu et al.,2003
	53.75	*Mucor rouxii*	Yan and Viraraghavan, 2003
	135.3	*Phanerochaete chryosporium*	Iqbal and Edyvean, 2004
	45	*Cunninghamella echinulata*	El-Morsy, 2004
	209.33	*Aspergillus niger RH18*	Faryal, 2007
	6500	Isolate MR40	This study
	7700	Isolate KYO	This study
	2200	Isolate WR3	This study
	8300	Isolate WR4	This study
	2900	Isolate WR5	This study

4.7 Wood-rotting fungi identification

4.7.1 Identification by morphological characteristics

Fruiting body of brown-rot fungal isolate MR40 (Fig. 4.30A and B); the size of basidiocarp was 5-6 cm broad, 0.5-1 cm thick; pileus with shell-shaped and bulgy; the fruit bodies usually had no stipe, grow shelf-like, and firmly attached to the bark; upper surface: rough surface, cream-white to light-brown when young and usually dingy blackish-gray when age; pore surface: rough and cream-white circular pores; soft and tough fruit bodies appear when young and hard in age; trimitic hyphal systems with clamps in the generative hyphae (Fig. 4.30C), nodose-septate, hyaline to brownish in KOH, smooth, thin- to slightly thick-walled, branched and some with short branches, nonamyloid; hyaline, cylindrical or allantoid and smooth thin-walled basidiospores (Fig. 4.30D), and cell contents or wall that no reaction in Melzer's Reagent (nonamyloid).

Fruiting body of white-rot fungal isolate WR3 (Fig. 4.30E and F); the size of basidiocarp was 5-6 cm broad, 1-1.5 cm thick; pileus with fan-shaped; the fruit bodies had brownish stipe, grow shelf-like, and firmly attached to the bark; upper surface: rough surface and slightly wavy zonate, brownish and usually white at the margin when fresh, and more shiny red-brown pileus surface, yellowish-brown to orange-brown at the margin and their surface is often covered by a cocoa-brown layer of spores when old; pore surface: flat and cream-colored circular pores; lathery-sticky fruit bodies appear when young, hard crust, and woody fruit bodies appear when old; trimitic hyphal systems with clamps in the generative hyphae (Fig. 4.30G), nodose-septate, hyaline to brownish in KOH, smooth, thin- to slightly thick-walled, branched and some with short branches, nonamyloid; brown spores, void-shaped and thick-walled basidiospore with apical germ pore (Fig. 4.30H), and nonamyloid.

Fruiting body of brown-rot fungal isolate KYO (Fig. 4.30I and J); the size of basidiocarp was 4-6 cm broad, 1-1.5 cm thick; pileus with shell-shaped and slightly convex; the fruit bodies usually had no stipe, grow shelf-like, and firmly attached to the bark; upper surface: rough surface, dull brown or grey-brown and usually light brown at the margin when fresh, dingy grayish-black in age; pore surfaces: flat and cream-brownish circular pores; soft and tough fruit bodies appear when fresh, hard and cracked when old; trimitic hyphal systems with clamps in the generative hyphae (Fig. 4.30K), nodose-septate, hyaline to brownish in KOH, smooth, thin- to thick-walled, branched and some with short branches, nonamyloid.

Growth character of brown-rot fungal isolate WR4 on malt extract agar (Fig. 4.31A); the mycelium was scanty, pale cream-white, at first silky and downy, and becoming more compact mycelium after 1 week. Hyphal character of this fungus (Fig. 4.31B); trimitic hyphal systems with clamps in the generative hyphae, nodose-septate, hyaline hyphae, smooth, thin- to thick-walled, branched and some with short branches.

Growth character of white-rot fungal isolate WR5 on malt extract agar (Fig. 4.31C); the mycelium was extensive, mat white, at first silky and downy, and becoming cottony, woolly and more compact mycelium after 1 week. Hyphal character of this fungus (Fig. 4.31D); trimitic hyphal systems with clamps in the generative hyphae, nodose-septate, hyaline hyphae, smooth, thin- to slightly thick-walled, highly branched and some with short branches.

In this study, 5 isolates of wood-rotting fungi were morphologically identified as basidiomycetes based on the presence of clamp connections or basidiospores.

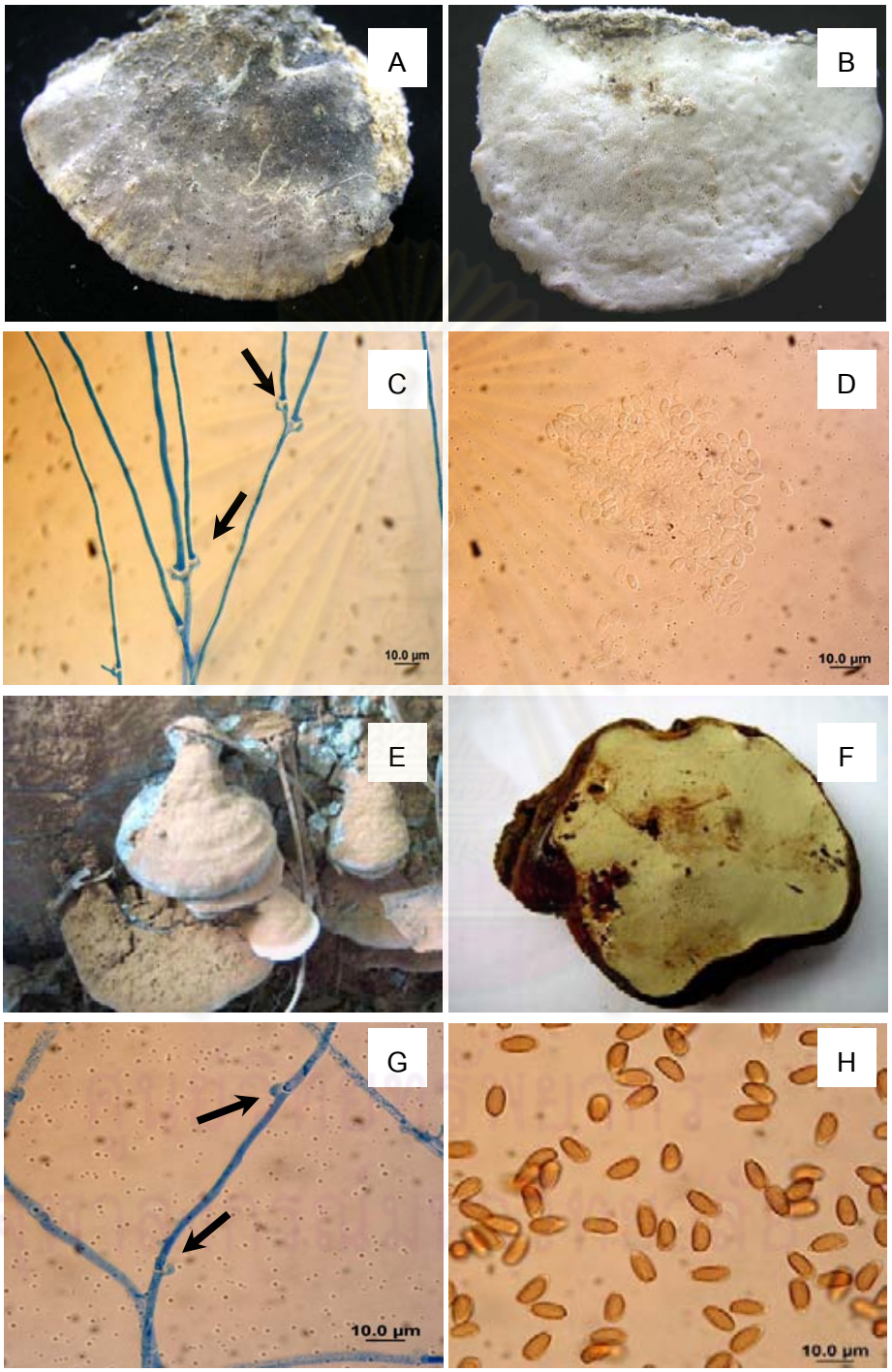


Fig. 4.30 Basidome (top view and bottom view), hyphal systems and basidiospores of brown-rot fungal (A to D) isolate MR40, and white-rot fungal (E to H) isolate WR3; basidome (top view and bottom view) and hyphal systems of brown-rot fungal (I to K) isolate KYO.

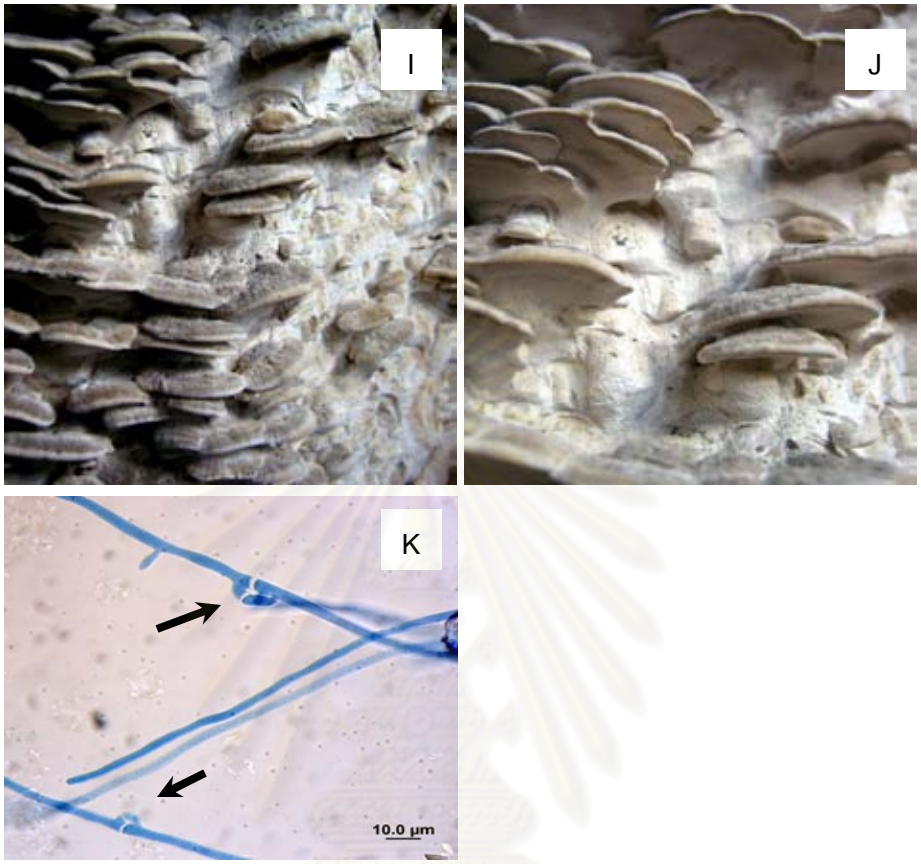


Fig. 4.30 (continued)

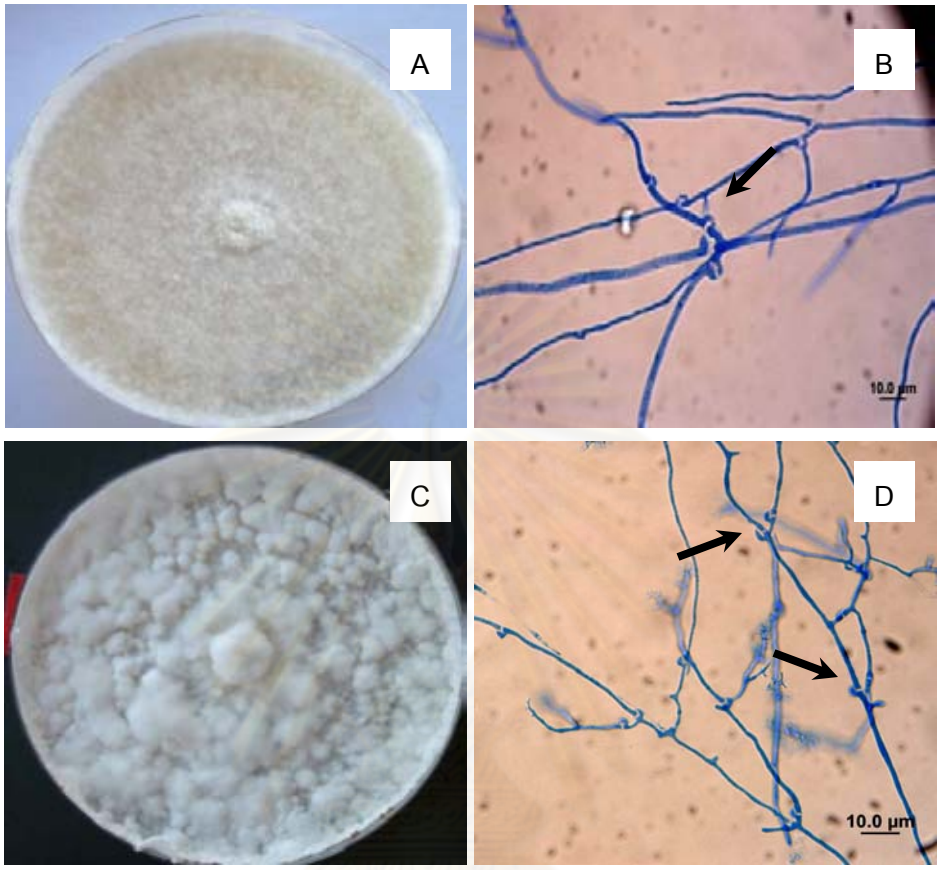


Fig. 4.31 Cultural characteristics and the hyphal systems of brown-rot fungal (A and B) isolate WR4 (A) and white-rot fungal (C and D) isolate WR5 when cultured on 2% MEA (7-10 days).

4.7.2 Confirmation by molecular methods

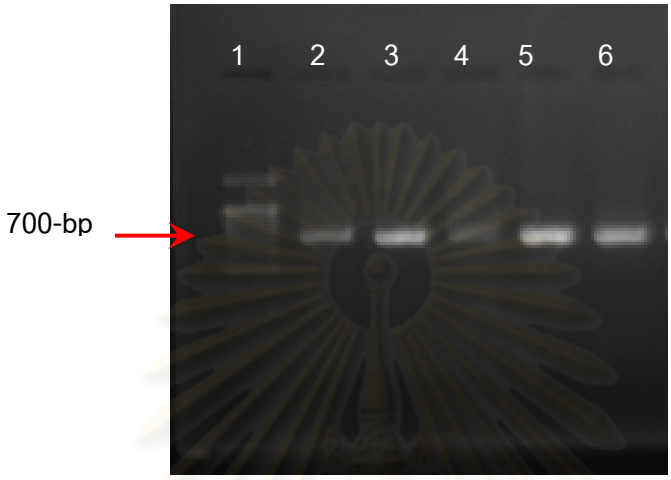


Fig. 4.32 Agarose gel electrophoresis of PCR products. Genomic DNA used from; lane 1 : 1-Kbp ladder DNA markers; lane 2-6 : PCR product of the isolate KYO, MR40, WR3, WR4, and WR5, respectively.

For the genotypic identification, approximately 700 bp of sequence information were obtained for the internal transcribed spacer regions between the small, 5.8S, and large rRNA genes (ITS1F and ITS4) of each fungal isolates (Fig. 4.32). The identities of all fungi were determined by comparing the sequence data with the National Center for Biotechnology Information (NCBI) Blast Library.

The sequence of brown-rot fungal isolate MR40 approximated size 552 bp exhibited the highest level of homology (99% of identity) with *Fomitopsis* sp. IMER2 (accession number EU015881) (Appendix F(F1)).

Comparing results that the sequence of brown-rot fungal isolate KYO approximated size 652 bp exhibited the highest level of homology (99% of identity) with *Fomitopsis cf. meliae* 8IV7/1 (accession number GQ982889) (Appendix F(F2)).

The sequence of white-rot fungal isolate WR3 approximated size 531 bp exhibited the highest level of homology (99% of identity) with *Ganoderma* sp. STK-2006a (accession number EF016754) (Appendix F(F3)).

The sequence of brown-rot fungal isolate WR4 approximated size 511 bp exhibited the highest level of homology (100% of identity) with *Fomitopsis cf. meliae* 1P_1_1 (accession number FJ372673) (Appendix F(F4)).

The sequence of white-rot fungal isolate WR5 approximated size 555 bp exhibited the highest level of homology (98% of identity) with *Ganoderma aff. steyaertanum* C16452 (accession number EU239386) (Appendix F(F5)).

Base on morphological characteristics or cultural characteristics, and molecular data indicated that brown-rot fungal isolate KYO and WR4 identified as *Fomitopsis cf. meliae*, which had not been reported in the heavy metal immobilization (precipitation) and –transformation ability. Therefore this research represented the first study to report the heavy metal immobilization (precipitation) and –transformation by the *Fomitopsis cf. meliae*. Similarly white-rot fungal isolate WR5, from the identification using cultural characteristics and molecular techniques indicated that this fungus might be the *Ganoderma aff. steyaertanum*, which had not been reported in the heavy metal immobilization (precipitation) and –transformation. So this research was the first study, which reported the heavy metal immobilization (precipitation) and –transformation by the *Ganoderma aff. steyaertanum*.

Identification using morphological characteristics and molecular techniques indicated that brown-rot fungal isolate MR40 and white-rot fungal isolate WR3 were classified as *Fomitopsis* sp. and *Ganoderma* sp, respectively. Genus *Fomitopsis* had been used in the heavy metal remediation study by some research such as Jarosz-Wilkolazka and Gadd (2003) which found that the brown-rot fungus *Fomitopsis pinicola*, grown on media containing high levels of toxic metal ions displayed the formation of crystals on zinc oxide, cobalt phosphate and calcium cabornate-amended plates. Kartal and Imamura (2003) and Kartal et al. (2003) showed that brown-rot fungus *Fomitopsis palustris*

remediation of CCA-treated sawdust for 10 days could remove about 72% copper, 87% chromium, and 100% arsenic. Similarly study by Son et al. (2003) also showed that remediation of CCA-treated wood by *Fomitopsis palustris* grown in a bioreactor resulted in 61% copper, 72% chromium, and 59% arsenic removal. In another study by Kim et al. (2009) showed that *Fomitopsis palustris* could remove copper, chromium and arsenic at the highest removal rate 96%, 92% and 98%, respectively.

The *Ganoderma* genus was reported high heavy metal uptake capacity such as the white-rot fungus, *Ganoderma applanatum* had been reported to be capable of accumulation cadmium 272 mg/g, aluminium 600 mg/g, and calcium 602 mg/g (Gabriel et al., 1994). Similarly Muraleedharan et al. (1995) reported that the maximum copper binding capacity 24 mg/g was obtained by *Ganoderma lucidum*. In another work by Javaid and Bajwa (2008) also showed that *Ganoderma lucidum* was removing heavy metal ions viz., copper (II), chromium (VI), nickel (II) and zinc (II) from electroplating industrial effluents. Biosorption assays indicated that the test fungus possess great ability for removal of heavy metal ions. However, the *Ganoderma* genus has not been reported in oxalic acid production and heavy metal precipitation, so the production of oxalic acid and heavy metal precipitation by the *Ganoderma* genus has been shown only in the present study.

CHAPTER V

CONCLUSIONS

Heavy metal pollution represents an important environmental problem because of the toxic effects of metals, and their accumulation throughout the food chain that could lead to serious ecological and human health problems. Unlike degradable organic contaminants and even short-lived radionuclides that could become less toxic over time, metals could be considered “conservative” because they were not decomposed in the environment (Fomina et al., 2005b). However, previous studies found that wood-rotting fungi could be extreme tolerance to the toxic metals (Schmidt and Zimemer, 1976; Gadd, 1993) and was capable to precipitate the soluble metal compounds by producing organic acid such as oxalic acid or citric acid. The formation of oxalates containing potentially toxic metals may provide a mechanism whereby oxalate producing fungi could tolerate environments containing high concentrations of toxic metals (Sayer and Gadd, 1997; Fomina et al., 2005c; Gadd, 2007b). Moreover, most metal oxalates produced by wood-rotting fungi were immobile and resistant to further solubilization, so no heavy metals releasing to the environment (Morris and Allen, 1994). However, the study of heavy metal remediation by wood-rotting fungi in Thailand has not been studied yet. Thus, the objective of this study was to select fungal strains which show high metal tolerance and able to remove heavy metals from the metal-amended media by precipitation as metal oxalate crystals (biominerals). Moreover, this experiment was to determine the effect of heavy metals on the fungal growth and heavy metal accumulation by wood-rotting fungi.

The study of screening of fungal tolerance to the heavy metal and detection of the crystals formation found that among sixty fungal isolates, only five isolates (brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3 and WR5) were able to tolerate and produce the metal crystals in the agar medium amended with various

concentrations of heavy metals. Especially brown-rot fungal isolate KYO was able to grow at high concentration of heavy metals and produced numerous metal crystals. Metal crystals formed by brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3 and WR5 consist mainly of oxalic acid which indicated that oxalic acid as an important metabolite elaborated in the response of wood-rotting fungi to toxic metal stress. Oxalate production by brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3 and WR5 could result in the transformation of zinc sulfate ($ZnSO_4.7H_2O$) into zinc oxalate hydrate ($C_2O_4Zn.2H_2O$), copper sulfate ($CuSO_4.5H_2O$) into copper oxalate hydrate (moolooite) ($C_2CuO_4.xH_2O$), cadmium sulfate ($3CdSO_4.8H_2O$) into cadmium oxalate hydrate ($C_2CdO_4.2.5H_2O$ and $C_2CdO_4.3H_2O$), and lead nitrate ($Pb(NO_3)_2$) into lead oxalate (PbC_2O_4), which were resistant to further solubilization and less inhibitory effect on the fungal growth.

An experiment was conducted to determine the effect of heavy metals on fungal growth and heavy metal accumulation by brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3 and WR5. The result showed that the presences of the metal compounds did not stimulate the pH reduction and/or acid production. Moreover, increasing concentrations of heavy metal compounds in the culture medium caused a significant decrease in the radial growth of the most fungal isolates, whereas the fungal biomasses were significantly increased. The analysis of metal content in the fungal mycelia cultivated on media containing heavy metals showed that the accumulated heavy metals significantly increased when the concentrations of heavy metals increased. These results provided the evidence that brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3 and WR5 could tolerate high concentrations of heavy metals and able to remove heavy metals from the metal-amended media by precipitation as metal oxalate crystals (biominerals). Moreover, these fungi were capable to accumulate heavy metals within their biomass during immobilization of the soluble metal compounds therefore fungal biomasses should be applied as biosorbent for heavy metals which might provide potential for metal removal and recovery of valuable elements.

The present study was the first study in which the heavy metal immobilization (precipitation) and –transformation by the *Fomitopsis cf. meliae*. and *Ganoderma aff. steyaertanum*. Finally, production of oxalic acid and heavy metal precipitation by the *Ganoderma* genus had been detected in the present study.

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Appendices

Appendix A

Media and chemical reagents

1. Malt Extract Agar (2% MEA)

Malt extract	20	g
Agar	15	g
Distilled water	1,000	ml
pH 5.5 ± 0.1		

Dissolve with distilled water 900 ml thoroughly and adjust pH with HCl to pH 5.5. After that the distilled water was added to reach 1000 ml. Sterilization in a autoclave at 121°C and pressure at 15 pounds/square inch for 15 minutes.

2. Malt Extract Agar (4% MEA)

Malt extract	40	g
Agar	25	g
Distilled water	1,000	ml
pH 5.5 ± 0.1		

Dissolve with distilled water 900 ml thoroughly and adjust pH with NaOH to pH 5.5. After that the distilled water was added to reach 1000 ml. Sterilization in a autoclave at 121°C and pressure at 15 pounds/square inch for 15 minutes.

3. Tannic Acid Agar (TA)

Tanic acid	5	g
Malt extract	15	g
Agar	20	g
Distilled water	1,000	ml

Autoclave malt extract and agar in 850 ml water. Sterilize other 150 ml water in separate flask. When done, add tannic acid to pure water while still hot, and dissolve it. Then add to agar-malt falk, mix, and pour plates. This medium is used to determine the oxidase production of wood rotting fungi.

4. 2X CTAB lysis buffer

CTAB	4	g
1N Tris-HCl (pH 8.0)	20	ml
0.5N EDTA (pH 8.0)	8	ml
Sodium chloride (NaCl)	16.86	g
2-Mercaptoethanol	1	ml

Mix CTAB, 0.5N EDTA, NaCl and 2- Mercaptoethanol. After that the distilled water was added to reach 200 ml and mix thoroughly. Keep at room temperature.

5. Choloroform/Isoamyl alcohol (24:1 v/v)

Choloroform	192	ml
Isoamyl alcohol	8	ml

Tris-HCl pH 8.0

Tris base	121	g
Distilled water	800	ml

Dissolve Tris base thoroughly and adjust pH with HCl to pH 8. After that the distilled water was added to reach 1000 ml. Autoclave at 121 °C and pressure at 15 pounds/square inch for 15 minutes. Keep at room temperature.

6. Tris-EDTA buffer (TE buffer)

1N Tris-HCl; pH 7.4, 7.5 or 8.0	10	ml
0.5N EDTA; pH 8.0	2	ml

Mix Tris-HCl; pH 7.4, 7.5 or 8.0 and EDTA; pH 8.0. After that the distilled water was added to reach 1000 ml. Autoclave at 121°C and pressure at 15 pounds/square inch for 15 minutes. Keep at room temperature.

9. Melzer's reagent

Iodine crystals	0.5	g
Potassium iodide	1.5	g
Chloral hydrate	20	g
Distilled water	20	ml

This solution is used on fungal tissues, especially ascus tips and basidiospores. A positive reaction gives a dark blue (amyloid) strain. Intermediate reactions of yellowish- or reddish-brown (dextrinoid) are also obtained with some species.

10. Lactophenol cotton blue strain

Phenol crystals	20	g
Lactic acid	20	ml
Glycerol	40	ml
Cotton blue	0.05	g
Distilled water	20	ml

Phenol crystals were dissolved in lactic acid, glycerol and distilled water and then melt in water bath. After that cotton blue was added to mix with the phenol crystal solution.

APPENDIX B

Table B1 Change in pH of MEA medium produced by brown-rot fungal isolates MR40, KYO and WR4 in the presence or absence of zinc sulfate

Isolates	Zinc concentration (ppm)/Initial pH				
	0 / 5.50	500 / 4.70	1000 / 4.60	2000 / 4.52	3000 / 4.46
BRF isolate MR 40	2.12 ± 0.11 (2.6)	2.24 ± 0.07 (2.1)	1.93 ± 0.08 (2.4)	1.81 ± 0.08 (2.5)	2.28 ± 0.07 (2.0)
BRF isolate KYO	1.95 ± 0.07 (2.8)	2.10 ± 0.12 (2.2)	2.08 ± 0.05 (2.2)	1.91 ± 0.01 (2.4)	n.g.[a]
BRF isolate WR4	2.05 ± 0.05 (2.7)	2.15 ± 0.07 (2.2)	2.05 ± 0.13 (2.2)	1.85 ± 0.04 (2.4)	n.g.

[a] Growth was not observed under these conditions

Means of three replicates were shown with ± SD

Numbers within parentheses represent the pH reduction values (units redution) with respect to the initial pH values

B1. Statistic analysis data by Oneway-ANOVA of the pH Reduction values of brown-rot fungal isolate MR40 in zinc sulfate-amended medium

ANOVA

pHReduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.864	4	.216	22.547	.000
Within Groups	.096	10	.010		
Total	.960	14			

Post Hoc Tests

Homogeneous Subsets

pHReduction

Duncan [a]

		Subset for alpha = .05		
Conc	N	1	2	3
3000.00	3	1.9600		
500.00	3	2.1067		
1000.00	3		2.3867	
2000.00	3		2.5000	2.5000
.00	3			2.5967
Sig.		.096	.187	.254

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B2. Statistic analysis data by Oneway-ANOVA of the pH Reduction values of brown-rot fungal isolate KYO in zinc sulfate-amended medium

ANOVA

pHReduaction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.746	3	.249	36.458	.000
Within Groups	.055	8	.007		
Total	.800	11			

Post Hoc Tests

Homogeneous Subsets

pHReduction

Duncan [a]

		Subset for alpha = .05	
Conc	N	1	2
1000.00	3	2.2067	
500.00	3	2.2400	
2000.00	3	2.3667	
.00	3		2.8300
Sig.		.052	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B3. Statistic analysis data by Oneway-ANOVA of the pH Reduction values of brown-rot fungal isolate WR4 in zinc sulfate-amended medium

ANOVA

pHReduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.444	3	.148	17.658	.001
Within Groups	.067	8	.008		
Total	.511	11			

Post Hoc Tests

Homogeneous Subsets

pH Reduction

Duncan [a]

		Subset for alpha = .05		
Conc	N	1	2	3
500.00	3	2.1900		
1000.00	3	2.2533		
2000.00	3		2.4467	
.00	3			2.6833
Sig.		.421	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table B2 Change in pH of MEA medium produced by brown-rot fungal isolates MR40, KYO and WR4 in the presence or absence of copper sulfate

Isolates	Copper concentration (ppm)/ Initial pH				
	0 / 5.50	500 / 3.73	1000 / 3.46	3000 / 3.20	5000 / 3.15
BRF isolate MR 40	2.12 ± 0.11 (2.6)	2.11 ± 0.05 (1.8)	2.02 ± 0.07 (1.7)	n.g.[a]	n.g.
BRF isolate KYO	1.95 ± 0.07 (2.8)	2.18 ± 0.03 (1.7)	1.99 ± 0.11 (1.7)	1.59 ± 0.13 (2.0)	1.77± 0.03 (1.8)
BRF isolate WR4	2.05 ± 0.05 (2.7)	1.98 ± 0.09 (1.9)	1.72 ± 0.12 (2.0)	1.26 ± 0.06 (2.5)	n.g.

[a] Growth was not observed under these conditions

Means of three replicates were shown with ± SD

Numbers within parentheses represent the pH reduction values (units redution) with respect to the initial pH values

B4. Statistic analysis data by Oneway-ANOVA of the pH Reduction values of brown-rot fungal isolate MR40 in copper sulfate-amended medium

ANOVA

pHReduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.467	2	.733	101.552	.000
Within Groups	.043	6	.007		
Total	1.510	8			

Post Hoc Tests

Homogeneous Subsets

pH Reduction

Duncan [a]

		Subset for alpha = .05	
Conc	N	1	2
1000.00	3	1.7133	
500.00	3	1.7700	
.00	3		2.5967
Sig.		.445	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B5. Statistic analysis data by Oneway-ANOVA of the pH Reduction values of brown-rot fungal isolate KYO in copper sulfate-amended medium

ANOVA

pHReduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.658	4	.664	72.643	.000
Within Groups	.091	10	.009		
Total	2.749	14			

Post Hoc Tests

Homogeneous Subsets

pH Reduction

Duncan [a]

		Subset for alpha = .05		
Conc	N	1	2	3
500.00	3	1.7133		
1000.00	3	1.7433		
5000.00	3	1.7767		
3000.00	3		2.0267	
.00	3			2.8300
Sig.		.457	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B6. Statistic analysis data by Oneway-ANOVA of the pH Reduction values of brown-rot fungal isolate WR4 in copper sulfate-amended medium

ANOVA

pHReduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.308	3	.436	35.219	.000
Within Groups	.099	8	.012		
Total	1.407	11			

Post Hoc Tests

Homogeneous Subsets

pH Reduction

Duncan [a]

		Subset for alpha = .05	
Conc	N	1	2
500.00	3	1.9033	
1000.00	3	2.0233	
3000.00	3		2.5367
.00	3		2.6833
Sig.		.223	.145

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table B3 Change in pH of MEA medium produced by brown-rot fungal isolates MR40, KYO and WR4, and white-rot fungal isolate WR3 in the presence or absence of cadmium sulfate

Isolates	Cadmium concentration (ppm)/ Initial pH						
	0 / 5.50	100 / 5.10	500 / 4.76	2000 / 4.39	5000 / 4.3	8000 / 4.23	10000 / 4.15
BRF isolate MR 40	2.12 ± 0.11(2.6)	3.40 ± 0.11(1.5)	2.86 ± 0.09(1.7)	n.g.[a]	n.g.	n.g.	n.g.
BRF isolate KYO	1.95 ± 0.07(2.8)	2.16 ± 0.04(2.4)	1.99 ± 0.05(2.4)	1.75 ± 0.03(2.5)	1.78 ± 0.02(2.4)	1.81 ± 0.04(2.3)	1.70 ± 0.01(2.4)
WRF isolate WR3	4.35 ± 0.12(1.3)	4.03 ± 0.12(1.3)	3.82 ± 0.08(1.2)	n.g.	n.g.	n.g.	n.g.
BRF isolate WR4	2.05 ± 0.05(2.7)	2.09 ± 0.07(2.4)	2.06 ± 0.05(2.3)	1.93 ± 0.06(2.3)	1.72 ± 0.04(2.5)	1.50 ± 0.03(2.8)	n.g.

[a] Growth was not observed under these conditions

Means of three replicates were shown with ± SD

Numbers within parentheses represent the pH reduction values (units redution) with respect to the initial pH values

B7. Statistic analysis data by Oneway-ANOVA of the pH Reduction values of brown-rot fungal isolate MR40 in cadmium sulfate-amended medium

ANOVA

pHReduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.087	2	1.043	141.649	.000
Within Groups	.044	6	.007		
Total	2.131	8			

Post Hoc Tests

Homogeneous Subsets

pH Reduction

Duncan [a]

		Subset for alpha = .05	
Conc	N	1	2
100.00	3	1.5033	
500.00	3	1.6667	
.00	3		2.5967
Sig.		.059	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B8. Statistic analysis data by Oneway-ANOVA of the pH Reduction values of brown-rot fungal isolate KYO in cadmium sulfate-amended medium

ANOVA

pHReduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.510	6	.085	30.185	.000
Within Groups	.039	14	.003		
Total	.549	20			

Post Hoc Tests

Homogeneous Subsets

pH Reduction

Duncan [a]

		Subset for alpha = .05			
Conc	N	1	2	3	4
8000.00	3	2.3333			
100.00	3	2.3633	2.3633		
500.00	3	2.4000	2.4000		
5000.00	3	2.4167	2.4167	2.4167	
10000.00	3		2.4400	2.4400	
2000.00	3			2.5100	
.00	3				2.8300
Sig.		.097	.124	.059	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B9. Statistic analysis data by Oneway-ANOVA of the pH Reduction values of white-rot fungal isolate WR3 in cadmium sulfate-amended medium

ANOVA

pHReduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	2	.000	.424	.673
Within Groups	.006	6	.001		
Total	.007	8			

Post Hoc Tests

Homogeneous Subsets

pH Reduction

Duncan[a]

Conc	N	Subset for alpha = .05
		1
500.00	3	1.2467
100.00	3	1.2633
.00	3	1.2700
Sig.		.420

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B10. Statistic analysis data by Oneway-ANOVA of the pH Reduction values of brown-rot fungal isolate WR4 in cadmium sulfate-amended medium

ANOVA

pHReduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.654	5	.131	30.461	.000
Within Groups	.052	12	.004		
Total	.706	17			

Post Hoc Tests

Homogeneous Subsets

pH Reduction

Duncan [a]

		Subset for alpha = .05			
Conc	N	1	2	3	4
2000.00	3	2.2800			
500.00	3	2.3133			
100.00	3		2.4433		
5000.00	3		2.5067		
.00	3			2.6833	
8000.00	3				2.8133
Sig.		.545	.259	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table B4 Change in pH of MEA medium produced by brown-rot fungal isolates MR40, KYO and WR4, and white-rot fungal isolates WR3 and WR5 in the presence or absence of lead nitrate

Isolates	Lead concentration (ppm)/ Initial pH						
	0 / 5.5	1000 / 4.5	4000 / 3.73	9000 / 3.6	12000 / 3.53	15000 / 3.48	18000 / 3.43
BRF isolate MR 40	2.12 ± 0.11(2.6)	2.65 ± 0.18(1.7)	1.99 ± 0.03(1.9)	1.89 ± 0.11(1.9)	1.82 ± 0.09(1.9)	2.09 ± 0.06(1.7)	2.64 ± 0.10(1.3)
BRF isolate KYO	1.95 ± 0.07(2.8)	1.44 ± 0.30(3.1)	1.68 ± 0.03(2.2)	1.63 ± 0.05(2.2)	1.58 ± 0.02(2.2)	1.58 ± 0.02(2.2)	1.60 ± 0.02(2.1)
WRF isolate WR3	4.35 ± 0.12(1.3)	4.08 ± 0.11(1.1)	3.36 ± 0.06(1.1)	n.g.[a]	n.g.	n.g.	n.g.
BRF isolate WR4	2.05 ± 0.05(2.7)	2.01 ± 0.01(2.2)	1.37 ± 0.09(2.7)	1.64 ± 0.02(2.2)	1.73 ± 0.05(2.0)	n.g.	n.g.
WRF isolate WR5	6.40 ± 0.31(1.2)*	4.13 ± 0.16(1.1)	3.44 ± 0.06(1.1)	n.g.	n.g.	n.g.	n.g.

[a] Growth was not observed under these conditions

Means of three replicates were shown with ± SD

Numbers within parentheses represent the pH reduction values (units redution) with respect to the initial pH values. The asterisk (*) indicate the pH increase values (units increase) with respect to the initial pH values.

B11. Statistic analysis data by Oneway-ANOVA of the pH Reduction values of brown-rot fungal isolate MR40 in lead nitrate -amended medium

ANOVA

pHReduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.782	6	.464	55.175	.000
Within Groups	.118	14	.008		
Total	2.900	20			

Post Hoc Tests

Homogeneous Subsets

pH Reduction

Duncan [a]

		Subset for alpha = .05			
Conc	N	1	2	3	4
18000.00	3	1.3000			
15000.00	3		1.6700		
1000.00	3		1.7033		
4000.00	3			1.8833	
9000.00	3			1.9100	
12000.00	3			1.9467	
.00	3				2.5967
Sig.		1.000	.663	.436	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B12. Statistic analysis data by Oneway-ANOVA of the pH Reduction values of brown-rot fungal isolate KYO in lead nitrate -amended medium

ANOVA

pHReduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.151	6	.525	7.851	.001
Within Groups	.936	14	.067		
Total	4.087	20			

Post Hoc Tests

Homogeneous Subsets

pH Reduction

Duncan [a]

		Subset for alpha = .05	
Conc	N	1	2
18000.00	3	2.1467	
15000.00	3	2.2033	
9000.00	3	2.2133	
4000.00	3	2.2233	
12000.00	3	2.2300	
.00	3		2.8300
1000.00	3		3.2233
Sig.		.725	.084

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B13. Statistic analysis data by Oneway-ANOVA of the pH Reduction values of white-rot fungal isolate WR3 in lead nitrate -amended medium

ANOVA

pHReduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.055	2	.027	34.125	.001
Within Groups	.005	6	.001		
Total	.059	8			

Post Hoc Tests

Homogeneous Subsets

pH Reduction

Duncan [a]

		Subset for alpha = .05	
Conc	N	1	2
1000.00	3	1.1000	
4000.00	3	1.1100	
.00	3		1.2700
Sig.		.680	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B14. Statistic analysis data by Oneway-ANOVA of the pH Reduction values of brown-rot fungal isolate WR4 in lead nitrate -amended medium

ANOVA

pHReduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.139	4	.285	36.707	.000
Within Groups	.078	10	.008		
Total	1.217	14			

Post Hoc Tests

Homogeneous Subsets

pH Reduction

Duncan [a]

		Subset for alpha = .05		
Conc	N	1	2	3
12000.00	3	2.0367		
9000.00	3	2.1933	2.1933	
1000.00	3		2.2400	
.00	3			2.6833
4000.00	3			2.7200
Sig.		.054	.531	.621

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B15. Statistic analysis data by Oneway-ANOVA of the pH Reduction values of white-rot fungal isolate WR5 in lead nitrate -amended medium

ANOVA

pHReduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.011	2	.005	2.630	.151
Within Groups	.012	6	.002		
Total	.023	8			

Post Hoc Tests

Homogeneous Subsets

pH Reduction

Duncan [a]

Conc	N	Subset for alpha = .05
		1
1000.00	3	1.0900
4000.00	3	1.0900
.00	3	1.1633
Sig.		.104

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B16. Statistic analysis data by Oneway-ANOVA of the mycelial diameter and the biomass of brown-rot fungal isolate MR40 in zinc sulfate-amended medium

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Biomass	Between Groups	131811.7	4	32952.914	74.497	.000
	Within Groups	4423.360	10	442.336		
	Total	136235.0	14			
Diameter	Between Groups	312.197	4	78.049	4294.712	.000
	Within Groups	.182	10	.018		
	Total	312.379	14			

Post Hoc Tests

Homogeneous Subsets

Diameter

Duncan [a]

		Subset for alpha = .05			
Conc	N	1	2	3	4
3000.00	3	.8467			
2000.00	3		2.7033		
500.00	3			9.6300	
1000.00	3			9.6300	
.00	3				12.8300
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Biomass

Duncan [a]

		Subset for alpha = .05		
Conc	N	1	2	3
3000.00	3	32.4000		
1000.00	3		183.7333	
2000.00	3		192.5000	
.00	3			286.3667
500.00	3			290.0667
Sig.		1.000	.621	.834

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B17. Statistic analysis data by Oneway-ANOVA of the mycelial diameter and the biomass of brown-rot fungal isolate KYO in zinc sulfate-amended medium

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Biomass	Between Groups	5689.063	3	1896.354	21.499	.000
	Within Groups	705.667	8	88.208		
	Total	6394.729	11			
Diameter	Between Groups	74.282	3	24.761	351.050	.000
	Within Groups	.564	8	.071		
	Total	74.846	11			

Post Hoc Tests

Homogeneous Subsets

Diameter

Duncan [a]

Conc	N	Subset for alpha = .05		
		1	2	3
2000.00	3	5.8067		
500.00	3		9.6300	
1000.00	3		9.6300	
.00	3			12.8300
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Biomass

Duncan [a]

Conc	N	Subset for alpha = .05		
		1	2	3
.00	3	78.8000		
500.00	3	84.1000		
1000.00	3		112.3667	
2000.00	3			132.5000
Sig.		.509	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B18. Statistic analysis data by Oneway-ANOVA of the mycelial diameter and the biomass of brown-rot fungal isolate WR4 in zinc sulfate-amended medium

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Biomass	Between Groups	1520.129	3	506.710	4.961	.031
	Within Groups	817.160	8	102.145		
	Total	2337.289	11			
Diameter	Between Groups	64.192	3	21.397	24.919	.000
	Within Groups	6.869	8	.859		
	Total	71.062	11			

Post Hoc Tests

Homogeneous Subsets

Diameter

Duncan [a]

		Subset for alpha = .05		
Conc	N	1	2	3
2000.00	3	6.4200		
1000.00	3		8.5600	
500.00	3		9.6300	
.00	3			12.8300
Sig.		1.000	.195	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Biomass

Duncan [a]

		Subset for alpha = .05	
Conc	N	1	2
.00	3	87.3333	
500.00	3	90.1000	
1000.00	3	101.0333	101.0333
2000.00	3		115.9667
Sig.		.150	.108

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B19. Statistic analysis data by Oneway-ANOVA of the the mycelial diameter and the biomass of brown-rot fungal isolate MR40 in copper sulfate-amended medium

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Biomass	Between Groups	9946.416	2	4973.208	6.156	.035
	Within Groups	4847.100	6	807.850		
	Total	14793.516	8			
Diameter	Between Groups	15.929	2	7.964	7.000	.027
	Within Groups	6.827	6	1.138		
	Total	22.756	8			

Post Hoc Tests

Homogeneous Subsets

Diameter

Duncan[a]

		Subset for alpha = .05	
Conc	N	1	2
1000.00	3	9.6300	
500.00	3	10.6967	
.00	3		12.8300
Sig.		.267	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Biomass

Duncan[a]

		Subset for alpha = .05	
Conc	N	1	2
.00	3	286.3667	
1000.00	3		356.4333
500.00	3		357.3333
Sig.		1.000	.970

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B20. Statistic analysis data by Oneway-ANOVA of the the mycelial diameter and the biomass of brown-rot fungal isolate KYO in copper sulfate-amended medium

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Biomass	Between Groups	45768.780	4	11442.195	71.071	.000
	Within Groups	1609.973	10	160.997		
	Total	47378.753	14			
Diameter	Between Groups	334.418	4	83.605	111.484	.000
	Within Groups	7.499	10	.750		
	Total	341.918	14			

Post Hoc Tests

Homogeneous Subsets

Diameter

Duncan [a]

		Subset for alpha = .05		
Conc	N	1	2	3
5000.00	3	1.8200		
3000.00	3	2.1900		
1000.00	3		9.6300	
500.00	3			11.7633
.00	3			12.8300
Sig.		.612	1.000	.162

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Biomass

Duncan [a]

		Subset for alpha = .05	
Conc	N	1	2
.00	3	78.8000	
500.00	3	92.8333	
3000.00	3		189.8333
5000.00	3		196.6000
1000.00	3		206.6000
Sig.		.205	.153

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B21. Statistic analysis data by Oneway-ANOVA of the mycelial diameter and the biomass of brown-rot fungal isolate WR4 in copper sulfate-amended medium

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Biomass	Between Groups	20753.980	3	6917.993	58.796	.000
	Within Groups	941.287	8	117.661		
	Total	21695.267	11			
Diameter	Between Groups	180.966	3	60.322	68.504	.000
	Within Groups	7.045	8	.881		
	Total	188.010	11			

Post Hoc Tests

Homogeneous Subsets

Diameter

Duncan [a]

		Subset for alpha = .05		
Conc	N	1	2	3
3000.00	3	2.8433		
1000.00	3		9.6300	
500.00	3			11.7633
.00	3			12.8300
Sig.		1.000	1.000	.201

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Biomass

Duncan [a]

		Subset for alpha = .05			
Conc	N	1	2	3	4
.00	3	87.3333			
500.00	3		118.2333		
1000.00	3			166.5333	
3000.00	3				194.5667
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B22. Statistic analysis data by Oneway-ANOVA of the mycelial diameter and the biomass of brown-rot fungal isolate MR40 in cadmium sulfate-amended medium

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Biomass	Between Groups	115410.6	2	57705.314	421.665	.000
	Within Groups	821.107	6	136.851		
	Total	116231.7	8			
Diameter	Between Groups	284.908	2	142.454	122103.3	.000
	Within Groups	.007	6	.001		
	Total	284.915	8			

Post Hoc Tests

Homogeneous Subsets

Diameter

Duncan [a]

		Subset for alpha = .05		
Conc	N	1	2	3
500.00	3	.5600		
100.00	3		1.2600	
.00	3			12.8300
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Biomass

Duncan [a]

		Subset for alpha = .05	
Conc	N	1	2
100.00	3	46.8000	
500.00	3	46.8333	
.00	3		286.3667
Sig.		.997	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B23. Statistic analysis data by Oneway-ANOVA of the the mycelial diameter and the biomass of brown-rot fungal isolate KYO in cadmium sulfate-amended medium

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Biomass	Between Groups	86031.345	6	14338.557	62.888	.000
	Within Groups	3192.033	14	228.002		
	Total	89223.378	20			
Diameter	Between Groups	369.365	6	61.561	12587.916	.000
	Within Groups	.068	14	.005		
	Total	369.434	20			

Post Hoc Tests

Homogeneous Subsets

Diameter

Duncan[a]

		Subset for alpha = .05						
Conc	N	1	2	3	4	5	6	7
10000.00	3	1.1100						
8000.00	3		1.5200					
5000.00	3			2.2067				
2000.00	3				4.2300			
500.00	3					7.7000		
100.00	3						9.6300	
.00	3							12.8300
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Biomass

Duncan[a]

		Subset for alpha = .05			
Conc	N	1	2	3	4
500.00	3	76.7667			
.00	3	78.8000			
100.00	3	83.0667			
10000.00	3		123.5333		
8000.00	3			201.3333	
5000.00	3			208.9000	208.9000
2000.00	3				234.9667
Sig.		.636	1.000	.549	.053

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B24. Statistic analysis data by Oneway-ANOVA of the the mycelial diameter and the biomass of white-rot fungal isolate WR3 in cadmium sulfate-amended medium

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Biomass	Between Groups	27942.740	2	13971.370	32.208	.001
	Within Groups	2602.720	6	433.787		
	Total	30545.460	8			
Diameter	Between Groups	148.378	2	74.189	14904.016	.000
	Within Groups	.030	6	.005		
	Total	148.408	8			

Post Hoc Tests

Homogeneous Subsets

Diameter

Duncan [a]

Conc	N	Subset for alpha = .05		
		1	2	3
500.00	3	.3567		
100.00	3		1.8800	
.00	3			9.6300
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Biomass

Duncan [a]

Conc	N	Subset for alpha = .05		
		1	2	3
500.00	3	47.5667		
100.00	3		99.6667	
.00	3			182.8667
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B25. Statistic analysis data by Oneway-ANOVA of the the mycelial diameter and the biomass of brown-rot fungal isolate WR4 in cadmium sulfate-amended medium

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Biomass	Between Groups	77531.263	5	15506.253	52.902	.000
	Within Groups	3517.380	12	293.115		
	Total	81048.643	17			
Diameter	Between Groups	204.573	5	40.915	204572.7	.000
	Within Groups	.002	12	.000		
	Total	204.575	17			

Post Hoc Tests

Homogeneous Subsets

Diameter

Duncan [a]

		Subset for alpha = .05				
Conc	N	1	2	3	4	5
8000.00	3	2.4200				
5000.00	3		3.5000			
2000.00	3			6.4200		
100.00	3				7.7000	
500.00	3				7.7000	
.00	3					12.8300
Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Biomass

Duncan [a]

		Subset for alpha = .05			
Conc	N	1	2	3	4
.00	3	87.3333			
500.00	3		119.0667		
100.00	3		138.2333		
8000.00	3			205.5667	
2000.00	3			217.6333	
5000.00	3				279.5333
Sig.		1.000	.195	.405	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B26. Statistic analysis data by Oneway-ANOVA of the mycelial diameter and the biomass of brown-rot fungal isolate MR40 in lead nitrate -amended medium

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Biomass	Between Groups	125609.9	6	20934.981	18.966	.000
	Within Groups	15453.320	14	1103.809		
	Total	141063.2	20			
Diameter	Between Groups	367.600	6	61.267	108.319	.000
	Within Groups	7.919	14	.566		
	Total	375.518	20			

Post Hoc Tests

Homogeneous Subsets

Diameter

Duncan[a]

		Subset for alpha = .05					
Conc	N	1	2	3	4	5	6
18000.00	3	.9500					
15000.00	3	1.2000					
12000.00	3		4.3133				
9000.00	3			6.1133			
4000.00	3				7.7000		
1000.00	3					10.6967	
.00	3						12.8300
Sig.		.690	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Biomass

Duncan[a]

		Subset for alpha = .05			
Conc	N	1	2	3	4
18000.00	3	21.7000			
15000.00	3		83.6667		
4000.00	3		100.0667		
9000.00	3		117.1667		
1000.00	3		144.1667	144.1667	
12000.00	3			178.4667	
.00	3				286.3667
Sig.		1.000	.058	.227	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B27. Statistic analysis data by Oneway-ANOVA of the the mycelial diameter and the biomass of brown-rot fungal isolate KYO in lead nitrate –amended medium

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Biomass	Between Groups	64164.031	6	10694.005	44.964	.000
	Within Groups	3329.660	14	237.833		
	Total	67493.691	20			
Diameter	Between Groups	269.890	6	44.982	87.431	.000
	Within Groups	7.203	14	.514		
	Total	277.093	20			

Post Hoc Tests

Homogeneous Subsets

Diameter

Duncan [a]

		Subset for alpha = .05				
Conc	N	1	2	3	4	5
18000.00	3	2.7133				
15000.00	3	3.5000				
12000.00	3		4.8100			
9000.00	3			6.4200		
4000.00	3				9.6300	
1000.00	3				10.6967	
.00	3					12.8300
Sig.		.201	1.000	1.000	.090	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Biomass

Duncan [a]

		Subset for alpha = .05			
Conc	N	1	2	3	4
1000.00	3	66.0000			
4000.00	3	72.9333			
.00	3	78.8000			
9000.00	3		126.8333		
18000.00	3			165.9000	
15000.00	3			184.8000	
12000.00	3				214.3333
Sig.		.351	1.000	.156	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B28. Statistic analysis data by Oneway-ANOVA of the mycelial diameter and the biomass of white-rot fungal isolate WR3 in lead nitrate –amended medium

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Diameter	Between Groups	102.973	2	51.486	118814.5	.000
	Within Groups	.003	6	.000		
	Total	102.975	8			
Biomass	Between Groups	46994.509	2	23497.254	46.067	.000
	Within Groups	3060.380	6	510.063		
	Total	50054.889	8			

Post Hoc Tests

Homogeneous Subsets

Diameter

Duncan [a]

		Subset for alpha = .05		
Conc	N	1	2	3
4000.00	3	1.4100		
1000.00	3		4.6200	
.00	3			9.6300
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Biomass

Duncan [a]

		Subset for alpha = .05	
Conc	N	1	2
4000.00	3	39.7667	
.00	3		182.8667
1000.00	3		201.5333
Sig.		1.000	.350

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B29. Statistic analysis data by Oneway-ANOVA of the mycelial diameter and the biomass of brown-rot fungal isolate WR4 in lead nitrate -amended medium

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Biomass	Between Groups	89492.587	4	22373.147	65.854	.000
	Within Groups	3397.387	10	339.739		
	Total	92889.973	14			
Diameter	Between Groups	189.112	4	47.278	1108.079	.000
	Within Groups	.427	10	.043		
	Total	189.539	14			

Post Hoc Tests

Homogeneous Subsets

Diameter

Duncan [a]

		Subset for alpha = .05			
Conc	N	1	2	3	4
12000.00	3	2.6933			
9000.00	3		5.5000		
1000.00	3			9.6300	
4000.00	3			9.6300	
.00	3				12.8300
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Biomass

Duncan [a]

		Subset for alpha = .05			
Conc	N	1	2	3	4
4000.00	3	43.8000			
1000.00	3	65.4667	65.4667		
.00	3		87.3333		
9000.00	3			155.7333	
12000.00	3				256.8333
Sig.		.181	.177	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B30. Statistic analysis data by Oneway-ANOVA of the mycelial diameter and the biomass of white-rot fungal isolate WR5 in lead nitrate -amended medium

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Biomass	Between Groups	58598.069	2	29299.034	90.183	.000
	Within Groups	1949.300	6	324.883		
	Total	60547.369	8			
Diameter	Between Groups	49.682	2	24.841	1689.878	.000
	Within Groups	.088	6	.015		
	Total	49.771	8			

Post Hoc Tests

Homogeneous Subsets

Diameter

Duncan [a]

		Subset for alpha = .05		
Conc	N	1	2	3
4000.00	3	1.0400		
.00	3		5.5000	
1000.00	3			6.4200
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Biomass

Duncan [a]

		Subset for alpha = .05		
Conc	N	1	2	3
4000.00	3	22.4333		
.00	3		107.0667	
1000.00	3			219.4333
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B31. Statistic analysis data by Oneway-ANOVA of zinc accumulation by brown-rot fungal isolate MR40, KYO and WR4

ANOVA

Content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	15660874	9	1740097.152	181.157	.000
Within Groups	192109.5	20	9605.474		
Total	15852984	29			

Post Hoc Tests

Homogeneous Subsets

Content

Duncan [a]

		Subset for alpha = .05		
Isolates	N	1	2	3
3.00	3	44.4667		
2.00	3	46.0867		
1.00	3	52.2533		
6.00	3	55.2067		
8.00	3	60.3000		
4.00	3	72.9600		
5.00	3	105.0467		
9.00	3	216.5733		
7.00	3		608.8333	
10.00	3			2485.3333
Sig.		.075	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B32. Statistic analysis data by Oneway-ANOVA of copper accumulation by brown-rot fungal isolate MR40, KYO and WR4

ANOVA

Content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1545802	8	193225.274	20.292	.000
Within Groups	171403.3	18	9522.404		
Total	1717205	26			

Post Hoc Tests

Homogeneous Subsets

Content

Duncan [a]

Isolate	N	Subset for alpha = .05				
		1	2	3	4	5
1.00	3	200.3333				
2.00	3		543.9333			
5.00	3		593.9333	593.9333		
4.00	3		604.9333	604.9333		
3.00	3		608.6000	608.6000		
6.00	3			763.0000	763.0000	
7.00	3				921.3333	921.3333
8.00	3				924.2667	924.2667
9.00	3					1032.6000
Sig.		1.000	.467	.065	.070	.202

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B33. Statistic analysis data by Oneway-ANOVA of cadmium accumulation by brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3

ANOVA

Content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2E+008	14	12797192.66	106.311	.000
Within Groups	3611248	30	120374.933		
Total	2E+008	44			

Post Hoc Tests

Homogeneous Subsets

Content

Duncan[a]

		Subset for alpha = .05					
Isolate	N	1	2	3	4	5	6
4.00	3	226.0000					
2.00	3	249.3333					
1.00	3	324.6667					
3.00	3	331.3333					
6.00	3	722.6667	722.6667				
8.00	3		1082.6667				
5.00	3		1232.0000	1232.0000			
7.00	3			1784.0000			
10.00	3				4186.6667		
11.00	3				4353.3333	4353.3333	
9.00	3				4600.0000	4600.0000	4600.0000
14.00	3				4620.0000	4620.0000	4620.0000
15.00	3				4800.0000	4800.0000	4800.0000
12.00	3					4893.3333	4893.3333
13.00	3						5073.3333
Sig.		.127	.099	.061	.060	.097	.145

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B34. Statistic analysis data by Oneway-ANOVA of lead accumulation by brown-rot fungal isolate MR40, KYO and WR4, and white-rot fungal isolate WR3 and WR5

ANOVA

Content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5E+008	19	24690414.89	136.749	.000
Within Groups	6680477	37	180553.445		
Total	5E+008	56			

Post Hoc Tests

Homogeneous Subsets

Content

Duncan [a, b]

Isolate	N	Subset for alpha = .05											
		1	2	3	4	5	6	7	8	9	10	11	12
2.00	3	49.60											
1.00	2	57.60											
9.00	3	655.67	655.67										
6.00	2		864.30	864.30									
5.00	3		878.67	878.67									
3.00	3		1054.80	1054.80									
4.00	3		1084.00	1084.00									
7.00	3			1537.80	1537.80								
8.00	3				2223.73	2223.73							
10.00	3					2867.87	2867.87						
19.00	3						3011.00						
11.00	3							4841.00					
17.00	2								6103.40				
20.00	3								6320.67	6320.67			
14.00	3								6491.80	6491.80	6491.80		
16.00	3								6851.53	6851.53	6851.53		
12.00	3									7061.13	7061.13	7061.13	
18.00	3										7161.47	7161.47	
15.00	3											7672.47	7672.47
13.00	3												8311.20
Sig.		.119	.298	.102	.064	.082	.693	1.000	.063	.066	.096	.116	.084

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 2.791.

b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

APPENDIX C

Energy-dispersive X-ray microanalysis (EDXA) of the extracted crystals

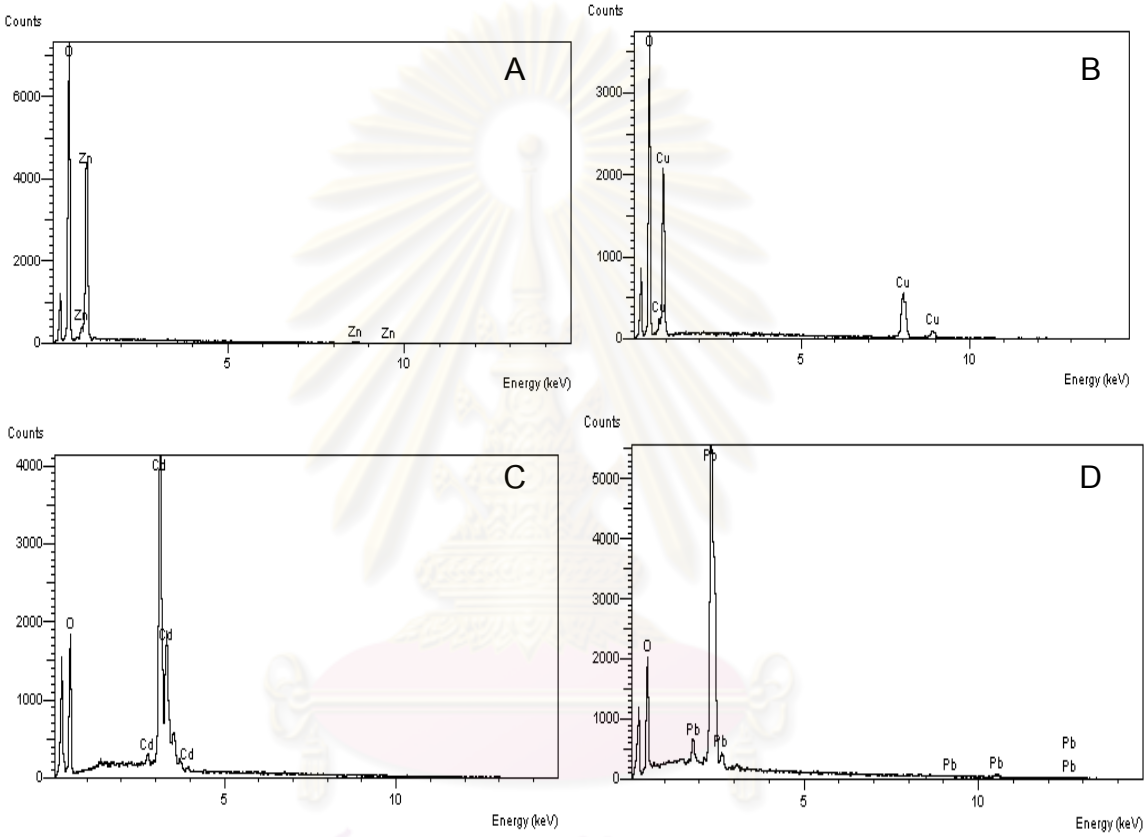

Fig.C1 Elemental composition of crystals produced by the isolate KYO during growth on zinc sulfate (A), copper sulfate (B), cadmium sulfate (C) and lead nitrate-amend medium (D).

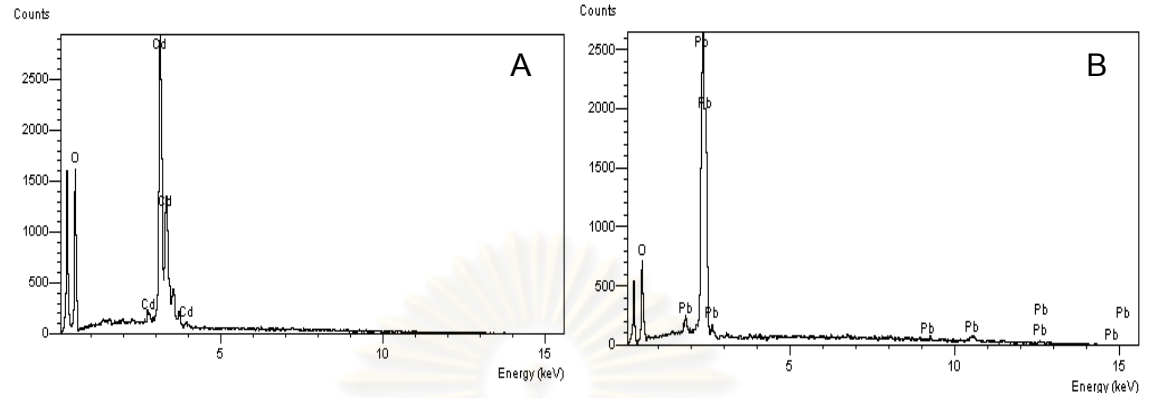

Fig. C2 Elemental composition of crystals produced by the isolate WR3 during growth on cadmium sulfate (A) and lead nitrate-amend medium (B).

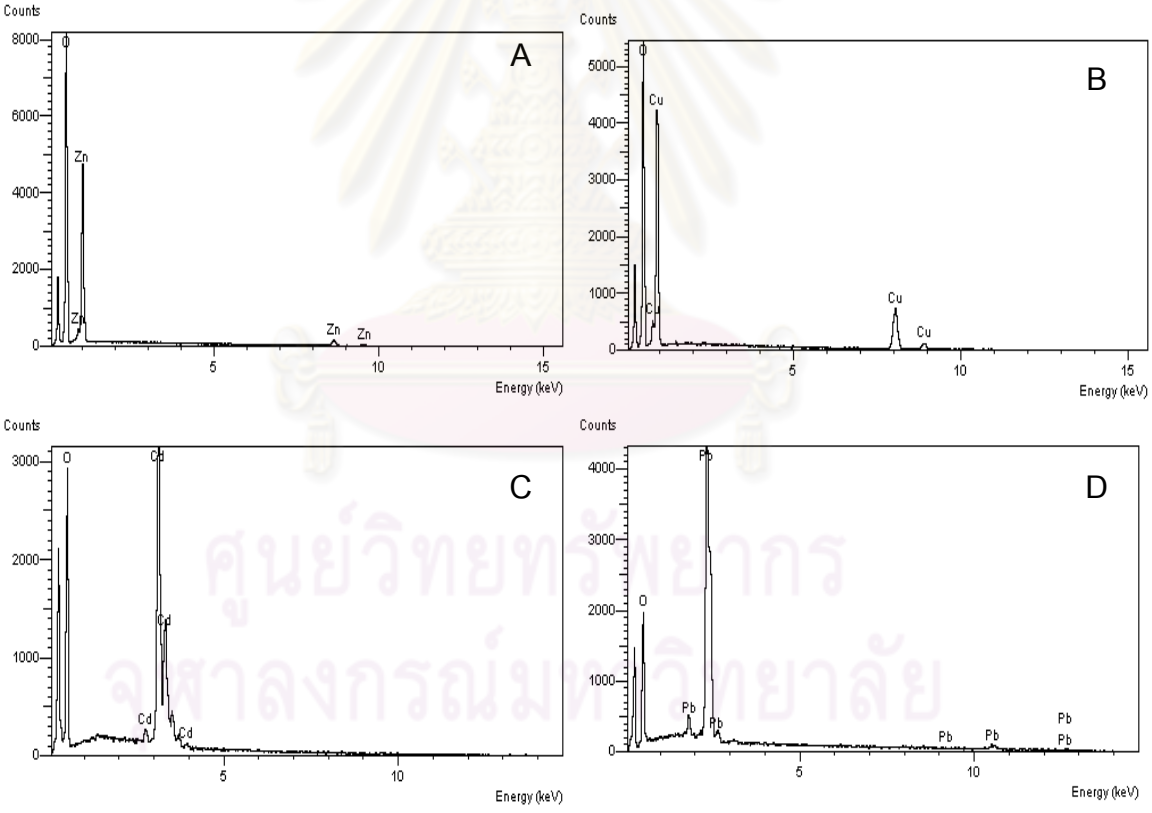

Fig. C3 Elemental composition of crystals produced by the isolate WR4 during growth on zinc sulfate (A), copper sulfate (B), cadmium sulfate (C) and lead nitrate-amend medium (D).

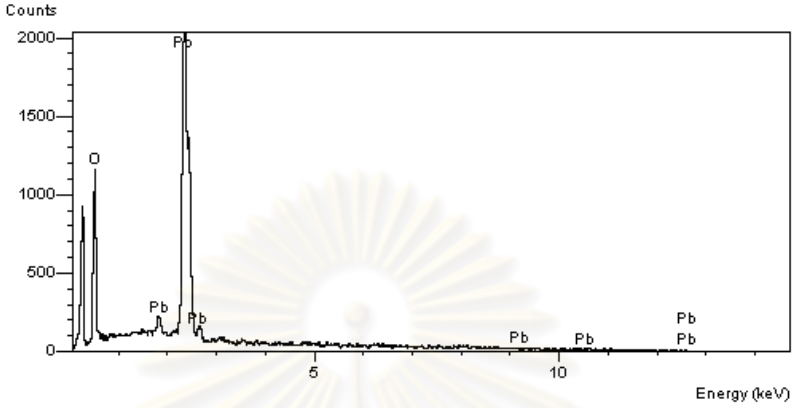

Fig. C4 Elemental composition of crystals produced by the isolate WR5 during growth on lead nitrate-amend medium.

APPENDIX D

HPLC chromatograms of the extracted crystals

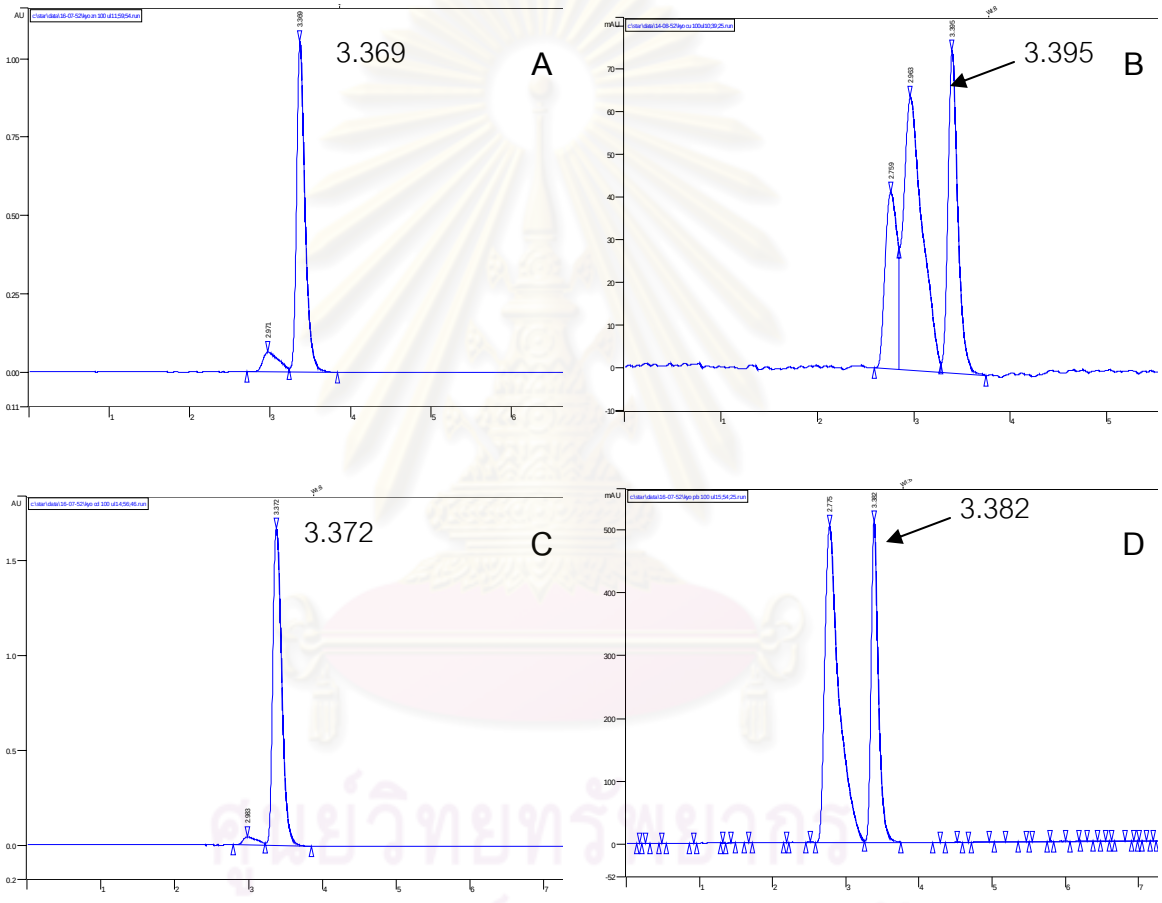

Fig. D1 HPLC chromatogram of the extracted crystals formed by the isolate KYO after grown on zinc sulfate (A); copper sulfate (B); cadmium sulfate (C), and lead nitrate-amended agar (D). X-axis—time (min), Y -axis—mAU.

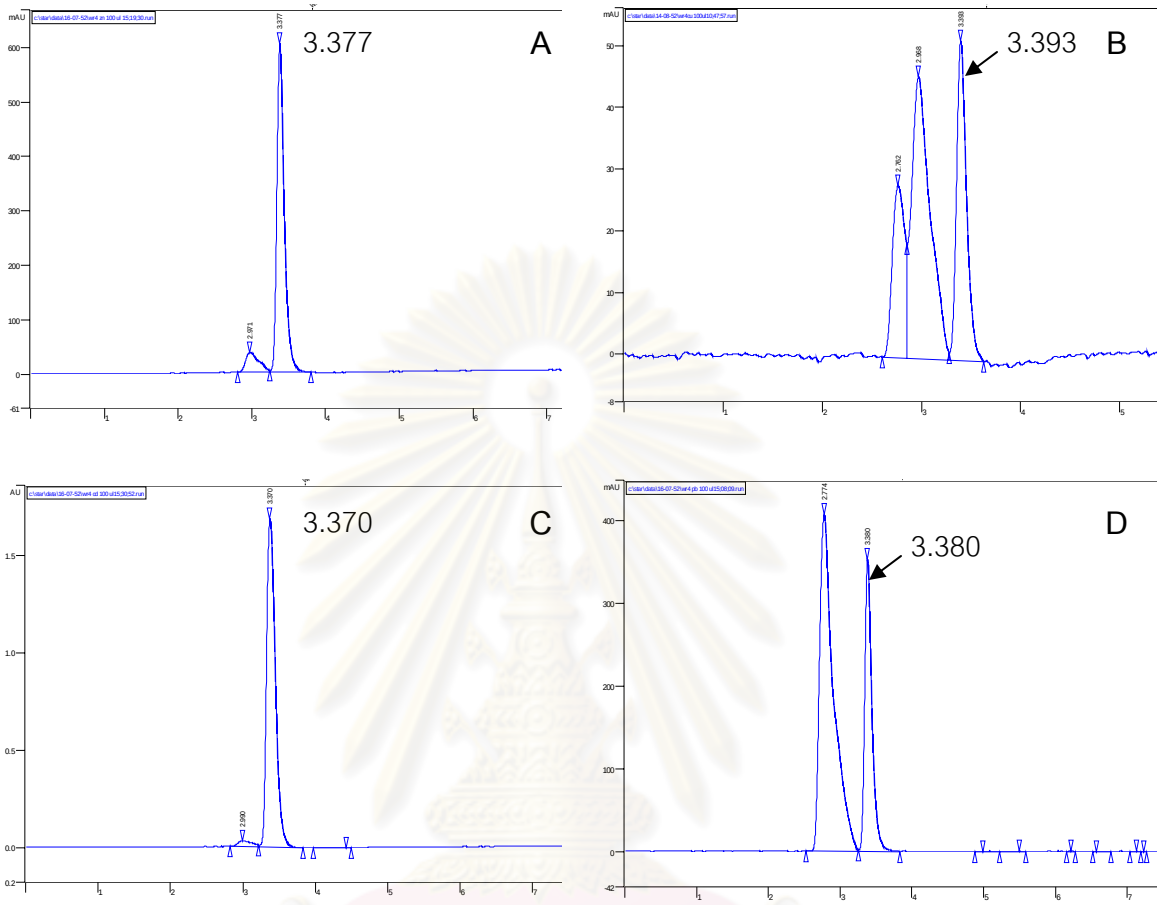

Fig. D2 HPLC chromatogram of the extracted crystals formed by the isolate WR4 after grown on zinc sulfate (A); copper sulfate (B); cadmium sulfate (C), and lead nitrate-amended agar (D). X-axis—time (min), Y -axis—mAU.

APPENDIX E

Typical XRPD patterns of metal oxalate crystals precipitated by wood-rotting fungi after growth on toxic metal medium

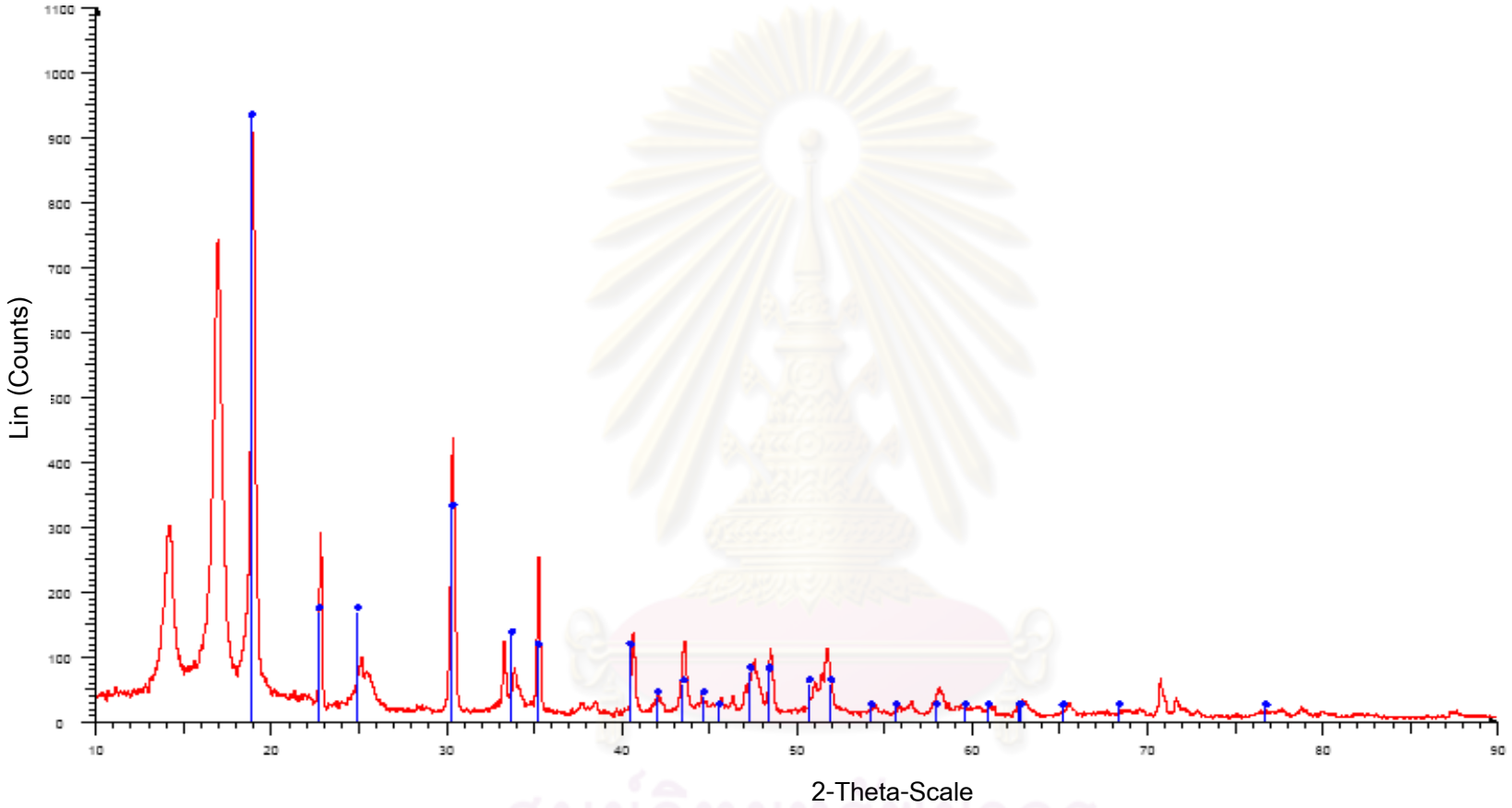

Fig. E1 Typical XRPD patterns for zinc oxalate hydrate crystals precipitated by the isolate KYO after growth on zinc sulfate-amended plate. The vertical bars indicate the peak positions and relative intensities for standard XRPD patterns of metal oxalates as given in the International Centre for Diffraction Data powder diffraction file. The standard used was zinc oxalate hydrate (PDF-14-0740).

198

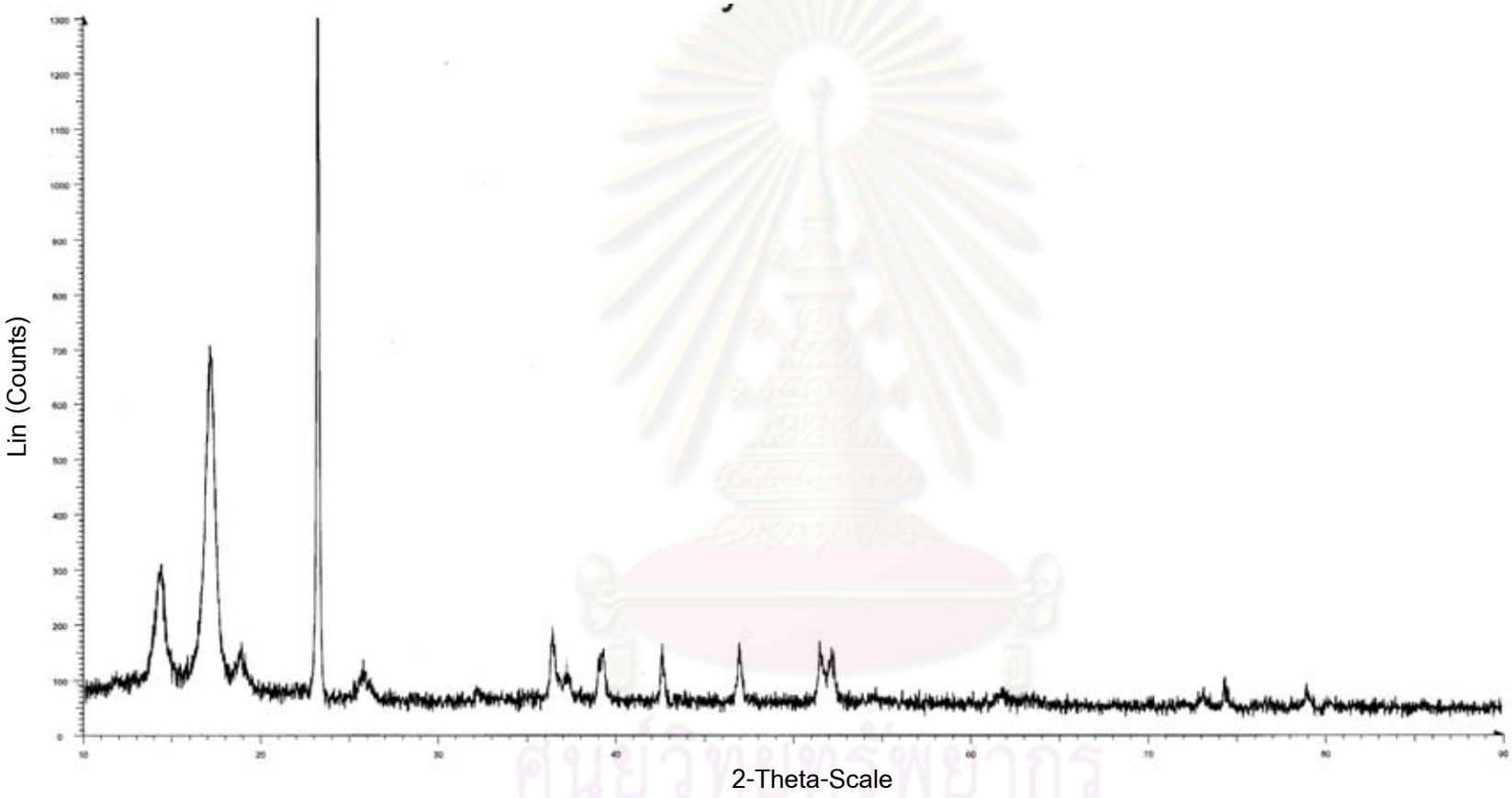

Fig. E2 Typical XRPD patterns for copper oxalate hydrate (moolooite) crystals precipitated by the isolate KYO after growth on copper sulfate-amended plate. The vertical bars indicate the peak positions and relative intensities for standard XRPD patterns of metal oxalates as given in the International Centre for Diffraction Data powder diffraction file. The standard used was copper oxalate hydrate (PDF-21-0297).

199

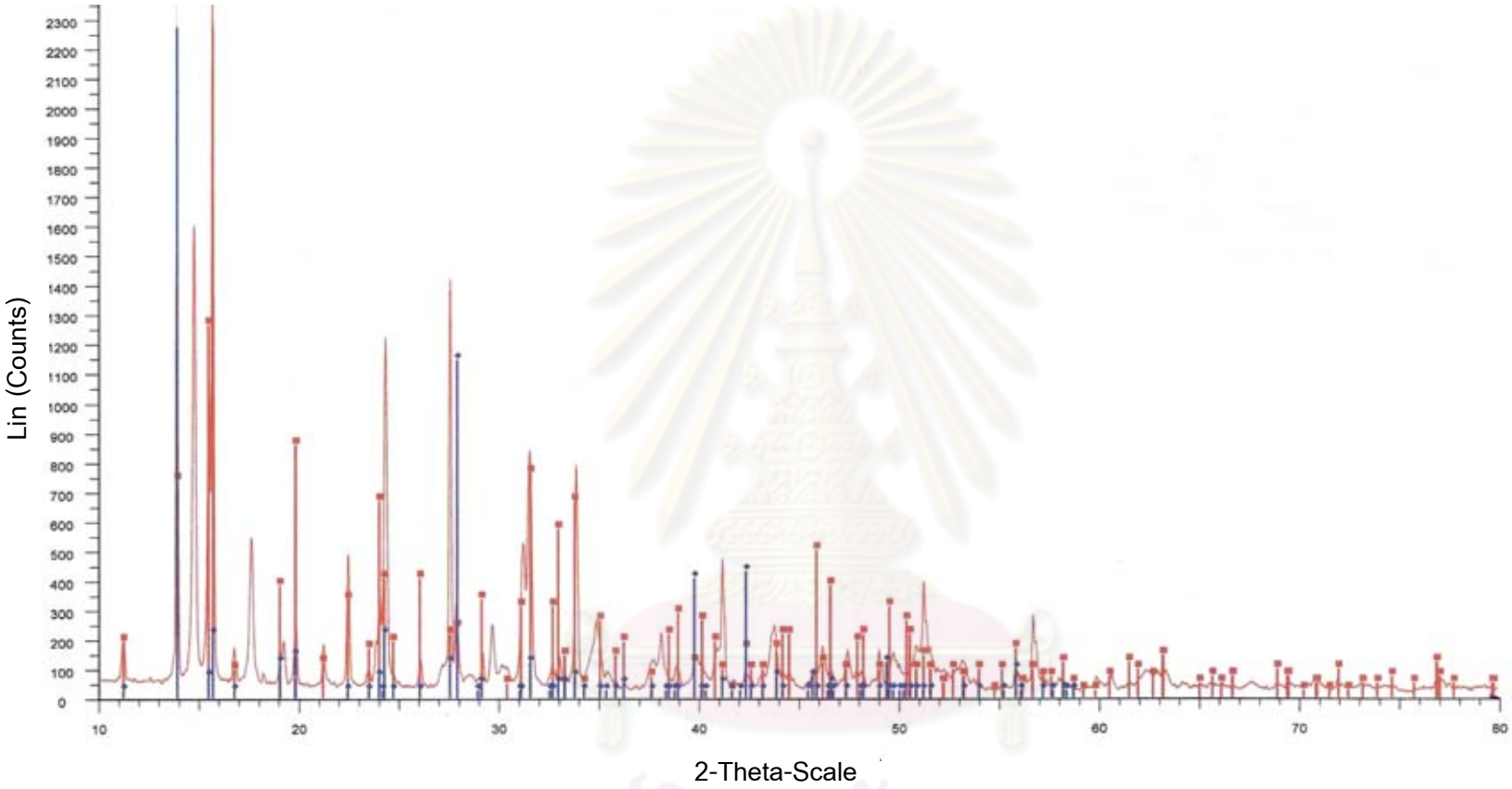

Fig. E3 Typical XRPD patterns for cadmium oxalate hydrate crystals precipitated by the isolate KYO after growth on cadmium sulfate-amended plate. The vertical bars indicate the peak positions and relative intensities for standard XRPD patterns of metal oxalates as given in the International Centre for Diffraction Data powder diffraction file. The standard used was cadmium oxalate hydrate (PDF-54-0317 and PDF-53-0085).

200

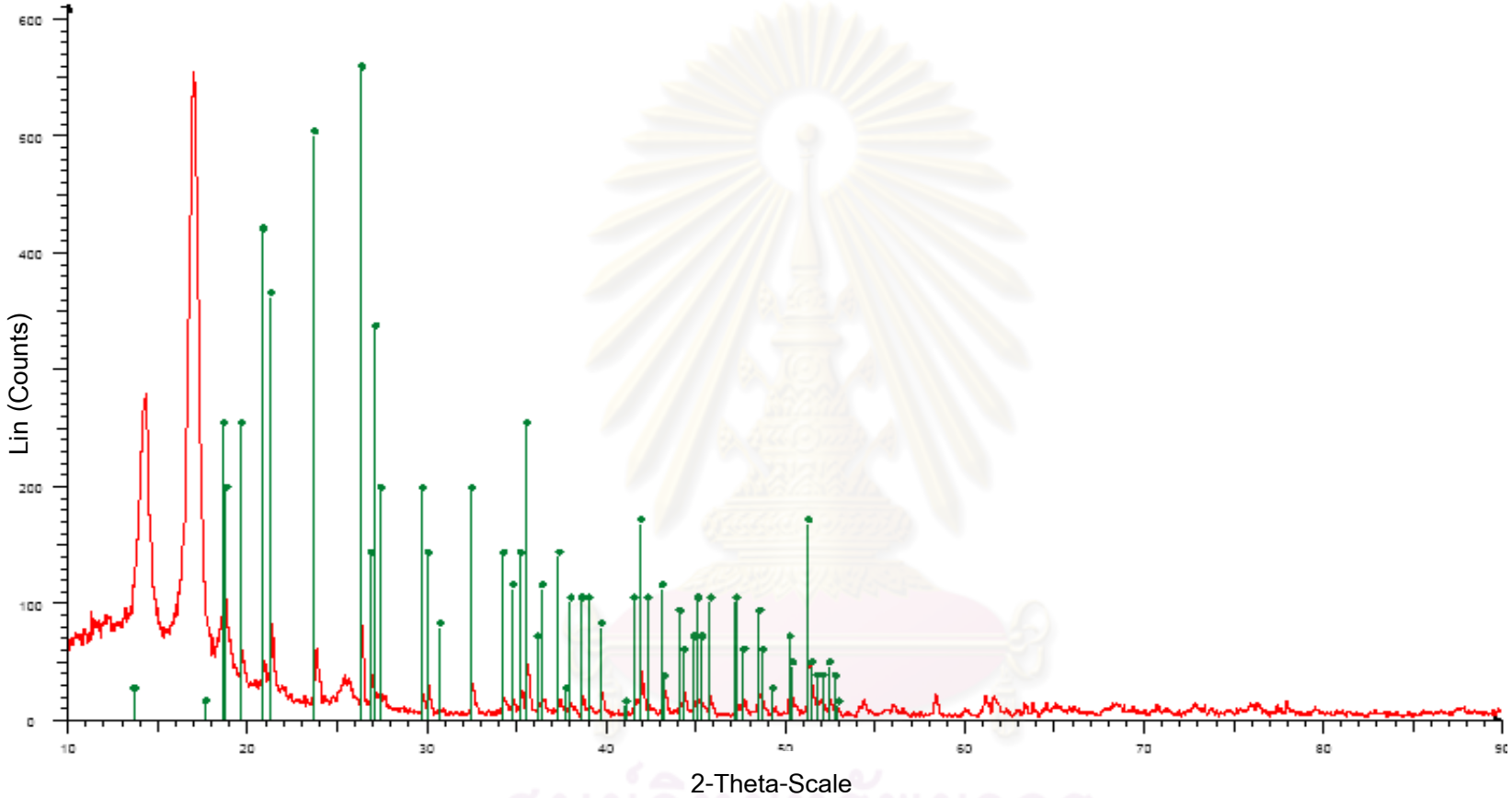

Fig. E4 Typical XRPD patterns for lead oxalate crystals precipitated by the isolate KYO after growth on lead nitrate-amended plate. The vertical bars indicate the peak positions and relative intensities for standard XRPD patterns of metal oxalates as given in the International Centre for Diffraction Data powder diffraction file. The standard used was lead oxalate (PDF-11-0723).

201

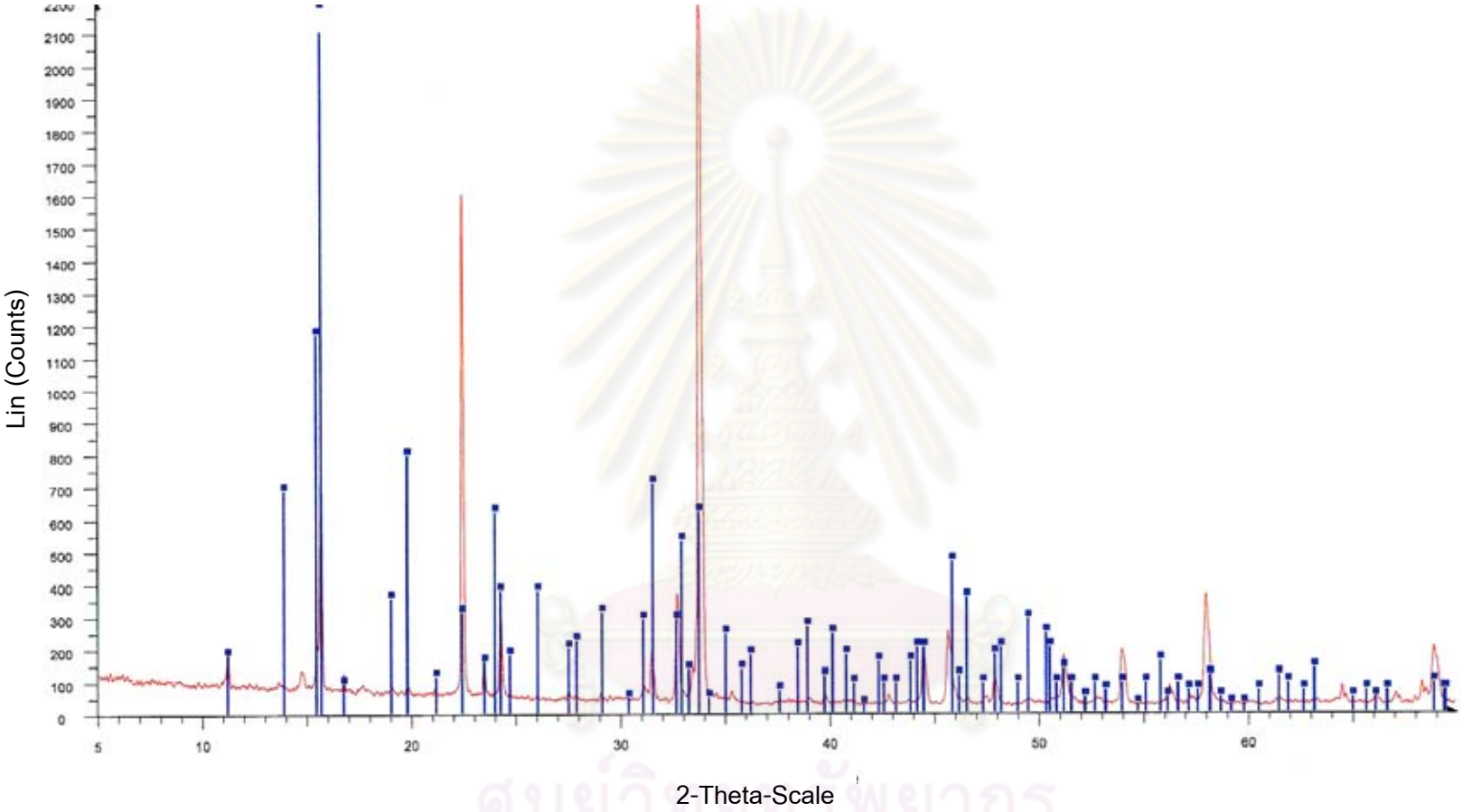

Fig. E5 Typical XRPD patterns for cadmium oxalate hydrate crystals precipitated by the isolate WR3 after growth on cadmium sulfate-amended plate. The vertical bars indicate the peak positions and relative intensities for standard XRPD patterns of metal oxalates as given in the International Centre for Diffraction Data powder diffraction file. The standard used was cadmium oxalate hydrate (PDF-54-0317).

202

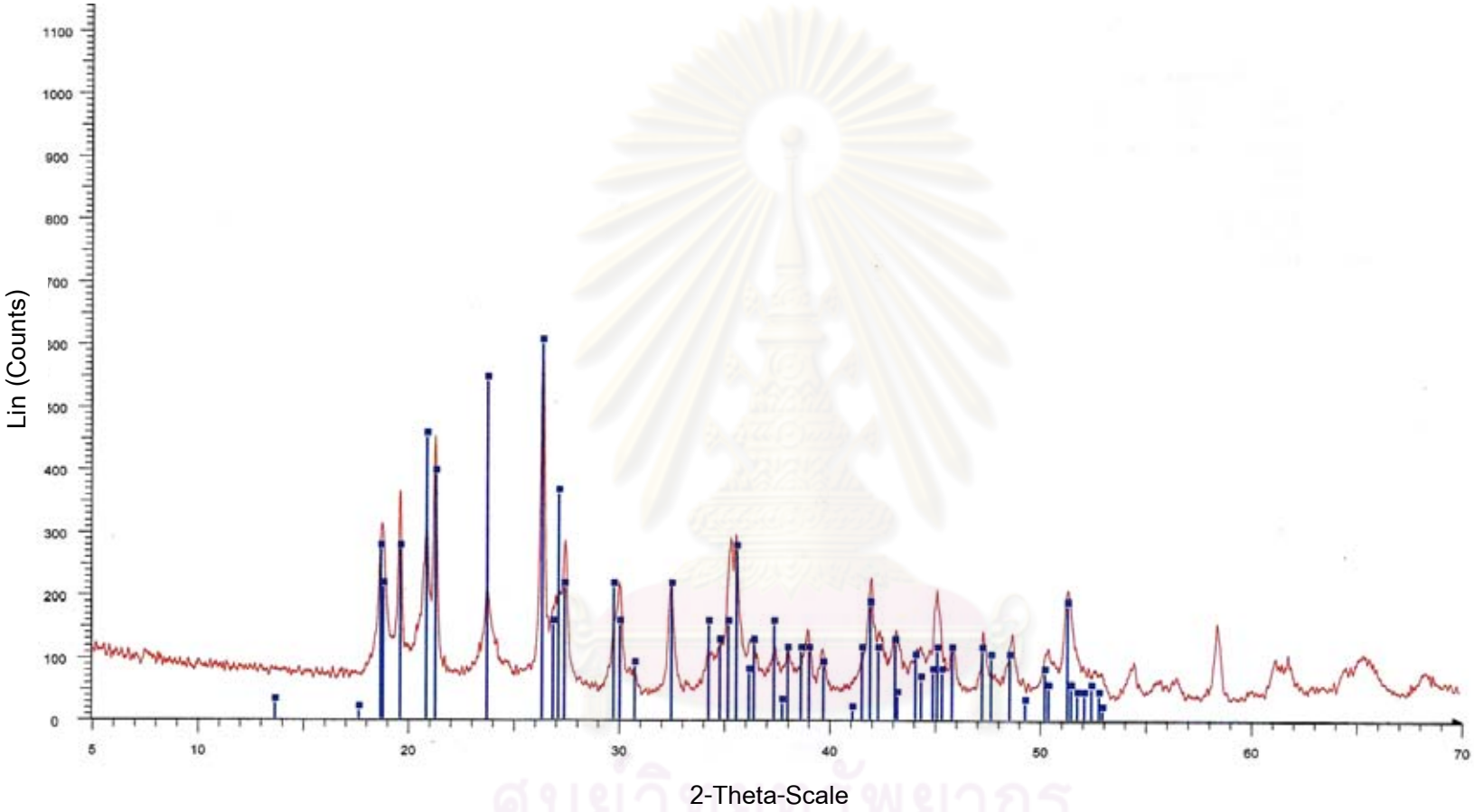

Fig. E6 Typical XRPD patterns for lead oxalate crystals precipitated by the isolate WR3 after growth on lead nitrate-amended plate. The vertical bars indicate the peak positions and relative intensities for standard XRPD patterns of metal oxalates as given in the International Centre for Diffraction Data powder diffraction file. The standard used was lead oxalate (PDF-14-0803).

203

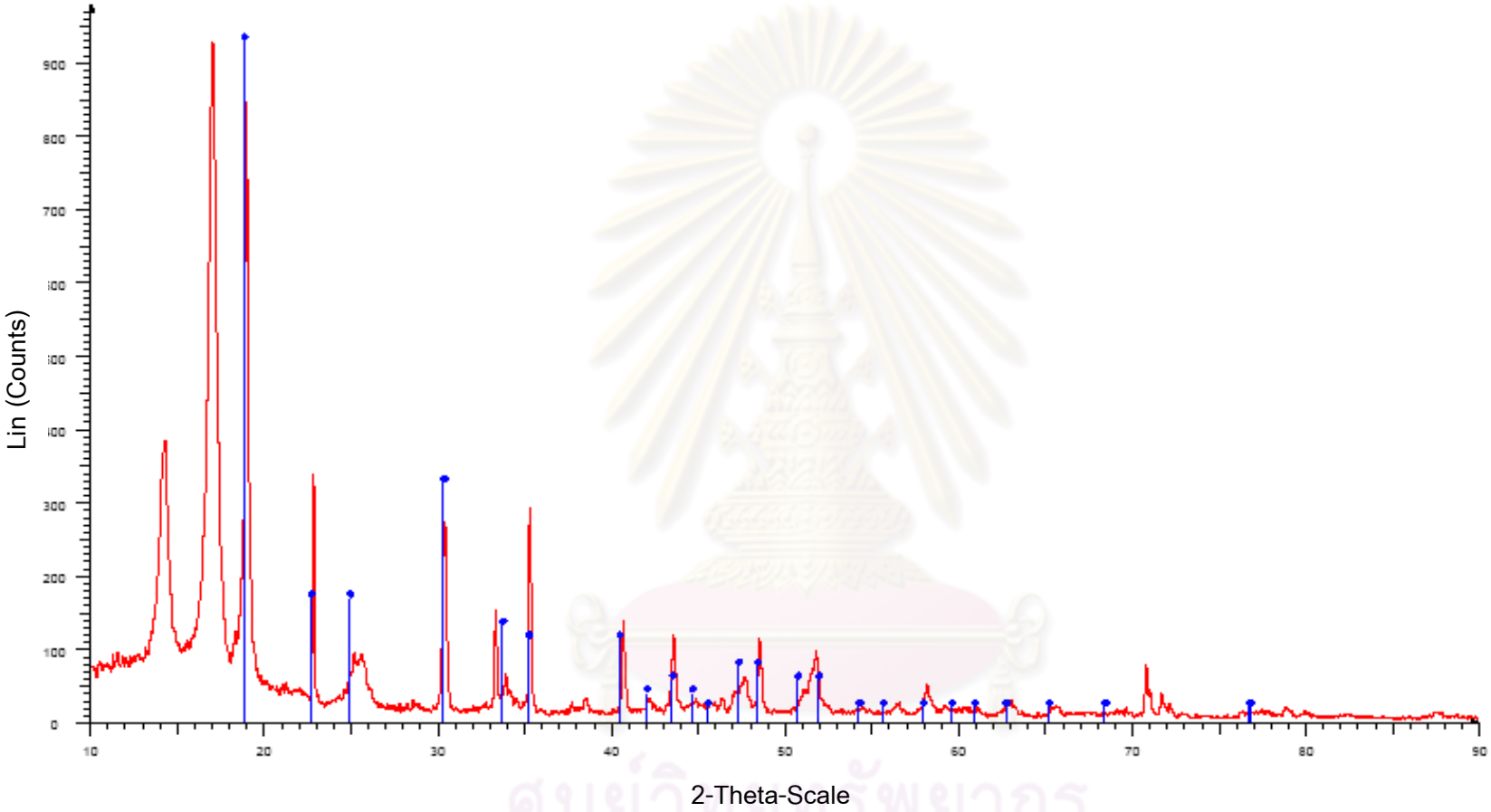

Fig. E7 Typical XRPD patterns for zinc oxalate hydrate crystals precipitated by the isolate WR4 after growth on zinc sulfate-amended plate. The vertical bars indicate the peak positions and relative intensities for standard XRPD patterns of metal oxalates as given in the International Centre for Diffraction Data powder diffraction file. The standard used was zinc oxalate hydrate (PDF-14-0740).

204

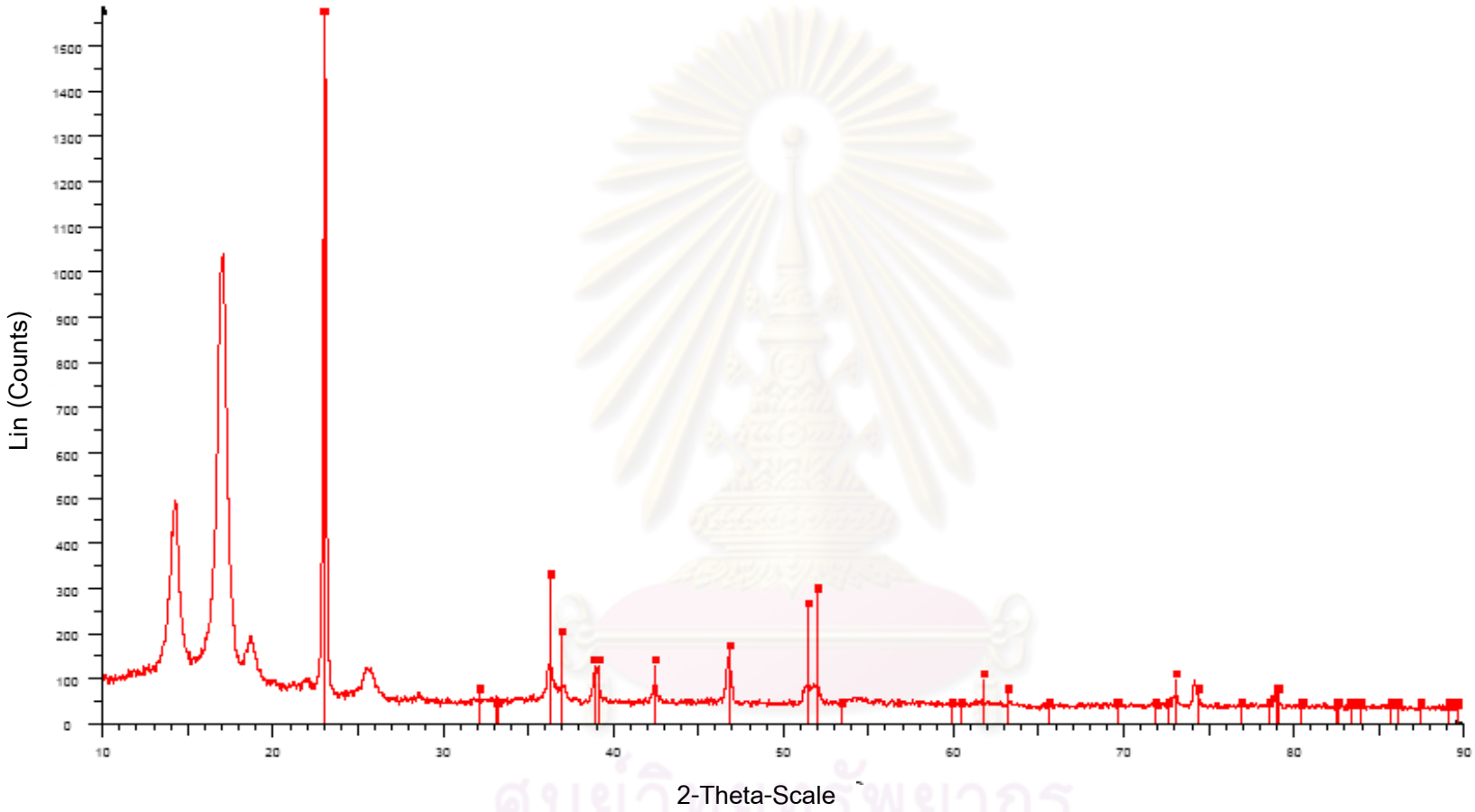

Fig. E8 Typical XRPD patterns for copper oxalate hydrate (moolooite) crystals precipitated by the isolate WR4 after growth on copper sulfate-amended plate. The vertical bars indicate the peak positions and relative intensities for standard XRPD patterns of metal oxalates as given in the International Centre for Diffraction Data powder diffraction file. The standard used was copper oxalate hydrate (PDF-21-0297).

205

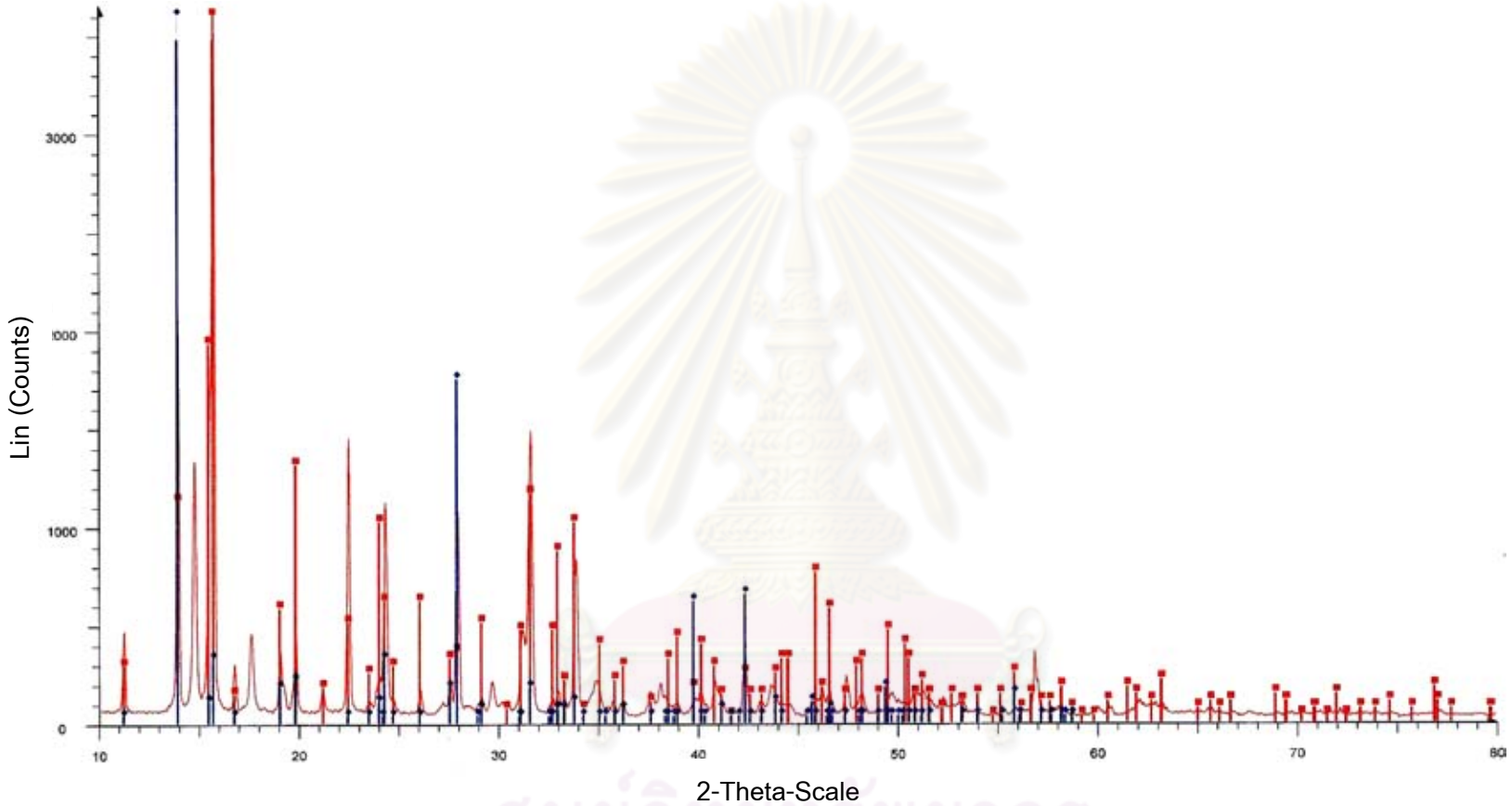

Fig. E9 Typical XRPD patterns for cadmium oxalate hydrate crystals precipitated by the isolate WR4 after growth on cadmium sulfate-amended plate. The vertical bars indicate the peak positions and relative intensities for standard XRPD patterns of metal oxalates as given in the International Centre for Diffraction Data powder diffraction file. The standard used was cadmium oxalate hydrate (PDF-54-0317 and PDF-53-0085).

206

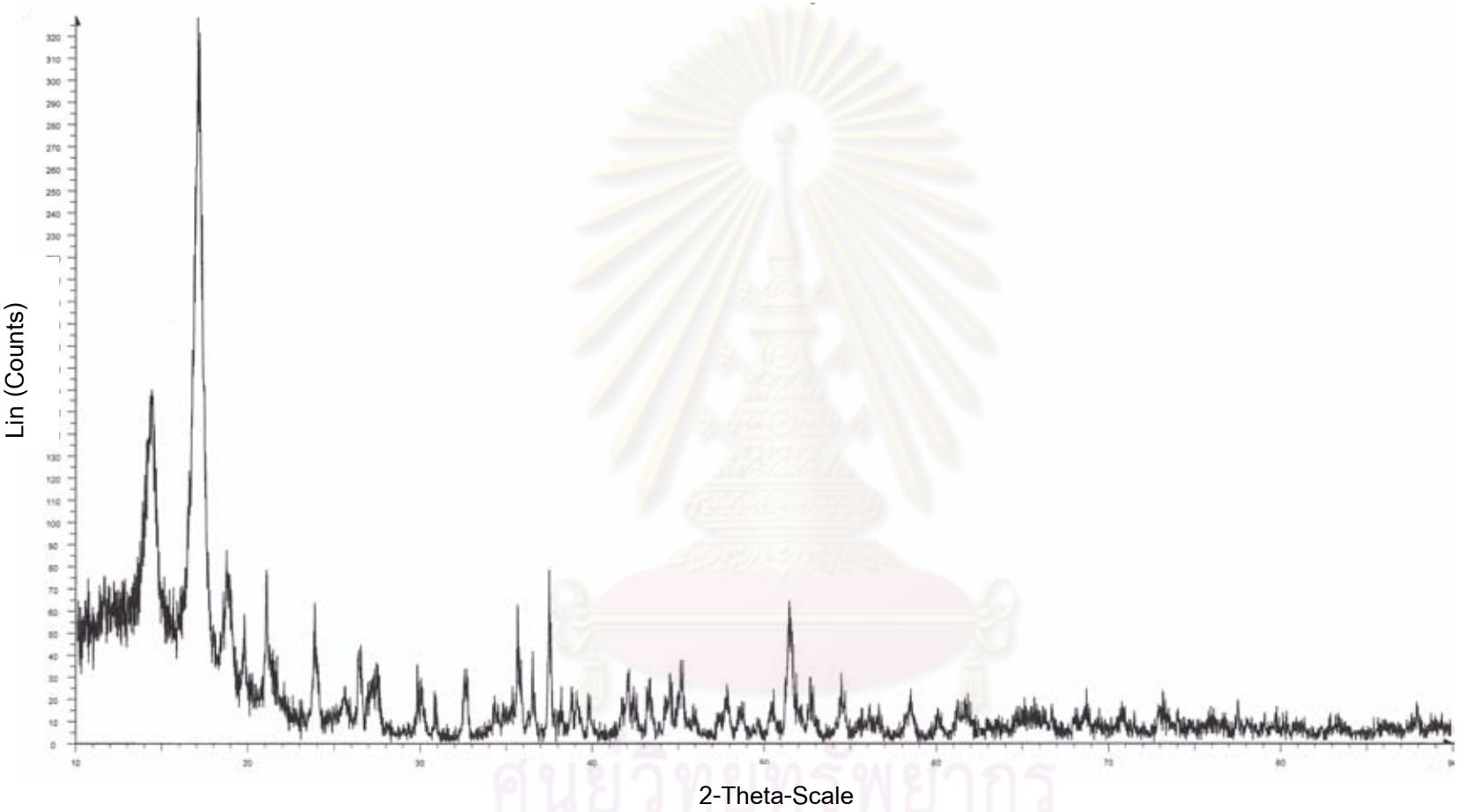

Fig. E10 Typical XRPD patterns for lead oxalate crystals precipitated by the isolate WR4 after growth on lead nitrate-amended plate. The vertical bars indicate the peak positions and relative intensities for standard XRPD patterns of metal oxalates as given in the International Centre for Diffraction Data powder diffraction file. The standard used was lead oxalate (PDF-11-0723).

207

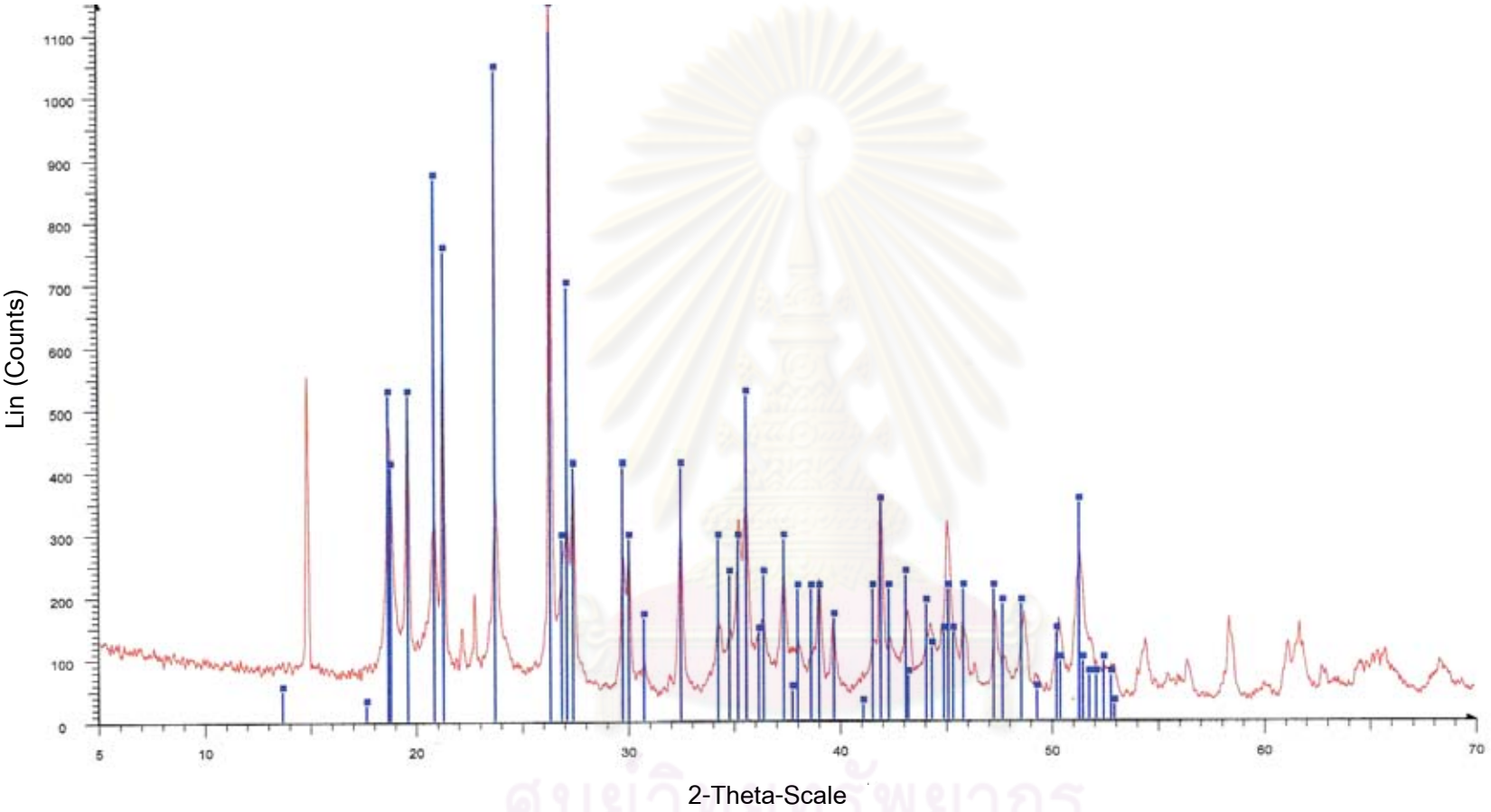

Fig. E11 Typical XRPD patterns for lead oxalate crystals precipitated by the isolate WR5 after growth on lead nitrate-amended plate. The vertical bars indicate the peak positions and relative intensities for standard XRPD patterns of metal oxalates as given in the International Centre for Diffraction Data powder diffraction file. The standard used was lead oxalate (PDF-14-0803).

208

APPENDIX F

Nucleotides have the important characteristic as following:

F1. Isolate MR40

LOCUS: EU015881; 631 bp DNA linear

DEFINITION: Fomitopsis sp. IMER2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

ACCESSION: EU015881

REFERENCE: 1 (bases 1 to 631)

AUTHORS: Xiong,Z., Zhang,X., Wang,H., Ma,F., Li,L. and Li,W.

TITLE: Application of brown-rot basidiomycete Fomitopsis sp. IMER2 for biological treatment of black liquor

JOURNAL: J. Biosci. Bioeng. 104 (6), 446-450 (2007)

REFERENCE: 2 (bases 1 to 631)

AUTHORS: Zhang,X., Ma,F. and Xiong,Z.

TITLE: Direct Submission

JOURNAL: Submitted (02-JUL-2007) The College of Life Science and Technology, Huazhong University of Science and Technology (HUST), 1037 Luoyu Road, Wuhan, Hubei 430074, China

ORIGIN

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301 cgaaatgcga taagtaatgt gaattgcaga attcagtgaa tcatcgaatc tttgaacgca

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421 ctatttgctt ttgtgaatag agcttggatt tggaggttta ttgctggtac ttgtgatcgg

481 ctcctcttga atgcattagc tcgaaccttt gcggatcagc tatcggtgtg ataattgtct

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601 gactttgacc tttgacctca aatcaggtag g

Nucleotide sequence of the isolate MR40 as following:

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CCCTGATCACTATCCATCTCACACACCTGTGCACACACTGTAGGTTGGCTTGTGATTGGA
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CCTGTTTGAGTGTCATGGAATTCTCAACTCTATTTGCTTTTGTGAATAGAGCTTGGATTTGG
AGGTTTATTGCTGGTACTTGTGATCGGCTCCTCTTGAATGCATTAGCTCGAACCTTTGCGG
ATCAGCTATCGGTGTGATAATTGTCTACGCCGTTGCAGTGAAGCATATCAATGGGCTCGG
CTTCC

F2. Isolate KYO

LOCUS: GQ982889; 643 bp DNA linear

DEFINITION: Fomitopsis cf. meliae 8IV7/1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

ACCESSION: GQ982889

REFERENCE: 1 (bases 1 to 643)

AUTHORS: Pinruan,U., Rungjindamai,N., Choeyklin,R., Lumyong,S. and Jones,G.

TITLE: Occurrence and diversity of basidiomycetous endophytes isolated from the oil palm, Elaeis guineensis from Thailand

JOURNAL: Unpublished

REFERENCE: 2 (bases 1 to 643)

AUTHORS: Pinruan,U., Rungjindamai,N., Choeyklin,R., Lumyong,S. and Jones,G.

TITLE: Direct Submission

JOURNAL: Submitted (21-SEP-2009) BIOTEC Bioresources Technology Unit, National Center for Genetic Engineering and Biotechnology, NSTDA, 113 Thailand Science Park, Paholyothin Road, Khlong 1, Khlong Luang, Pathum Thani 12120, Thailand

ORIGIN

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Nucleotide sequence of the isolate KYO as following:

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TATTCCGAGGAGCATGCCTGTTTGAGTGTCATGGAATTCTCAACTCTATTCACTTTTGTGAA
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TAGCTCGAACCTTTGTGGATCAGCTATCGGTGTGATAATTGTCTACGCCGTTGCTGTGAAG
CATGTTAATGGGATCGGCTTCCAATCGTCCTTTACTTGGGGACAATGCCTTTGACCTTTGA
CCTCAAATCAGGTAGGATTACCCGCTGAACTTAAGCATATCAA

F3. Isolate WR3

LOCUS: EF016754; 656 bp DNA linear

DEFINITION: Ganoderma sp. STK-2006a internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

ACCESSION: EF016754

REFERENCE: 1 (bases 1 to 656)

AUTHORS: Sheena Kumari,T.K., Rohini,I., Gunasekaran,M. and Rajesh,M.K.

TITLE: Identification of Ganoderma sp., causal organism of basal stem rot of arecanut (Areca catechu L.)

JOURNAL: Unpublished

REFERENCE: 2 (bases 1 to 656)

AUTHORS: Sheena Kumari,T.K., Rohini,I., Gunasekaran,M. and Rajesh,M.K.

TITLE: Direct Submission

JOURNAL: Submitted (21-SEP-2006) Pathology Department, CPCRI, Kudlu, Kasaragod, Kerala 671124, India

ORIGIN

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241 aactttcagc aacggatctc ttggctctcg catcgatgaa gaacgcagcg aaatgcgata

301 agtaatgtga attgcagaat tcagtgaatc atcgtatctt tgaacgcacc ttgcgctcct

361 tggtattccg aggagcatgc ctgtttgagt gtcatgaaat cttcaaccta caatctcttt

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481 aatgcattag cttggttcct ttgcgaatcg gctgtcggtg tgataatgtc tacgccgcga

541 ccgtgacgcg tttggcgagc ttctaaccgt cccgttattg ggacaactct tatgacctct

601 gacctcaaat caggtaggac tacccgcgga acttaagcat atcaataagc ggagga

Nucleotide sequence of the isolate WR3 as following:

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AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTG

AACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGAAATCTT

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GATCGGCTCCTCTCAAATGCATTAGCTTGGTTCCTTTGCGAATCGGCTGTCGGTGTGATAA

TGTCTACGCCGCGACCGTGACGCGTTTGGCGAGCTTCTAACCGT

F4. Isolate WR4

LOCUS: FJ372673; 625 bp DNA linear

DEFINITION: Fomitopsis cf. meliae 1P_1_1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

ACCESSION: FJ372673

REFERENCE: 1 (bases 1 to 625)

AUTHORS: Rungjindamai,N., Pinruan,U., Choeyklin,R., Hattori,T. and Jones,G.

TITLE: Molecular characterization of basidiomycetous endophytes isolated from leaves, rachis and petioles of the oil palm, Elaeis guineensis, in Thailand

JOURNAL: Fungal Divers. 33, 139-161 (2009)

REFERENCE: 2 (bases 1 to 625)

AUTHORS: Rungjindamai,N., Pinruan,U., Choeyklin,R. and Jones,G.

TITLE: Direct Submission

JOURNAL: Submitted (06-OCT-2008) Phylogenetics and Mycology, Bioresources Technology, National Center for Genetic Engineering and Biotechnology (BIOTEC), 113 Thailand Science Park, Klong Laung, Pathum Thani 12120, Thailand

ORIGIN

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361 agcatgcctg tttgagtgtc atggaattct caactctatt cacttttgtg aatagggctt

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481 aacctttgtg gatcagctat cggtgtgata attgtctacg ccgttgctgt gaagcatgtt

541 aatgggatcg gcttccaatc gtcctttact tggggacaat gcctttgacc tttgacctca

601 aatcaggtag gattacccgc tgaac

Nucleotide sequence of the isolate WR4 as following:

GCTGGCCGTCAGCGGCATGTGCACGCCCTGATCATTATCCATCTCAAACACCTGTGCACA
CACTGTAGGTCGGTTTGTGGCTGGAGTGGGCGCGCTCTGTGTCCGCTTTGGTTGTAGGC
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TAAATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAA
TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGC
GCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGGAATTCTCAACTCTATTCA
CTTTTGTGAATAGGGCTTGGACTTGGAGGTTTATTGCCGGTACACCTGTGATCGGCTCCTC
TTGAATGCATTAGCTCGAACCTTTGTGGATCAGCTATCGGTGTGATAATTGTCTACGCCGT
TGCTGTGAAGCATGTTAATGGGATC

F5. Isolate WR5

LOCUS: EU239386; 596 bp DNA linear

DEFINITION: Ganoderma aff. steyaertanum C16452 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence.

ACCESSION: EU239386

REFERENCE: 1 (bases 1 to 596)

AUTHORS: Glen,M., Bougher,N.L., Francis,A., Nigg,S.Q., Lee,S.S., Irianto,R., Barry,K. and Mohammed,C.L.

TITLE: Molecular differentiation of Ganoderma and Amauroderma species associated with root rot disease of Acacia mangium plantations in Indonesia and Malaysia

JOURNAL: Unpublished

REFERENCE: 2 (bases 1 to 596)

AUTHORS: Glen,M.

TITLE: Direct Submission

JOURNAL: Submitted (20-OCT-2007) Ensis FBP, CSIRO, College Rd, Sandy Bay, Tas 7005, Australia

ORIGIN

1 gatcattatc gagttttgac tgggttgtag ctggccttcc gaggcatcgt gcacgccctg

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121 tcgtttgacg agtttgcgaa gcgcgtctgt gcctgcgttt tatcacaaac actataaagt

181 attagaatgt gtattgcgat gtaacgcatc tatatacaac tttcagcaac ggatctcttg

241 gctctcgcat cgatgaagaa cgcagcgaaa tgcgataagt aatgtgaatt gcagaattca

301 gtgaatcatc gaatctttga acgcaccttg cgctccttgg tattccgagg agcatgcctg

361 tttgagtgtc atgaaatctt caacctacaa tctctttgcg gtttttgtag gcttggactt

421 ggaggcttgt cggtctttta ttgatcggct cctctcaaat gcattagctt ggttcctttg

481 cgaatcggct gtcggtgtga taatgtctac gccgcgaccg tgacgcgtgt ggcgagcttc

541 taaccgtccc gttattggga caactcttta tgacctctga cctcaaatca ggtagg

Nucleotide sequence of the isolate WR5 as following:

ATGTATCGAGTTTTGACTGGGTTGTAGCTGGCCTTCCGAGGCATCTTGCACGCCCTGCTC
ATCCACTCTACACCTGTGCACTTACTGTGGGTTATAGATCGTGTGGAGTGAGCTCGTTCGT
TTGACTAGTTTGTGAAGCGCGTCTGTGCCTGCGTTTTTTTCATCACAAACACTATAAAGTAT
TAGAATGTGTATTGCGATGTAACGCATCTATATACAACTTTCAGCAACGGATCTCTTGGCTC
TCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGA
ATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGA
GTGTCATGAAATCTTCAACCTACAATCTCTTTGCGGTTTTTGTAGGCTTGGACTTGGAGGCT
TGTCGGTCTTTTATTGATCGGCTCCTCTCAAATGCATTAGCTTGGTTCCTTTGCGAATCGGC
TGTCGGTGTGATAATGTCTACGCCGCGACCGTGACGCGTTTGGCGAGCTTCTAACCGTCC
CGTTA

BIOGRAPHY

Miss Benjawan Kaewdoung was born in June 18, 1985 in Nonthaburi province, Thailand. She graduated from Department of Biology, Faculty of Science, Srinakharinwirot University in 2007 with the Bachelor Degree of Science (Biology). Recently, she has pursued her Master degree of Program in Industrial Microbiology and expected to finish by the academic year of 2009.

Scientific Presentation

Benjawan Kaewdoung, Geoffrey Michael. Gadd, Jittra Piapukiew, Nongnuj Muangsin and Prakitsin Sihanonth. Heavy resistance and oxalate crystallization in wood-rotting fungi. Proceedings of the second conference on Environmental Science, Engineering and Management-CESEM2010, 18-19 March 2010, Chulalongkorn University, Bangkok, Thailand.

Research Fund

This work was supported by CU.Graduate School Thesis Grant