BIOSORPTION OF CHROMIUM, COPPER, LEAD AND ZINC BY ENDOPHYTIC FUNGI ISOLATED FROM WETLANDS OF SARAWAK

By

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Abstract

In order to treat heavy metal pollution, biosorption has emerged as an alternative solution to remove heavy metal ions from aqueous solutions as it can overcome several limitations of conventional physico-chemical methods used such as electrochemical treatment, ion exchange and reverse osmosis. This study aims to isolate endophytic fungi from wetlands of Sarawak and assess their potential use as biosorbents to remove heavy metals Cu(II), Cr(VI), Pb(II) and Zn(II). The wetlands harbour many unique species and are also known for their high ion-exchange potential which contributes to their ability to sequester metal ions. Three hundred and forty-two (342) endophytic fungi were successfully isolated from *Nypa fruticans* in Kuching Wetland National Park, Sarawak, and ninety-three (93) isolates were chosen for preliminary screening using media supplemented with heavy metals (up to 1000 ppm) revealed that nine (9) of the isolates were resistant against Cu(II), Cr(VI), Pb(II) and Zn(II). The most heavy-metal resistant endophytic fungus was identified as *Pestalotiopsis* sp. using fungal primers ITS 1 and ITS 4. This is to our knowledge that this is the first study reporting on the ability of *Pestalotiopsis* sp. to resist Cu(II), Cr(VI), Pb(II) and Zn(II) at high concentrations. Several studies have shown that immobilised and/or dead fungal biomass can be more efficient than living fungi, so we immobilized the most resistant strain in calcium alginate beads to study and compared the removal efficiency of heavy metal ions between live and dead immobilized fungal biomass under a pH range of 4 –6. Immobilized live fungal biomass displayed higher efficiency in removing Cr(VI) at pH 5 and 6, while both live and dead immobilized biomass were able to remove Pb(II) at pH 4 and 5 with a moderate significant difference value of P=0.5 at an incubation period of 30 minutes. In order to further understand the biosorptive mechanism, a proteomic study was conducted to investigate down-regulation and up-regulation expression levels of proteins under the treatment of the heavy metals. Seven (7) proteins were identified via MALDI-ToF analysis and among the proteins identified; protein tRNA-specific 2-thiouridylase MnmA, tryptophan synthase alpha chain, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, putative aminopeptidaseYhfE, and glutamyl-tRNA reductase were found to be newly-induced component of heavy metal stress proteins reported in the presence of the heavy

metals studied, while multidrug resistance protein (MRP homolog) was found to be up-regulated in the presence of Pb(II). Thus, the data found in this study gives us a new line of evidence in the biosorptive and defensive mechanisms of *Pestalotiopsis* sp. under heavy metal stress.

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Declaration

I hereby declare that this research entitled “Biosorption of Chromium, Copper, Lead and Zinc by endophytic fungi isolated from wetlands of Sarawak” is original and contains no material which has been accepted for the award to the candidate of any other degree or diploma, except where due reference is made in the text of the examinable outcome; to the best of my knowledge contains no material previously published or written by another person except where due reference is made in the text of the examinable outcome; and where work is based on joint research or publications, discloses the relative contributions of the respective workers or authors.

(JENNY CHOO CHENG YI)

Date: 26th March 2015

Publications Arising from this Thesis

The work described in this thesis has been submitted as described in the following:

Choo J. C. Y., Mohd. Sabri N., Mujahid A. & Müller M. 'Heavy metal resistant endophytic fungi isolated from *Nypa fruticans* in Kuching Wetland National Park'. *Ocean Science Journal* (Manuscript ID: OSJO-D-14-00054R1).

Early work has been presented in the following conferences and contributed to the content presented in Chapters 3 and 4 of this thesis:

Choo J. C. Y., Tan D. &Müller M. 'Biosorption of chromium, copper, lead and zinc by endophytic fungi isolated from wetlands of Sarawak'. IOC/WESTPAC 9th International Scientific Symposium, 22-25 April 2014, Nha Trang, Khanh Hoa, Vietnam. (Poster presentation)

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Introduction

1.1 Heavy metal pollution

Heavy metal pollution is regarded as a major environmental issue due to its toxic effect to our human health through the food chain and its high persistence in our environment from various industrial activities (Wang & Chen 2009a). Although there is no clear definition of what heavy metal term is, the density of the metal is said to be mostly attributed as its primary factor (Vijayaraghavan & Yun 2008). Hence, heavy metals are commonly classified as metals of having a specific density of more than 5 g/cm^3. Although heavy metals have been used and required by humans, various adverse effects of heavy metals have been reported with continual exposure and accumulation of large amounts (Vijayaraghavan & Yun 2008). It is also been reported that continual exposure of heavy metals has been increasing in some parts of the world, especially the less developing countries. With the increasing heavy metal pollution, the effects towards human health has been regularly studied and reviewed by various international bodies such as World Health Organization (WHO).

Heavy metals such as chromium, lead, zinc and copper are among the most common pollutants being discharged from industrial and agricultural activities which cause major environmental problems and serious health hazards to human. (Petrini 1986). Increasingly, most of the industrial and agricultural activities such as mining and smelting metalliferous, electroplating, coating and metal surface, electrolysis, electro-osmosis, energy and fuel production, automotive and steel industry, fertilizer and pesticide industry, leatherworking, and electric appliance manufacturing produce and discharge large amount of wastewater effluent containing heavy metals into the environment as shown in Figure 1. These heavy metals are toxic when reaching a certain level of concentrations and the concentrations are reported to be too low to be treated by standard methods (Clay & Schardl 2002). Hence, the accumulation of the heavy metals by the increase discharge of the waste effluents has inevitably led to major environmental and health problems worldwide.

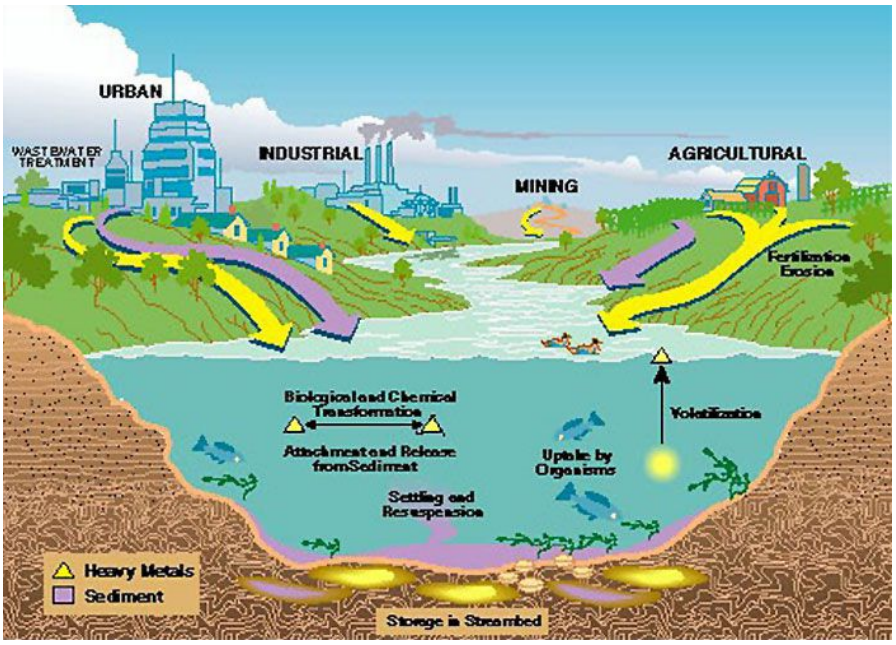

Figure 1.Picture taken from:
https://embryology.med.unsw.edu.au/embryology/images/3/35/Metal_contamination.jpg

1.2 The application and effects of heavy metals

1.2.1 The application of Lead and its effects

Lead (Pb) is inevitably one of the most abundant toxic heavy metals and a common contaminant in our environment (Zayed, Gowthaman & Terry 1998). The early applications of lead include building materials, pipes for transporting water and pigments for glazing ceramics; lead acetate was even used to sweeten the old wine by the Ancient Roman and been reported to be consumed as much as 1 gram per day by the Romans (Vijayaraghavan & Yun 2008). If accumulated in a large amount, lead poses a serious threat not only to humans but to the aquatic life.

Lead has been discharged into our environment via various anthropogenic activities; mining and smeltering activities, paper and pulp, gasoline and explosives materials, disposal of municipal sewage sludge enriched with lead, and paper and pulp being the main factors of lead pollution (Men et al. 2008). Besides, contamination of lead in the waters through anthropogenic activities is the primary factor of fish poisoning (Shendure et al. 2004). Furthermore, as estimation of 60% of total lead emission is attributed from the petrol combustion worldwide (Zhu et al. 1999). Moreover, the lead pollution found in soil and vegetation is primarily found to be caused via automobile exhaust pollution (Crini 2006). Apart from that, lead is known to be widely used in

manufacturing of batteries, photographic materials, paint materials, printing, fuel and pigments (Vieira & Volesky 2010; Volesky 2007).

Among the various organs affected, the most severe effect is the human Central Nervous System (CNS). Lead poisoning can lead to the damage of the peripheral and central nervous system (CNS), the kidneys and also affect our blood pressure. Patients with high blood lead levels may experience severe, intractable colic, motor impairment, blurred conscious, paralysis and weakness of the body (Ahmaruzzaman 2008). Besides, long term exposure of lead can also affect both male and female reproduction, hypertension and renal failure with acute lead poisoning.

1.2.2 The application of Chromium (VI) and its effect

Chromium (VI) is one of the heavy metals that are of major concern as it is carcinogenic to human and much more toxic than Chromium (III) (McCallan & Miller 1958). Hexavalent chromium is highly soluble and is non-degradable in nature. It is classified as group 'A' carcinogen for humans due to its carcinogenic and mutagenic properties (Babel & Kurniawan 2003). While chromium (VI) is known to be toxic to both humans and plants, chromium (III) is less toxic to humans and instead more toxic to plants at a high concentrations (Aksu 2005; Bhatnagar & Minocha 2006).

Chromium (VI) is commonly discharged into the environment through electroplating, leather tanning, steel manufacturing, pulp processing, wood preservation and as biocide in cooling water of power plants (Lesage et al. 2007; Strobel & Daisy 2003). The untreated chromium effluent can contain approximately 100mg/l of chromium (VI), which was much higher than the permissible discharge concentrations (Park, Yun & Park 2010). According to the US EPA regulation, the permissible discharge of chromium (VI) to surface water is regulated to below 0.05mg/ml, while the total permissible discharge for all other chromium types which include chromium (III), chromium (VI) and other forms are regulated below 2mg/ml (Modak & Natarajan 1995).

Chromium (VI) is known widely to cause allergic dermatitis and being toxic and carcinogenic towards human and animals. Besides that, chromium (VI) can also cause lung carcinoma, kidney dysfunction, reproductive system, severe diarrhea and ulcer (Manahan 1994) Furthermore, chromium (VI) compounds have been reported to be

approximately 1000-fold more cytotoxic and mutagenic than Chromium (III) in cultured human fibroblasts (Volesky & Tsezos 1982). According to Veglio and Beolchini (1997), the hexavalent chromium has been studied to demonstrate neurotoxicity, dermatotoxicity, immunotoxicity, genotoxicity, and carcinogenicity. Moreover, morphological and neoplastic transformation and mutagenicity in murine and human cells have been studied to be induced by soluble and insoluble hexavalent chromium salts (Andres, Texier & Le Cloirec 2003).

1.2.3 The application of Copper and its effect

Copper (Cu) is considered as one of the heavy metals which is less toxic as compared to the other metals. Although exposure to copper is not hazardous, exposure to high concentrations of copper is harmful to humans (Kapoor & Viraraghavan 1995).

Copper is one of the heavy metals used in construction, machinery, transportation, military weapons and imitation jewellery; commonly used as a metal itself or as alloy (Gadd & White 1993; González-Fernández, Prats & Jorrín-Novo 2010). It is also an important element in white gold and is also used in dental equipment, intrauterine devices, and cosmetics (Grinyer et al. 2005; Grinyer et al. 2004).

Based on the literature studies, copper is generally found in seawater and freshwater at a low concentrations (µg/L); copper concentrations in natural waters are reported to be average of 0.066 mg/L and 0.075 mg/L for surface waters and ground waters in North America respectively. Moreover, the unpolluted waters in Asia reported a reading of 0.0008mg/L to 0.010mg/L of copper concentration, while a mean of 0.006 mg/L of copper concentration in the rivers of United Kingdom (WHO 2003). On the other hand, copper concentrations in drinking water has a more significant range differences, as this is due to the corrosion of copper pipes and the differences in pH, dissolved oxygen, hardness, complexing and oxidizing agents, and stagnation period in the pipes. According to a survey done on 70 Canadian municipalities, it is reported that 20% of the tap waters value of copper concentrations was higher than the corresponding treatment plant (WHO 2003).

Although copper is one of the essential micronutrients needed in a human diet, consuming more than 30 mg/L of copper in contaminated water or beverages can result in copper-related illness (Strobel & Daisy 2003). The liver is primarily affected by the

high concentrations of copper, as liver is the first organ of copper deposition after entering the bloodstream (Kapoor & Viraraghavan 1995). Besides, its toxicity can also cause tissue damage and significant oxidative stress. Its oxidative stress which related to copper toxicity is part of a consequence in its redox reactivity, in which the free or low molecular weight copper complexes to catalyse the production of the hydroxyl radical by the reaction between superoxide anion and H_2O_2. (Yıldırım et al. 2011). Moreover, impaired activity in humans is reported when the copper binds with the free thiols of cysteines which result in oxidation and subsequent crosslinks between proteins (Khoa Pham & Wright 2007).

The largest literature highlighting the toxicity of copper is comprised of single oral exposure towards high concentrations of copper. Symptoms of high exposure to copper include nausea and vomiting, headache, abdominal pain, lethargy, diarrhea and tachycardia. The more serious symptoms even include haemolytic anaemia, respiratory problem, gastrointestinal bleeding and also liver and kidney damage (Bae & Chen 2004; Gillet et al. 2006; Vido et al. 2001; Wilkins 1994). Furthermore, it has also been reported that acute toxemia and possible death could happen when ingestion of more than 1g copper salts by adult human (NRC 2000). However, the mechanisms which involve the toxicity of copper in the human body is yet to be fully understood, but it is most probably due to the combination of significant oxidative stress at multiple points in the body with marked perturbations in several components in the endocrine system (Kapoor & Viraraghavan 1995; Lueking et al. 1999; Wolters, Washburn & Yates 2001).

1.2.4 The application of Zinc and its effect

Zinc is one of the essential minerals needed in the homeostatic mechanisms of the human body, which include specific inflammation, immunity and oxidative stress (Redman et al. 2002). Compared to the other metals with similar chemical properties, zinc is considered to be comparatively harmless to humans. Only upon drastic exposure towards zinc will it lead to its toxic effects (Bacon & White 2000).

According to the U.S. Geological survey, 12 million tons of zinc is produced annually worldwide, and this is mostly due to the increasing zinc mine production in China and Australia (Bandara, Seneviratne & Kulasooriya 2006). The production of zinc has caused major pollution of the environment in the metal industry. Zinc (Zn) is one of the smelting furnace products and zinc is mostly incorporated in iron silicates, zinc oxides

and zinc sulphide (Jalgaonwala, Mohite & Mahajan 2011). Besides, Zn and Pb ores are also part of the metallurgical dumps. Zinc has been widely used in the automobile industry as the alloy has the ability to improve corrosion protection (Kharwar et al. 2009).

Generally, there are 3 main routes of entry for zinc into the human body, which include ingestion, through the skin and by inhalation and each types of exposure consequently affects different types of the organs, and allow different uptake of zinc concentrations into the body (Sathe & Raghukumar 1991).

As an essential trace element, oral uptake of zinc at a small amount is vital to the human body. It is reported that 11 mg/day of zinc for men and 8 mg/day for women is the recommended dietary allowance (RDA) (Peterson et al. 2005). On the other hand, as estimation of 27 g/day of zinc for human is reported to be the LD_{50} value based on the comparison studies done on rats and mice (Sathe & Raghukumar 1991). Based on a published report, a woman was reported to have died after ingesting approximately 28 g of zinc, with symptoms of vomiting and development of tachycardia and hyperglycemia, and experiencing hemorrhagic pancreatitis and renal failure (Azevedo et al. 2000). Immediate signs of toxic amounts uptake of zinc include nausea, vomiting, and abdominal pain (Zhang, Becker & Cheng 2006).

1.3 Biosorption

In order to treat wastewater effluent contaminated with heavy metals, biosorption is an emerging and attractive alternative solution to remove heavy metals from aqueous solution. As heavy metals are non-renewable, the removal and recovery of metals is crucial from the point of resource (Puranik, Modak & Paknikar 1999). Thus, it is regulated that every metal ions must be treated till its permissible concentration before being discharged (Machado, Soares & Soares 2010). This is due to the fact that the toxicity of the heavy metals can occur even at a low concentration of 0.001-0.1 mg/l (Alkorta et al. 2004; Volesky 1990a). Moreover, the toxicity of the heavy metals can remain in nature for a period of time and can even be transformed into toxic forms, such as mercury, in a certain environment (Wang & Chen 2006). Furthermore, the heavy metals could not be degraded even by using biotreatment but can only be transformed and changed in valence and species (Wang & Chen 2006). The normal physiological activity and human life could also be severely affected through the bioaccumulation of the heavy metals by food chain in the environment (Meitei & Prasad 2013).

There are several conventional methods which are used to treat and removal heavy metals from wastewater effluents, such as ion-exchange, reverse osmosis, chemical precipitation, electrochemical treatment, adsorption on activated carbon and membrane technologies (Carmona, da Silva & Leite 2005; Kadirvelu et al. 2002; Wang & Chen 2009b). However, conventional methods for treating industrial wastewater effluents containing heavy metals are reported to be uneconomical and ineffective (Saikkonen, Gyllenberg & Ion 2002). This is due to the fact that large amount of sludge is being produced when removing heavy metals ions in industrial effluents as low as 100 mg/l using chemical precipitation technique and hence it is highly ineffective (Wang & Chen 2006). Besides, it is reported that solvent extraction techniques are not recommended when the heavy metal concentrations in the industrial effluent is less than 1 g/l (Wang & Chen 2009b). Moreover, other conventional methods like ion exchange, adsorption on activated carbon and membrane technologies are highly costly due to the expensive cost of the synthetic resins used and when large amount of water and wastewater containing low concentration of heavy metals (Volesky 2001).

On the other hand, biosorption process offers an advantageous alternative compared to the conventional methods due to its low cost and efficiency in removing heavy metals

from aqueous solution (Jumpponen 2001; Zhou et al. 2010). Biosorption process utilizes various biological materials such as bacteria, fungi, algae, and yeast to adsorb the heavy metal ions and hence remove and reduce the metal ions from the aqueous solution as shown in several studies done by Strobel et al. (2002), Huang et al. (2008), Verma et al. (2007), and Guo et al. (2000). The process involves the ability of the microorganisms to bind and sequester the heavy metal ions through the several physical-chemical process of its cell wall with the metal ions (Saikkonen, Gyllenberg & Ion 2002). Biomass metabolism does not always necessary involved and hence dead and live biological biomass can be used for the process (Gangadevi & Muthumary 2009; Verma et al. 2007). Furthermore, agricultural waste products which contain cellulose such as rice and wheat bran, rice and wheat husk, saw dust of different plants and bark of the tree have also been studied and showed potential as biosorbents for heavy metal removal (Kapoor & Viraraghavan 1997a).

Based on several studies, it is important to highlight that the research of biosorption process emphasises on three main fields, and firstly is the biosorbent (Kratochvil & Volesky 1998). Types of biomass as the most promising and efficient biosorbents is the drive to expand the knowledge and research of the biosorption technology. Thus, it is crucial to continue researching for suitable types of biomass from an extremely large pool of inexpensive and readily available biological materials (Kapoor & Viraraghavan 1995). The second main field is the understanding of the biosorption mechanism process. It is important to have a better understanding of the metal uptake mechanisms by the various biosorbents and the interaction of the metal and the different microorganisms as this can help to enhance the biosorption technology. However, the mechanisms involve in the metal biosorption is yet to be fully understood to date. Thirdly is the scale of the biosorption process being studied as the studies are mostly done at a laboratory scale. Thus, it is a great challenge of applying biosorption process into practice (Volesky 1990b) and therefore improvement of the biosorption process through immobilization of the biosorbents for more rapid and uniform adsorption process, and regeneration and reuse of the biosorbent have been studied (Horikoshi, Nakajima & Sakaguchi 1981).

1.4 Endophytic fungi

Endophytic fungus has been a growing study interest since the discovery of *Pestalotiopsis microspora*, the world's first billion dollars anticancer drug, which produces paclitaxel (Taxol) that is widely used in cancer treatment (Bayramoğlu, Bektaş & Arıca 2003). The endophytic fungi *Pestalotiopsis microspora* colonizes the Himalayan yew tree, *Taxus wallichiana*, which does not bring any harm to the host plant. Thus, endophytic fungi are termed as fungus which grows within the plant tissue, especially the leaves, stems and roots for all or part of their lives without causing any apparent harm to the host plant, which simply acts as a harmless parasite (Azevedo et al. 2000; Ilhan et al. 2004). Endophytes are ubiquitous in plants as nearly all classes of plants and grasses are found to be the host for the endophyte organisms (Zhang, Becker & Cheng 2006).

In literal meaning, the word endophyte is derived from Greek, in which the word 'endo' or 'endon' simply means within, and 'phyte' or 'phyton' means plant (Jalgaonwala, Mohite & Mahajan 2011). Not only fungi has been found to inhabit plants, but also different types of organisms, such as bacteria, actinomycetes and mycoplasma are also endophytes of plants (Bandara, Seneviratne & Kulasooriya 2006). Endophytes have been known for their existence over a hundred years and the study and research on endophytic fungi has continually being studied over the past years and the diversity found among plants has been considerably huge and each plant harbours one or more endophytes (Kharwar et al. 2009). Furthermore, the plant endophyte relationship has captured much interest to be studied and one the endophyte asexual cycles through seed infection is summarized in Figure 2. Other types of infection include colonization through roots, stems and leaves and transmitted via seed coats and/or rhizomes (Rodriguez et al. 2009).

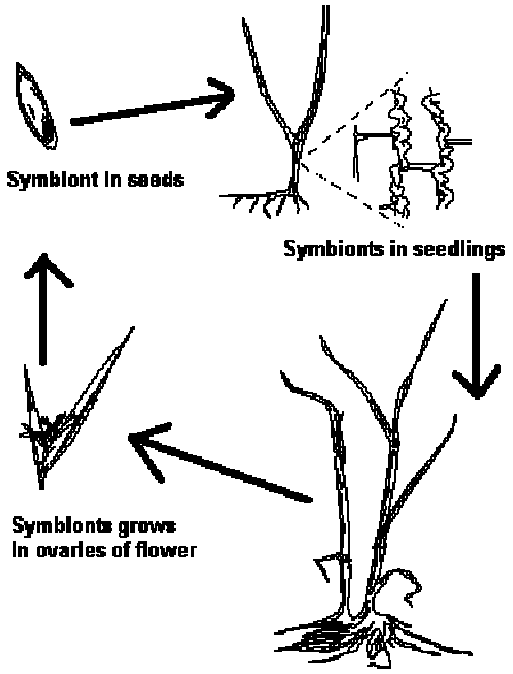

Figure 2.The endophytes asexual life cycle. Picture retrieved from Jalgaonwala, Mohite and Mahajan (2011).

Several studies have reported that the endophytes are not host specific (Cohen 2006). It is found that a single endophyte can inhabit a wide range of host. Besides, it is important to highlight that some strains of the same fungus isolated from different parts of the same host plant exhibited different ability in utilizing various substances (Carroll & Petrini 1983). Therefore, endophytic fungi can be isolated from different families and classes of plant and grow under various ecological and geographical conditions (Petrini 1986), and thus the relation between host to host and endophyte may be variable.

Endophytic fungi can enter the host plant mainly through two methods; (1) gain entry into host plant tissues via wounds, natural cracks, lenticels caused by rain water flowing down or air current, (2) through the animals, insects, beetles, and mites which live and breed in the plants and trees (Bayramoğlu, Bektaş & Arıca 2003). According to Bacon and White (2000), the term 'endophyte' should be applied to microorganisms which have been living in the plant for a period of time, and therefore the minimal requirement before a fungus is termed an endophyte should be the demonstration of its hyphae in the living tissue. In order to validate the possibility of endophytic fungi in living plant, Sathe and Raghukumar (1991) demonstrated the use of bleaching and staining technique to identify intracellular hyphae in seagrass. While identification of endophytic fungi from hyphal feature alone is insufficient, the identification techniques require methods

of immunofluorescence and DNA sequencing and comparison to the homologous sequences registered in the GeneBank (Peterson et al. 2005).

It is understood that the symbiotic relationship between the endophytes and the plant has a profound effect on the host plant. Generally, endophytes have been divided into 4 classes. The Class I endophyte such as the *Clavicipitaceous* endophyte and its phylogenetically related organisms are fastidious in culture and limited to some cool and warm season grasses (Bischoff et al. 2005). The transmission of Class I endophyte is mainly vertical, in which the fungi is passed on to its offspring plant via seed infections by the maternal plant (Saikkonen, Gyllenberg & Ion 2002). Futhermore, class II endophyte comprises of the *Dikarya* (*Ascomycota* and *Basidiomycota*) members of the endophyte species. Class II endophytes are known to confer environmental specific stress tolerance to host plants (Rodriguez et al. 2008). In addition, Class III endophytes are known for its vast diversity within the host tissues, plants and populations. The endophytes are transmitted in a vertical manner which include the nonvascular and vascular plants, woody and herbaceous angiosperms in the tropical and antarctic environmental conditions (Davis & Shaw 2008). A single leaf may harbour up to one isolate in each $2mm^2$ area of the leaf and a number of species, and thus a single plant itself may eventually harbour numerous types of endophytic fungi. Class IV endophytes are generally the ascomyceteous fungi, which have darkly melanised septate and found only in plant roots (Jumpponen 2001). This class of endophytes are conidial or sterile and form the melanised structures of the intercellular and intracellular hyphae and microsclerotia in the roots and is found in plants from the tropical, antarctic, arctic, alpine and temperate environment.

Numerous studies had been done on endophytic fungi and its bioactive compounds. Several species of endophytic fungi have been identified to be highly useful in agricultural and pharmaceutical industry, as it has become the source of anticancer, antidiabetic, immunosuppressive compounds, insecticide, while some even reported to produce metabolites with a thermoprotective property (Strobel & Daisy 2003). It is interesting to highlight that some plants have associated endophytes which produce the same bioactive compounds. For instance is the production of Taxol, a well-known anticancer agent which is found in the world's yew tree species, *Taxus* sp., has been found to be produced by more than 20 genera endophytic fungi so far and the range of Taxol producing fungi include the various *Taxus* and non-*Taxus* species (Zhou et al.

2010). The fungal producing taxol, *Taxomyces andreanae* from the yew *Taxus brevifolia* and *Pestalotiopsis microspora* from *Taxus wallachiana* are a few of the examples which clearly show the potential of endophytic fungi in pharmaceutical industry (Huang et al. 2008; Strobel et al. 1996).

Furthermore, an endophytic fungus *Curvularia* sp. is also found to be inhabiting the plants which are growing in the volcanic areas in the United States (Redman et al. 2002). Moreover, endophytes have also been reported to involve in the stress tolerance of the host plants. Studies have shown that endophytic fungi are able to protect the host plant from drought and salty condition and even increase heat tolerance in the plant (Clay & Schardl 2002; Jalgaonwala, Mohite & Mahajan 2011). Thus, the endophytes act as a biological trigger to activate the stress response in the host plant (Redman et al. 2002). Due to the endophytic fungi's ability in conferring the host plant to adapt and survive in harsh condition, and also their proven ability in producing unique secondary metabolites, endophytic fungi has undeniably become one of the important and attractive components of fungal biological study to date.

Apart from that, endophytic fungi have also been known to produce antioxidant, antiviral and antimicrobial compounds. Endophytic fungi have been the main source of antioxidant compounds, such as Pestacin and Isopestacin. These antioxidant compounds are found to be isolated from *Pestalotiopsis microspora*, from the *Terminalia morobensis* plant, originated from Papa New Guinea (Strobel et al. 2002). Furthermore, endophytic fungi are an undeniably a rich source of novel antimicrobial compounds in which it produces metabolites that help to induce resistance and acts as a protection against pathogens for the plants. Thus, several endophytic fungi exhibit antibacterial and antifungal properties which are useful in the pharmaceutical industry. For example, the endophytic fungus *Phomopsis* sp. YM 311483 is found to produce lactones, which exhibit antifungal properties that are able to inhibit the growth of *Aspergillus niger* and *Botrytis cinere*(Huang et al. 2008). Besides, the endophytic fungus *Fusarium* sp. isolated from the *Selaginella pollescens* plant are found to exhibit antifungal activity against *Candida albicans*(Strobel & Daisy 2003). Apart from antifungal properties, endophytic fungi such as *Cloridium sp*. has proven to exhibit strong antibacterial activity against *Pseudomonas* sp. (Verma et al. 2007). Moreover, novel antiviral compounds have been reported to be produced by endophytic fungi. For instance, the endophytic fungus *Cytonaema* sp. has found to produce two novel antiviral compounds,

the cytonic acid A and B (Guo et al. 2000). These compounds inhibit the human cytomegalovirus (hCMV) protease.

Endophytic fungi have also been proven to be useful to the agricultural industry as several are known to demonstrate insecticidal properties. For example, the nodulisporic acid compound which is isolated from the endophytic *Nodulispotium* sp.and *Bontia daphnoides*, has potent insecticidal properties against the larvae of the blowfly (Verma et al. 2007). In addition, the nematicidal and insecticidal compounds isolated from the endophytic fungus *Geotrichum* sp. AL4, which is found in the leaves of *Acalypha. indica*, possessed good bioactivity against the nematodes *Bursaphelenchus xylophilus* and *Panagrellus redivivus* (Gangadevi & Muthumary 2009).

From numerous studies, it has clearly proven that endophytic fungi are excellent sources of novel bioactive compounds, which can satisfy the demand of the pharmaceutical, medicinal, and agricultural industries. Besides, due to the highly novel bioactive compounds being isolated, endophytes particularly endophytic fungi, have garnered much attention and interest from researchers to explore and study more on its metabolites and its properties of the endophytic fungi, such as its potential as biosorbents in removing heavy metals ions from aqueous solution.

However, with the understanding and realization that the endophytic fungi are capable of producing novel bioactive compounds, the question as to how the endophytes that reside in the plant formed a relationship with is still yet to be fully understood. Therefore, the endophytes' physiology, defensive role, and also its biochemical pathways and secondary metabolite production are yet to be studied further in order to unearth and discover more of the vast abilities and potentials of the endophytic fungi.

1.4.1 Fungal cell wall structure and its constituents

The fungal cells are mostly multicellular and protected by a rigid cell wall, and the cell wall is reported to play an important site in sequestering the metal ions. According to the review by Svecova et al. (2006), it is explained that the fungal cell wall is mainly made of various polysaccharides, which are made of 90% of the cell wall. The polysaccharides on the cell wall include lipids, proteins, pigments, polyphosphates and inorganic ions (Tan & Cheng 2003). The thin outer layer of the cell wall consists of mixed glycans; consisting of glucans, mannans, or galactans, while the thick inner

microfibrillar layer consists of chitin chains in a parallel manner and sometimes of cellulose (D-glycopyranose) chains as shown in Figure 3. Chitin is a common constituent found on the fungal cell wall. It is a strong nitrogen-containing polysaccharide yet flexible, and it consists of the N-acetylglucosamine residues (Tan & Cheng 2003). The structures of the chitin and cellulose chains, glucans and mannans of the fungal cell wall are shown in Figure 3 below. Moreover, polyphosphates, inorganic ions and pigments are also found in the fungal cell wall.

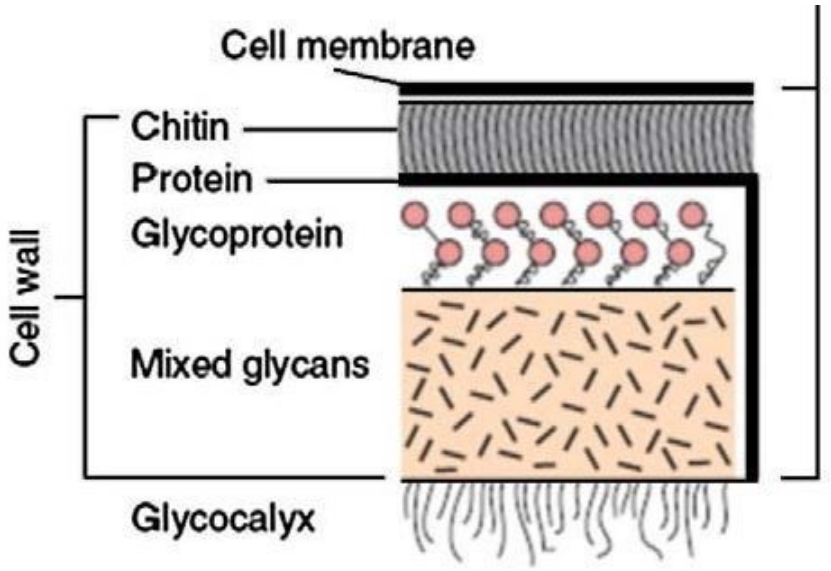

Figure 3.The rigid fungal cell wall composed of complex layers of various polysaccharides and proteins. Picture taken from Skowronski, Pirszel and Pawlik-Skowronska (2001).

However, different fungi harbour chemical and structural properties which are relatively different. For instance, the *Aspergillus niger* fungal cell wall consists of 73 to 83% of carbohydrates, 9 to 13% of hexamine, 2 to 7% of lipids and less than 1% of phosphorus; with an observation that denotes the lack of chitosan in the cell wall (Svecova et al. 2006). The *Aspergillus niger* fungus is reported to exhibit better biosorption efficiency as compared to *Rhizopus arrhizus*, which contains higher chitin content in the cell wall.

However, the potential biosorbent *Saccharomyces cerevisiae* only contains 1% of chitin on its cell wall. Hence, this suggests that the chitin on the fungal cell wall is not the only component which is responsible for metal sequestering. Based on Svecova et al. (2006) review, the large amounts of complex glucuronic acid and phosphate, and the chitin-chitosan constituents in the fungal cell wall provide huge possibilities of metal ions

binding through ion exchange and coordination mechanisms. In summary, the main constituents of the fungal cell wall: chitin-chitosan complex, phosphate and carboxyl groups on uranic acids and proteins, and nitrogen-containing ligands on proteins are believed to be the ionisable sites which influence and affect the metal ion uptake (Bapat, Kundu & Wangikar 2003; Filipović-Kovačević, Sipos & Briški 2000).

cellulose

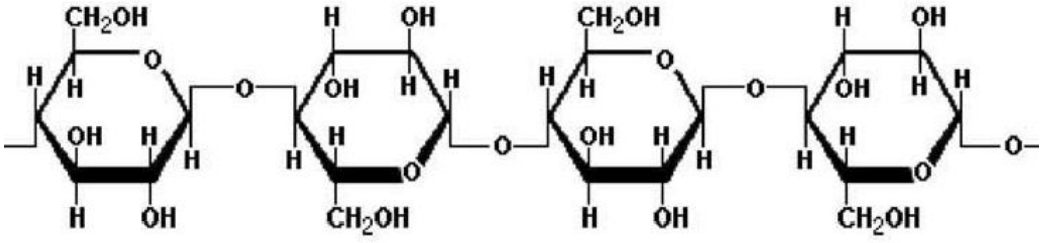

Figure 4.The structure of cellulose. Retrieved from http://www.scientificpsychic.com/fitness/carbohydrates2.html

chitin

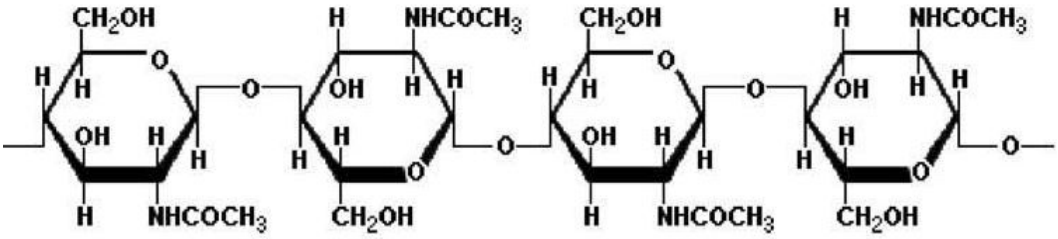

Figure 5.The structure of chitin. Retrieved from http://www.scientificpsychic.com/fitness/carbohydrates2.html

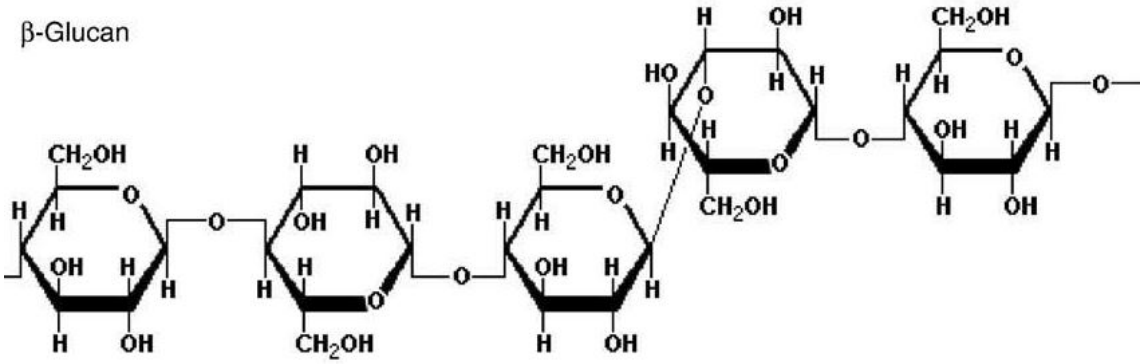

Figure 6.The structure of β-glucan. Retrieved from http://www.scientificpsychic.com/fitness/glucan.gif

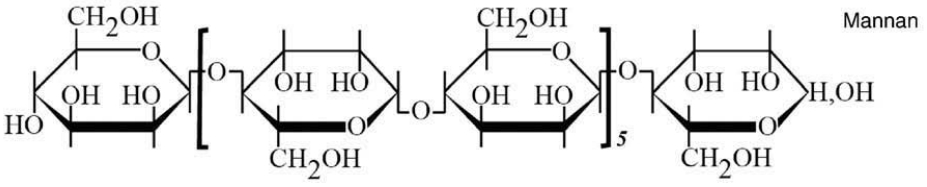

Figure 7.The structure of mannan. Retrieved from Arıca, Kaçar and Genç (2001a)

1.5 Fungi as biosorbents

1.5.1 Introduction

Fungi are ubiquitous in our natural environment and have proven to produce novel bioactive compounds which are highly useful to various agricultural, medicinal and pharmaceutical industries. Nevertheless, fungi have been a growing interest of study as potential biosorbents as fungi are observed to bind metallic elements. The importance of metallic ions towards the fungal cells has been known for a period of time and the presence of heavy metal ions are observed to influence the metabolism of the fungal activities and can subsequently affect the fermentation processes(Gadd 1994). Thus, this leads to the interest in relating the behaviour of fungi in the presence of heavy metal. Hence, researchers have been investigating the role of fungi as potential biosorbents and had led Kapoor and Viraraghavan (1997a) in the study of using fungi and yeasts in removing lead and cadmium ions from wastewater and recovery of precious gold and silver from process waters. It showed that both living and dead fungal biomass showed potential in adsorbing the heavy metals and precious metals.

Fungal organisms have been widely used in large industrial scale fermentation processes as most fungi are easy to grow and produce large amount of biomass, and it can also be manipulated morphologically and genetically to suit the interest of study (Wang & Chen 2009a). For example, *Aspergillus* strains have been used in the production of gallic acid, kojic acid, ferrichrome, itaconic acid, citric acid, glucose and enzymes such as amylase and lipases. Besides, the fungal biomass can also be easily and cheaply procured and also from by-product of various industrial processes, which made the fungal organisms as a growing interest of suitable and potential biosorbents.

Furthermore, the application of fungal biomass as biosorbent in removing heavy metal ions in aqueous solution can help to generate revenues from various industries which are currently wasting the biomass. Besides, the growing of fungal culture is relatively cheap as the biomass can be grown in inexpensive growth and fermentation media (Kapoor & Viraraghavan 1995). It has also been studied and investigated that the fungal cells can be altered genetically and morphologically to produce better raw biosorbent materials (Volesky 1990b).

1.5.2 Fungal biosorbents

Various groups of fungi have been proven to be efficient, economical and popular raw material chosen in removing heavy metal ions from dilute aqueous solution through biosorption process. This is due to the fact that fungal biomass offers several advantages as the fungal biomass provides high percentage of cell wall content which play an important role in the metal-binding process (Horikoshi, Nakajima & Sakaguchi 1981). Besides, large quantity of fungal biomass is easily obtainable and crucially, fungal biomass offers an eco-friendly environmental alternative solution for removal of heavy metal ions.

The fungus *Penicillium* has not only proven to produce the novel bioactive compound, penicillin, it has also been shown to be a potent adsorbent material in removing heavy metal ions. *Penicillium* has been studied and shown to remove various heavy metals such as Au, Cu, Zn, Cd, Mn, U and Th from diluted aqueous solution. Examples of *Penicillium* in heavy metal removal include *Pencillium oxalicum*, *Penicillium italicum*, and *Penicillium chrysogenum* (Mendil, Tuzen & Soylak 2008; Svecova et al. 2006; Tan & Cheng 2003). Among the *Penicillium* species, *P. chrysogenum* has been studied the most and *P. chrysogenum* was demonstrated to adsorb Cr(III), Ni, and Zn, as well as Pb, Cd, and Cu (Skowronski, Pirszel and Pawlik-Skowronska (2001); Tan and Cheng (2003).

Apart from *Penicillium* species, *Aspergillus niger* has also been studied as its role in adsorbing heavy metal ions. *Aspergillus niger* is a popular microorganism used in the biotechnological applications (Bapat, Kundu & Wangikar 2003). It is known to produce various extracellular enzymes and organic acids such as the pectinase, glucoamyls, acidic lipase, feruloyl esterase, gluconic acid and citric acid. Despite its role and ability in producing citric acid and various enyzmes, *Aspergillus niger* has the ability to

remove various heavy metal such as cadmium, copper, lead and chromium from aqueous solution (Wang & Chen 2009a). For example, *Aspergillus niger* 405 showed potential affinity for binding of Cu, Zn and Ni ions in a single composition system, while it only showed binding properties for Cu and Zn in a multi-metal solution as studied by Filipović-Kovačević, Sipos and Briški (2000).

In addition, the live and the dead white-rot fungus *Trametes versicolor* biomass entrapped in Ca-alginate beads showed metal binding ability towards Cd (II), with the maximum experimental adsorption capacity of 102.3±3.2 and 120.6±3.8 mg/g respectively (Arıca, Kaçar & Genç 2001a). Moreover, the white-rot fungus species *Lentinus sajorcaju* also showed potential in removing heavy metals. The entrapment of the *Lentinus sajorcaju* biomass into alginate beads has reported to adsorb a maximum of 104.8±2.7 mg/g of Cd (II) by the live entrapped biomass, and 123.5±4.3 mg/g Cd (II) by the dead entrapped biomass (Bayramoğlu, Bektaş & Arıca 2003). Furthermore, *Rhizopus nigricans* was studied and it showed maximum biosorption capacity from a range of 160 to 460 μmol/g for various heavy metals; Li^{+}, Ag^{+}, Pb^{2+}, Cd^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Sr^{2+}, Fe^{2+}, Fe^{3+} and Al^{3+}(Kogej & Pavko 2001).

The role played by the functional groups on the cell wall was studied. The fungal biomass was subjected to various chemical treatments in order to study the role of its functional groups to the biosorption process through the modification of functional groups, carboxyl, and phosphate and amino. A study had been done on the pretreatment of *Penicilllium lanosa-coeruleum* on the adsorption capacity of the heavy metals, in which it was found that heat, detergent pretreatmens and NaOH found to enhance the biosorption of Cu and Pb, whereas glutaraldehyde was found to improve the adsorption capacity of Ni(Ilhan et al. 2004). In addition, the pretreatment of *A. versicolor* was also being studied with pretreatment of glutaraldehyde, dimethyl sulfoxide and H_2O_2 and the results showed that the biosorption of Pb was increased (Çabuk et al. 2005). Thus, it clearly shows that the functional groups on the fungal cell wall play a crucial role in the heavy metal biosorption capacity.

In the field of biosorption, fungi have been a growing interest of many researches as fungi not only have proven to produce novel bioactive compounds, but also possess the ability in the adsorption of heavy metal from dilute aqueous solution based on several reviews mentioned above. Hence, it is of great interest in the continual study and

investigation on fungi, particularly endophytic fungi in the removal of heavy metals from aqueous solution.

1.6 Wetlands

Wetlands are referred as lands which are submerged with water for part or all of the year. Wetlands are divided into two types; the natural and the artificial/constructed/man-made. As defined by Convention (Secretariat 2004), the wetlands comprise a huge variety of habitats, which include the peatlands, marshes, floodplains, rivers and lakes. Moreover, coastal areas like mangroves, seagrass beds, and saltmarshes, and also marine areas and costal reefs which do not exceed six metres during low tide are classified as wetlands as well; including the man-made wetlands such as the wastewater treatment ponds and reservoirs.

Soil, hydrology, and vegetation form the major parts of the wetlands while aquatic plants play a major role in both the natural and artificial wetlands, which involve in oxygen production, sediment stabilization, water quality improvement and nutrient cycling (Mohan & Hosetti 1999). Aquatic plants are more favourable over the biological agents due to their frequent abundance in the aquatic ecosystems, easy handling and its low cost (Dhir 2013).

Wetlands have gained much interest and attention over the years due to their ability and capacity in removing pesticides, surfactants, PCPs, pharmaceuticals and microcontaminants (Matamoros & Salvadó 2012). Besides, wetlands are known to be the natural sinks for contaminants. The rhizosphere of the wetland plants provide an enriched culture zone for the microbes involved in degradation while the sediment zone provides the reducing conditions that are conducive to the metal removal pathway. The soil is the main supporting element for plant growth and microbial films and it influences the hydraulic processes. A mixture of sand and gravel produces the best results for the removal of contaminants and hydraulic conditions.

The removal of contaminants is influenced by the interstitial pore spaces and grain size in wetlands. The removal of contaminants occur through several mechanisms; filtration, sedimentation, plant uptake, sorption and microbial breakdown. The organic and inorganic constituents are removed via several ways, which is through physical removal via filtration and degradation to non-toxic form biologically. The constituents are also

being removed by the plants via adsorption and also being absorbed to media surfaces. The organic and inorganic constituents are also being chemically transformed and stored within the wetland matrix.

Macrophytes play a major role in wetlands, while free-floating aquatic species and submerged plants form a major part in both the natural and constructed wetlands (Brix 1997). The common aquatic species in wetlands include the salt marsh bulrushes (*Scirpus maritimus, Scirpus cyperinus, and Scirpus robustus*), rabbitfoot grass (*Polypogon monspeliensis*), cat-tail (*Typha angustifolia*, *Typha latifolia*), Irish-leaved rush (*Typha orientalis*, *Typha minima*), *Juncus xiphioides*, *Elodea sp*., *Cyperus alternifolius*, *Myriophyllum spicatum*, *Arundo donax*, *Lemna sp*., *Eichhornia crassipes*, and *Pontederia cordata.*. The submerged aquatic vegetation species include *Elodea muttalli*, *Ceratophyllum demersum*, *Najas guadalupensis*, and *Potamogeton pusillus*, which are present in numerous natural and constructed wetlands (Chen 2011).

1.6.1 Metal removals from wetlands

Metal removal from wetlands has been widely reported. It is found that the removal of metals in wetlands depend on various factors: (1) the types of element and (2) their ionic forms, (3) the season, (4) plant species and the (5) substrate conditions (Dhir 2013). Metal such as aluminium, cobalt, copper, iron, nickel, uranium and even lead have been reported to be removed from wetlands (Dhir 2013). The metal removal is due to the uptake from the plants, the soil adsorption, cation exchange through the plants which induced chemical changes in rhizophere and even through precipitation.

The sorption process by the plants occurs through the process of adsorption and precipitation. It can happen through either chemisorption or cation exchange. The cation exchange process involves the physical attachment of the ions to the organic matter particles in the soil and the surfaces of clay. The positively charged ions are believed to be bonded to the soil by electrostatic attraction.

It is said that adsorption plays an important mechanisms in the removal of metals in wetlands. Adsorption involves the transfer of ions from soluble phase to a solid phase and thus leads to short-term retention period or long term stabilization (Dhir 2013). In addition, the metals are absorbed to fine-textured sediments of particles and organic matter through ion exchange. This process occurs upon the presence of competiting

elements towards the adsorption sites and the types of elements presence (Seo, Yu & DeLaune 2008).

According to Sheoran and Sheoran (2006), metals such as Fe, Mn and Al upon going through hydrolysis and/or oxidation form insoluble compounds, and this leads to formation of a range of hydroxides, oxides and oxyhydroxides. Moreover, root surfaces of aquatic macrophytes are reported to deposit Fe (III) which has been oxidised from Fe (II) by the Fe(II) -oxidizing bacteria, and this forms plaques with a huge capacity to adsorb metals (Cambrolle et al. 2008). Furthermore, metals in wetlands are reported to form insoluble compounds through reduction and some metals may also form metal carbonates (Dhir 2013). The carbonates are believed to contribute to the initial trapping of metals (Sheoran & Sheoran 2006).

It is strongly believed that pH affects the metal removal efficiency in wetlands. The macrophytes in wetlands promote the nitrification process by releasing oxygen. The protons produced through the nitrification process may not be completely neutralized by HCO3- ions, which results in a decrease in pH. On the other hand, alkaline conditions are believed to promote coprecipitation of cationic metals, like nickel, copper, zinc and cadmium. Thus, the high rate of nitrification may reduce the efficiency of cationic metal removal.

Based on several studies, macrophytes such as *Phragmites australis*, *Phragmites karka*, *Phalaris arundinacea*, *Typha domingensis* and *Typha latifola* are believed to have the ability in removing metals in wetlands (Lesage et al. 2007). Besides, floating aquatic plants such as *Eichhornia crassipes*, *Pistia stratiotes* and *Salvinia herzogii* are reported to provide good metal absorption sites in the wetlands (Maine, Duarte & Suñé 2001; Maine, Suñé & Lagger 2004). The macrophytes are efficient in removing metals by immobilizing the rhizophere and store the metals in the below-ground biomass (Baldantoni, Ligrone & Alfani 2009; Zhang et al. 2010a). According to Lee and Scholz (2007), the macrophytes only take up small amount of metals and mostly are taken up by their roots and only a small amount is taken up to the shoots. While for the floating aquatic plants, they do not actively promote the metal adsorption to the substrate, but instead store the metals into their biomass (Dhir 2013). For the submerged aquatic

plants like *Potamogeton* spp. and *Hydrilla verticillata*, they are believed to have high potential to decontaminate water according to Bunluesin et al. (2007).

Apart from that, wetland plants are also reported to accumulate and store heavy metals in their tissues. Studies have shown that certain wetland plants have the potential to uptake >0.5% dry weight of an element and accumulate the elements in its tissue to a thousand-fold the initial concentration. For instance, the water hyacinth (*Eichhornia crassipes*) and duckweed (*Lemna minor*) are reported to be excellent bioaccumulators of cadmium with an accumulation of 6,000-130,000 mg/kg of dry weight and an approximate of 7,000 mg/kg of dry weight of copper accumulation (Zayed, Gowthaman & Terry 1998; Zhu et al. 1999). It clearly shows that the plants in wetlands have the ability in storing huge amounts of metals in its biomass and it is interesting to highlight that most wetland plants accumulate higher concentrations of metals in its roots than in shoots (DeBusk, Peterson & Reddy 1995; Deng, Ye & Wong 2004, 2006; Ye et al. 1997).

Besides, it is important to note that the major approach for immobilization of metals in the wetland plants is through phytostabilization, in which the metals are immobilized in the plants and stored in its root and soil. On the other hand, phytoextraction involves the use of hyperaccumulators to remove the metals. It is also reported that the site of metal accumulation and the types of elements in which they are absorbed varied from the different types of wetland plants species. Furthermore, the plants species in wetlands are reported to be able to alter the speciation of metals in the uptake and removal process. For instance, the *Eichhornia crassipes* uptakes the toxic Cr(VI) and subsequently reduce the ions to the less toxic Cr(III) ions.

1.7 Proteomics

The term proteome has been defined as the 'protein complement to a genome' by Wilkins in 1994 (Wilkins 1994). Hence, proteomics is a study of the proteome and this concept has only been introduced in the 1990s. Due to the fact that studying of proteins enable deeper understanding of cellular biochemistry which can then be employed in various biotechnological processes, proteomic technologies have been developed to measure large numbers of proteins in a qualitative and quantitative manner and it has since then acquired great significance in the research of biological systems (Bhadauria et al. 2007) . Most proteomic analyses are aimed to study and analyse the generation of quantitative data on differential protein expression in response to various environmental stresses and thus give an insight on the mechanisms involved at a molecular level (Carberry & Doyle 2007).

Generally, proteomic can be defined as the study of the total set of protein species found in a biological unit such as the organelle, cell, tissue, organ, species and ecosystem at any developmental stage and under certain environmental conditions (González-Fernández, Prats & Jorrín-Novo 2010). In proteomics, high-throughput processes have been incorporated in which larger numbers of proteins can be analysed in a short period of time (Lueking et al. 1999; Wolters, Washburn & Yates 2001). To date, proteomics have served not just an appendix of genomics or an approach, but has recognised as a complex scientific discipline exploiting the cell proteome.

Numerous proteomic studies have been conducted on plant pathogenic fungi (González-Fernández, Prats & Jorrín-Novo 2010). Several mycoparasitic fungi like *Trichoderma harzianum* and *Trichoderma atroviride* have been studied at the proteomic level and thus can provide useful information and insight to understand the mechanisms involved in the biological control of pathogenic fungi (Grinyer et al. 2005; Grinyer et al. 2004). However, the knowledge and understanding on the heavy metal stress towards fungi at the proteomic level is still limited to date.

Proteomics has allowed the study of heavy metal response and stress towards fungi on a much wider scale. Elucidating how the proteome expresses in response to stresses like heavy metal stress is highly interesting and crucial to understand the molecular mechanisms underlying the microorganism and heavy metal response and interaction.

Through proteomic studies, it enables us to exploit more of the fungal gene function, regulatory networks and biochemical pathways activated in the presence of heavy metals. Based on several research papers, heavy metal stress proteomics has been conducted on *Saccharomyces cerevisiae*, *Schizosaccharomcyces pombe*, *Chlamydomonas reinhardtii* and *Phanerochaete chyrosporium* (Bae & Chen 2004; Gillet et al. 2006; Hu et al. 2003; Vido et al. 2001). Thus, with proteomic technologies, heavy-metal resistant fungal genes can be analysed and characterized on a biochemical and molecular level and hence provides a further understanding of the biosorptive mechanisms involved.

1.8 Significance and aims of the present study and dissertation outline

To our present knowledge, biosorption process has been emerging as an alternative and attractive tool to treat heavy metal pollution from polluted wastewater. From numerous biosorption studies, limited studies have been done on endophytic fungi as biosorbent which are proven to produce novel bioactive compounds. Hence, the present study hopes and aims to discover and explore the abilities of the endophytic fungi isolated from wetlands, to study the influence of pH towards the heavy-metal resistant endophytic fungus in the biosorption process and to further understand the underlying mechanisms and intracellular activities involved of the endophytic fungi in response to heavy metal stress through proteomic analysis.

In chapter 2, we describe the methodologies applied in this study to (i) isolate, screen and identify heavy-metal resistant endophytic fungi from wetlands of Sarawak and (ii) conduct biosorption and proteomics analyses of the heavy-metal resistant fungus. In chapter 3, the experiment and findings on the isolation, identification and screening of the heavy-metal resistant fungi towards the heavy metals Cu(II), Cr(VI), Pb(II), and Zn(II) are discussed. In chapter 4, we report and discuss the biosorption experiments in the hope to study the influence of pH towards the heavy-metal resistant fungus and compare the removal efficiency between immobilized live and dead fungal biomass. Besides, the findings of the different protein expression levels of the endophytic fungus in response to heavy metal stress is reported and discussed.

Overall, the objectives of this study are to:

(i) screen and isolate endophytic fungi from *Nypa fruticans* collected in the Kuching Wetland National Park

(ii) assess their resistance to grow under elevated concentrations of Cu(II), Zn(II), Pb(II), and Cr(VI).

(iii) determine the effect of pH on the adsorption of Cu (II), Pb (II), Cr (VI) and Zn (II) by immobilized living and dead fungal biomass.

(iv) study the expression levels of heavy-metal resistant fungal protein under the treatment of the heavy metals

Chapter 2

2.0 Methodology

2.1 Isolation of endophytic fungi

The leaves of the *Nypa fruticans* sp. were washed with running water to wash off external contaminant such as dirt. Leaf dics (approximately 1cm^2) were cut which include the vein and the intervein tissues from each leaf sample, using a sterile razor blade. After cutting the leaf into smaller sections, the leaf dics and the surfaces were sterilized with 75% ethanol for approximately 1 minute. 70%-80% of ethanol for surface sterilization was found to be the best solution and the standard ethanol-water mixture concentration used to disinfect microorganisms as used in numerous studies (Guo, Hyde & Liew 2000; Lu et al. 2000; Rehman et al. 2008).. After washing with ethanol, the leafs discs were washed with 1% sodium hypochlorite for 3 minutes, and 95% ethanol for 30 seconds. Then, the leaf dics were washed with sterilized water to wash off the sterilant before drying them on a sterilized paper towel. The triple sterilized procedure was found to be optimum for isolating endophytes (Photitat et al. 2001).

The leaf dics were then transferred onto sterile Yeast extract Glucose Chloramphenicol Agar (YGCA) plates and left for incubation at 25 °C. YGCA (5.0 g/L yeast extract; 20.0 g/L dextrose; 0.10 g/L chloramphenicol; 14.9 g/L agar at pH 6.6±0.2) is a selective nutrient medium that inhibits the growth of organisms apart from yeast and molds due to the presence of chloramphenicol, and thus is suitable to be used for the first incubation medium for the isolation of endophytic fungi. Once the growth of the endophytic fungi on the YGCA plates was observed, the cultures were then transferred onto sterile Potato Dextrose Agar (PDA) to obtain pure cultures. The PDA (200g/L Potato infusion, 20g/L dextrose, 15g/L agar at pH 5.6±0.2 at 25 °C) is the ideal medium for growth of fungi as it contains potato infusion and dextrose which promote optimum fungal growth (Downes & Ito 2001).

2.2 Screening of fungal isolates against heavy metals

The axenic fungal cultures were screened against the selected heavy metals on a solid medium (PDA) for preliminary screening. The fungal cultures were transferred onto the PDA plates supplemented with the selected concentration of heavy metals. For the preparation of the medium with heavy metals, the selected heavy metal solutions is prepared and in order to eliminate contaminant which can affect the screening, the prepared heavy metal solutions were filtered using sterile syringe filter with pore size of 0.2 µm. The selected 0.2 µm pore size rather than the 0.45 µm due to the fact that the former pore size has a better ability in filtering out even the smallest size bacteria (Corporation 2014). It is found that initially the use of 0.45 µm syringe filter pore size is not sufficient to strain out the *Brevundimonas diminuta* bacteria. Hence, the pore size 0.2 µm is more favourable to strain out contaminants and microorganisms from the heavy metal solution.

After the preparation of the heavy metal stock solution, the solution is then transferred and mixed with the autoclaved PDA at approximately 50 °C before it solidified. The PDA supplemented with the heavy metals is then poured onto the sterilized petri plates for it to solidify. After solidification of the agar, the pure endophytic fungi cultures were transferred onto the plate for testing its tolerance against the heavy metals. The fungus culture was left for incubation at 25 °C and the growth of the fungi was examined daily for a period of 7 days. After the incubation period, the fungal culture which was able to grow on the PDA supplemented with heavy metals was then chosen for identification via morphological and molecular methods.

2.3 Identification of endophytic fungi isolate

2.3.1 Morphological identification

The morphological identification of the endophytic fungal isolates is conducted based on the morphology of the hyphae or colony culture, the spores' characteristics and the reproductive features if the structures can be observed (Barnett & Hunter 1972). The theoretical approach to describe the fungal morphology by using the microscopic image was reported in 1970 (Metz, De Bruijn & Van Suijdam 1981). During that period of time, the analysis of the image was not automated, and hence the measurement of the fungal culture was a challenge and time-consuming. Later in the late 1980s, automated

digital analysis was introduced to enhance the process of image analysis (Adams & Thomas 1988). Subsequently, the quantification of internal fungal structures and the documentation of the morphogenesis of single mycelia in growth chambers are reported (Spohr et al. 1998; Vanhoutte et al. 1995).

Prior to the molecular identification, each of the fungal isolates is inoculated onto the PDA separately to induce sporulation. Identification of the fungal characters is according to Huang et al. (2008) with modifications. The spore or the mycelia of the fungal culture is transferred and mounted onto a slide using sterile wire loop. The inoculated fungal cultures are subsequently stained with crystal violet before viewing it under the optical light microscope and the image is then captured as shown in Figure 8. Crystal violet stained is used to give a better visualization of the fungal culture upon viewing.

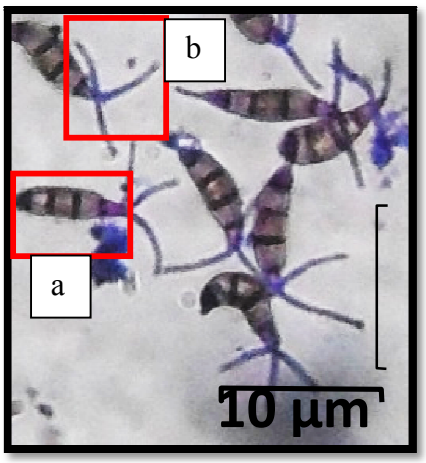

Figure 8.The captured image of selected heavy-metal resistant fungal spore stained with crystal violet and viewed under 40X optical light microscope. The fungal spore is observed to have five conidial cells with three brown median cells and hyaline cells (see a) and have three apical appendages arising from apical cells (see b).

2.3.2 Molecular Identification

2.3.2.1 DNA extraction

The fungal DNA is extracted prior to DNA amplification using the thermolysis method by Zhang et al. (2010b). The thermolysis method is considered a rapid and easy method to extract fungal genomic DNA and provides several advantages.

One of the advantages of applying the thermolysis method is that it is less time-consuming compared to the other extraction methods, as it does not require phenol/chloroform solution in the mechanical breaking of the fungal cell wall or the DNA purification. According to Zhang et al. (2010b), the 100 fungal samples can be processed in a day by using the thermolysis method. Secondly, the application of thermolysis method for fungal DNA extraction can eliminate the use of liquid nitrogen or an ultra-low temperature centrifuge. Instead, the thermolysis method only requires a water bath and a standard centrifuge. Thirdly, no toxic chemical such as phenol or chloroform is needed and hence the protocol eliminates the disposal of harmful chemicals and hence it is safe for operators. Apart from that, only small amount of fungal biomass (≈ 0.01 g) and thus the strains do not require being cultivated for a long period of time, and this is extremely beneficial for slow-growing fungi and also during the study of fungal genetics when large number of transformants can be cultivated in a short period of time. Lastly, the thermolysis method can reduce the chance of contaminants because it omits the surface contacts between the fungal DNA and equipment, mortar and pestle, and even spatula, and hence problems with PCR caused by contaminant DNA can be avoided (Kwok & Higuchi 1989).

On the other hand, the thermolysis method does have some disadvantages and should be discussed in order to avoid the certain conditions for the thermolysis application. As the thermolysis method does not require purification procedure, PCR product may be inhibited by certain chemicals released from the cells of some species and the inhibitors vary with species of the fungi, the types of media used for cultivation and growth of the fungi (Min et al 1995; Paterson 2007, 2008). Besides, it is important to note that eventhough thermolysis method has its disadvantages for extraction of DNA from fungal tissues, weak amplification can be obtained even if diluted genomic DNA is used. This is due to the fact that those cell walls of the fungal tissues are tougher to break compared to those using mycelia and also pigments and other chemical inhibitors of PCR amplification are usually present.

In order to extract the fungal DNA, the pure fungal cultures are inoculated onto PDA to form a fungal colony. Once it is formed, the small amount of fungal mycelia is transferred into the 1.5 ml of sterile centrifuge tube containing 100 µl of pure water by using sterile toothpick. The mixture was vortexed and subsequently centrifuged at 10,000 rpm for 1 minute at room temperature. This is conducted to wash off any

contaminant presence of the outer layer of the fungal culture. After centrifugation, the supernatant is discarded carefully using a pipette tip and 100 µl of the lysis solution is added into the microcentrifuge tube. The ingredients to prepare the lysis solution include 50 m/mol sodium phosphate at pH 7.4, 1 m/mol of EDTA, and 5 % glycerol. The lysis solution has to be autoclaved at 121 °C before use and can be stored at 4 °C.

2.3.2.2 DNA Amplification

2.3.2.2a Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a technique widely used for DNA amplification and it was developed by Kary Mullis in the 1980s (Kubista et al. 2006). The PCR is conducted using a thermocycler. Very small amounts of DNA (even in picograms range) can be amplified using PCR despite the presence of various contaminants (Cenis 1992). The several examples on the application of PCR include prenatal diagnosis of genetic diseases, genomic fingerprinting and genetic fingerprinting of forensic samples, direct nucleotide sequencing of genomic DNA and cDNA, direct cloning from the genomic DNA or cDNA, analysis of allelic sequence variations and RNA transcript structure, assays for the presence of the infectious agents and in vitro mutagenesis and engineering of DNA (Coen 2009).

The PCR theoretical structure is summarised in Figure 9. The components for the PCR include the double-stranded nucleic acid of interest to be amplified, two single strands oligonucleotide primer (forward and reverse primers), DNA polymerase, suitable deoxyribonucleoside triphosphates (dNTPs), salts and buffer. With the use of PCR, it allows the characterization of the fungi through amplification of specific sequences and the identification of the fungi can be achieved through the analysis of the PCR amplified products by sequencing, oligonucleotide probing and Restriction Fragment Length Polymorphisms (RFLP) analysis (Gardes & Bruns 1993). In order to perform these analyses, specific amplification of the fungal DNA from mixture of plants and fungal DNA has to be performed.

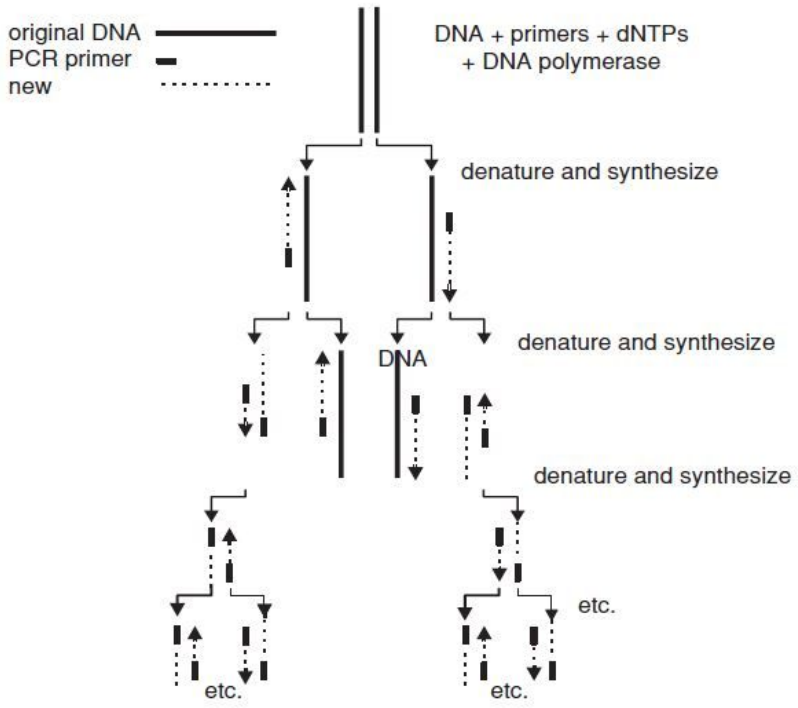

Figure 9A schematic representation of the Polymerase Chain Reaction. The thermostable DNA polymerase, oligonucleotide primers, dNTPs, and DNA template are required to bind to the target sequence and yield new DNA template. Each cycle is repeated and can lead to amplification to a million-fold amplification of the DNA fragment over the period of 20 to 30 cycles. Picture taken from Coen (2009).

2.3.2.2b The Principle of Polymerase Chain Reaction

The PCR occurs firstly with the denaturation of the targeted double-stranded nucleic acid (dsDNA) to form two single-stranded DNA (ssDNA) templates. The targeted DNA template is denatured by heating it at 95 °C for 15 seconds to 2 minutes, which vary with the different samples being studied. In the denaturation step, the breaking of the hydrogen bond of the double-stranded DNA occurs and yields a single-stranded DNA template.

The second step involves the annealing of the primers to the single-stranded DNA templates. The temperature is reduced to the range of approximately 40 °C and 60 °C, depending on the melting temperature (Tm) of the primers used. The concentration of the Guanine/Cytosine (G/C) and Thymine/Adenine (T/A) is known to affect the Tm value. It is reported that the sequence with rich G/C has a higher Tm value compared to those sequence with T/A rich (Rodríguez-Lázaro, Cook & Hernández 2013). The oligonucleotide primers used typically consist of short sequences (15 – 25 nt). At the

optimum temperature, it allows the oligonucleotide primers to anneal with the DNA templates at the complementary targeted sites and flank the region of target DNA for amplification. If the annealing of the primers to the template is sufficient, strong ionic bond between the template and the primer is formed and thus allows the DNA polymerase to attach and begin the copying of the targeted template.

The last step is the elongation process in which it is carried out across the target sequence by using a thermostable DNA polymerase in the addition of dNTPs and magnesium chloride ($MgCl_2$). The frequently and commonly used DNA polymerase enzyme is the Taq DNA polymerase (from *Thermis Aquaticus*), while Pfu Polymerase isolated from *Pyrococcus furiosus* is also widely used due to its fidelity when copying the DNA template (Sunil Kumar 2012). Temperature is increased to its optimum temperature for the thermostable DNA polymerase and the polymerase adds the dNTPs, known as the building block for new DNA strands, from the 5' to 3' of the template. The newly synthesised DNA template can serve as a new template for the next cycle. The cycles will be repeated and at the end of the cycle and the amount of the DNA product is doubled. The amplification of the final number of the targeted sequence copies is expressed by the following equation:

(2^n-2n) X

n = the number of cycles

2n = the first product attained after the first cycle and the second product attained after the second cycle with undefined length

X = the number of the original template copies

Hence, a 2^{30} –fold amplification of the DNA product will be produced after every 30 cycles. The analysis of the PCR product is conducted using Agarose Gel electrophoresis.

2.3.2.2c Internal Transcribed Spacer (ITS)

Internal Transcribed Spacer (ITS) regions of fungal rRNA genes have been identified as the formal DNA barcoding region for the molecular identification of fungi (Schoch et al. 2012). The ITS region contains two variable non-coding regions that are nested within the rRNA repeat between the highly conserved 18S, 5.8S and 28S rRNA genes

as shown in Figure 10 (Gardes & Bruns 1993). One of the features that made the ITS region favourable for amplification is that it provides a convenient target region for fungal molecular identification. This is due to the fact that the entire ITS region in a fungus is often in between approximately 450 and 750 base pair (bp) in length and hence can be readily amplified with the complementary sequences within the rRNA genes by using the ITS primers (Blaalid et al. 2013). Secondly, the ITS regions can be easily amplified from small, dilute or highly degraded DNA samples due to the multicopy nature of the rDNA repeats. Apart from that, the ITS regions have been demonstrated that it is highly variable due to the high sequence variability of the flanking rRNA genes which makes them suitable for identification on species and genus level (Buchan et al. 2002).

Studies have reported on the success of soil fungal community identification in using the ITS analysis and even species diversity within the endophytic fungi of the common reed (*Phragmites australis*) (Heinonsalo, Jørgensen & Sen 2001; Wirsel et al. 2001). Besides, the ITS analysis is able to demonstrate a different fungal community structure view compared to the culture-based methods (Viaud, Pasquier & Brygoo 2000).

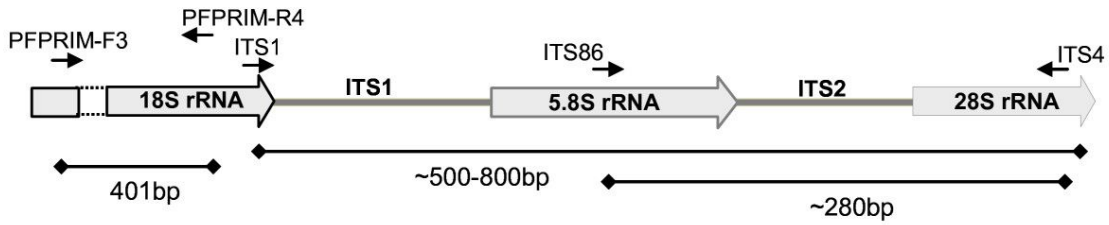

Figure 10.The schematic representation of the fungal ribosomal 18S rRNA genes and the ITS regions with the ITS 1 and ITS 4 primer binding locations. Picture taken from Embong et al. (2008).

2.4 Gel electrophoresis

The most widely used method for analysis of PCR product is by using Agarose gel electrophoresis, which separates the DNA product according to its charge (Garibyan & Avashia 2013). Agarose gel electrophoresis offers the easiest method for analysing and visualizing the PCR product. It allows the detection of the presence and size of the PCR product as shown in Figure 11. Standardized molecular marker is run alongside with the

DNA product on the gel simultaneously in order to determine the size of the PCR product.

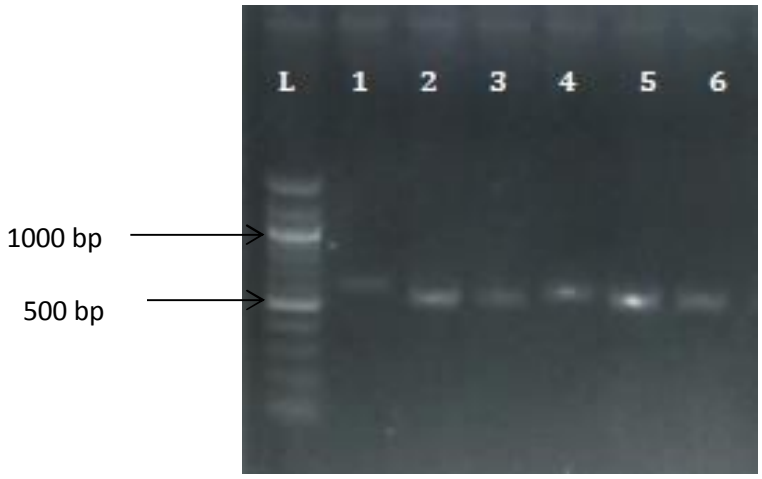

Figure 11. 1% Agarose gel electrophoresis showing PCR products with gene sequences Internal Transcribed Spacer (ITS) run in 1X TAE buffer with 100 bp DNA ladder (L). PCR products are inserted in lane 1-6 individually. High molecular weight DNA was observed at the range of 500-600 bp.

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There are two main methods for visualising the PCR products. The first method of visualization is the application of chemical dye such as ethidium bromide, as it intercalates between the two strands of duplex. The second method is the application of florescent dyes (fluorophores) for the labelling of the PCR primers and the DNA template prior to PCR amplification and/or molecular marker prior to running of gel electrophoresis.

2.5 DNA Sequencing

DNA sequence production has been widely carried out with the capillary-based, semi-automated implementations of the Sanger sequencing biochemistry since the early 1990s (Men et al. 2008). The Sanger enzymatic dideoxy method was described firstly in 1977 by Sanger, Nicklen and Coulson (1977) and since then, the Sanger chain termination sequencing method was able to determine the nucleic acid polymer of approximately 10^{11} bases (Shendure et al. 2004).

Sanger sequencing has evidently come a long and productive way for the past three 30 years. According to Men et al. (2008), Sanger sequencing was firstly used in the

deciphering the pioneer genomic study of the phiX174 bacteriophage genome led by Sanger's group in 1977, which only consists of over just 5000 bases of contiguous DNA. The technology was further enhanced to sequence multiple mega-sized genomes of bacteria by The Institute of Genomic Research (TIGR) team in the early 1990s, and subsequently to the characterization of the first eukaryotic genome of budding yeast *Saccharomyces cerevisiae* in 1996, and even the Human Genome Project sequencing relied on the automation of the sequencing technology firstly introduced by Sanger (Rizzo & Buck 2012). Hence, this clearly shows that the Sanger sequencing technology has become the main innovative tool for characterization of biological systems over the past years and has inevitably shifted the pathway of modern biology. To date, the Sanger technology has continuously been rapidly improving, in which the phenotypic data acquired can be combined with computed DNA sequence and thus precisely linked even small DNA changes such as the single-nucleotide polymorphisms (SNPs) to biological phenotypes (Men et al. 2008).

2.5.1 Sanger Sequencing (First Generation Sequencing)

The automated Sanger sequencing has been widely used for the characterization of numerous biological system and is considered the "first-generation" of DNA sequencing technology. The standard Sanger sequencing technically works by identifying the linear sequences of nucleotides by electrophoretic separation of the single-stranded terminated extension products in a capillary-based polymer gel and the methods are summarised in Figure 13(Sanger, Nicklen & Coulson 1977). The automated sequencing utilizes the laser excitation of fluorescent labels of fragments exiting the capillary and the detection of four-colour-labelled termination reaction, and the readout is then represented in a Sanger sequencing 'trace'as shown in Figure 12 (Shendure & Ji 2008). These traces acquired will then be translated into DNA sequence, in which error probabilities for each base-call can be generated (Ewing & Green 1998; Ewing et al. 1998).

The Sanger sequencing reactions can read DNA fragments of 500 bp to 1 kb in length, and this method is still widely used for small DNA fragments sequencing and it is reported as the standard for clinical cytogenetics (Kingsmore & Saunders 2011). Hence, the Sanger sequencing still remains the most routinely used technology today for

characterization of biological systems and its well-defined chemistry makes it the main application for sequencing (Rizzo & Buck 2012).

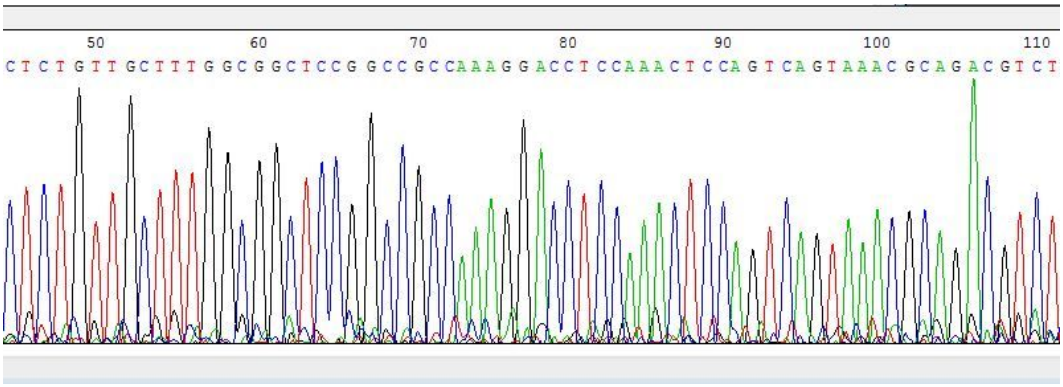

Figure 12.Representation of heavy-metal resistant fungal DNA sequence amplified using Internal Transcribed Spacer 1 and showing the fluorescently labelled dideoxynucleotides (ddNTPs) which are read using Chromas software.

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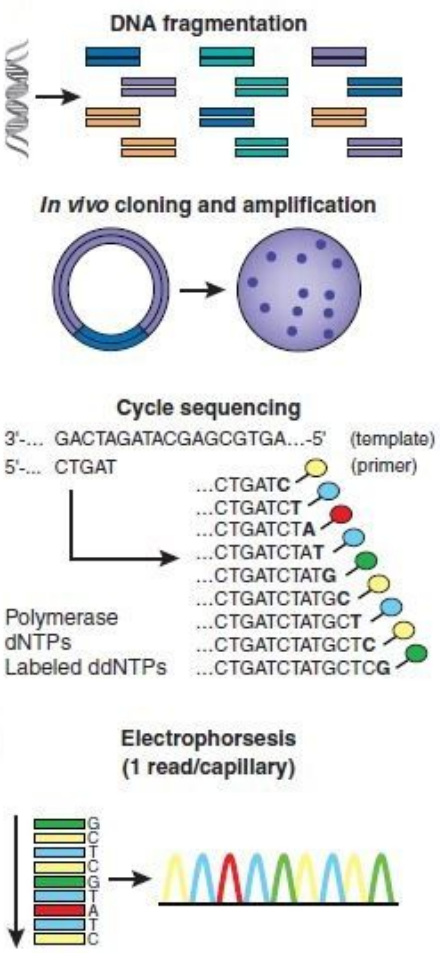


Figure 13. The schematic work flow of the conventional Sanger sequencing technology. The DNA is fragmented and is cloned to a plasmid vector for amplification. In each cycles sequencing, the fragments are subjected to high resolution electrophoretic separation within one of 96 or 384 capillaries in each run of a sequencing instrument. The fragments which are fluorescently labelled pass a detector and the sequencing trace is generated via the four-channel emission spectrum. Picture is taken from Shendure and Ji (2008).

2.6 Building Phylogenetic tree from molecular data

A phylogenetic tree is constructed to estimate the relationship among the biological taxa (sequence) and their hypothetical common ancestors (Felsenstein & Felenstein 2004; Hall 2013; Nei & Kumar 2000). Most phylogenetic tree is built by using molecular data (DNA or protein sequences) using software tools. Generally, phylogenetic tree is used to represent evolutionary relationships among biological taxa, while the NCBI database is used to hold the diverse molecular sequence data (Huson et al. 2007). Initially, most of the molecular phylogenetic trees are constructed to study the relationships among the species from the sequences acquired. However, nowadays, the construction of

phylogenetic tree has been expanded to understand the relationships among the sequences without regard to the host species, indicating the functions of genes that have not been studied experimentally (Hall, Pikis & Thompson 2009). Besides, phylogenetic analysis is also used as a tool in molecular epidemiology of infectious diseases to understand the mechanisms which can lead to microbial outbreaks (Hall & Barlow 2006).

To construct a phylogenetic tree, 4 main steps are needed: (1st step) A set of homologous DNA or protein sequences is identified and acquired. (2nd step) Alignment of those acquired sequences (3rd step). Estimation of a tree from the aligned sequences (4th step). Presentation of the phylogenetic tree in such a way it is clear to convey the data to others. An overview of a typical flow of a phylogenetic tree construction is shown in Figure 14.

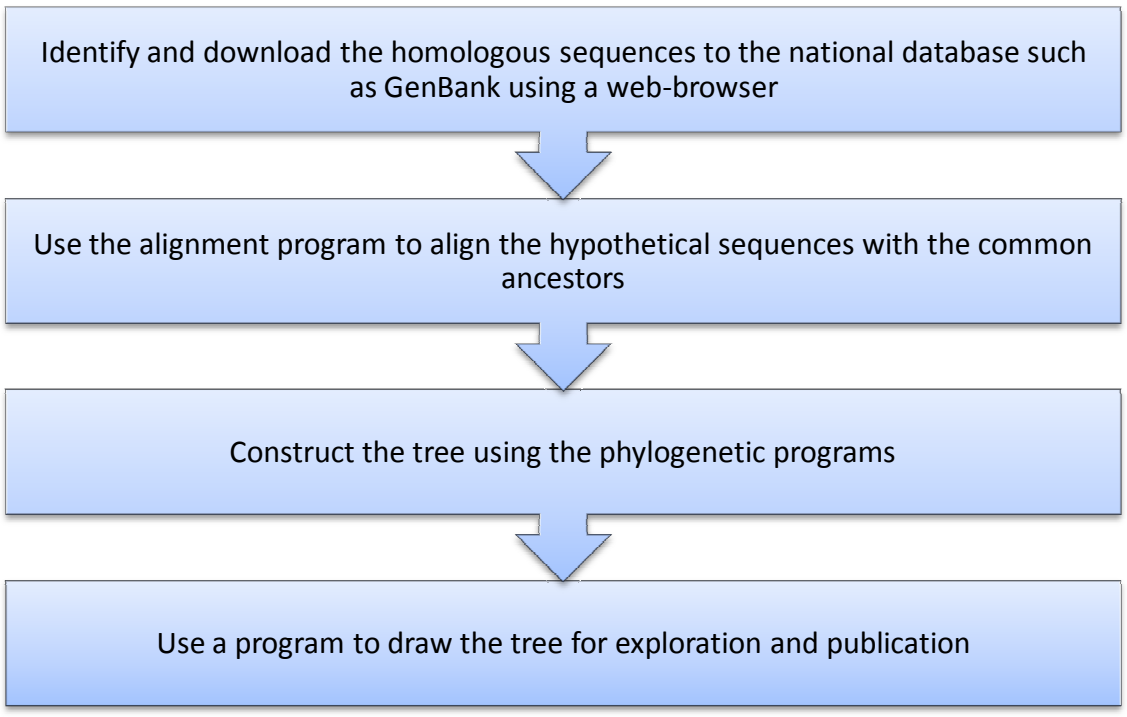


Figure 14. A flow chart of a typical phylogenetic tree construction to estimate the relationship among the sequences.

One of the programs that can eliminate the need to interconvert files from one program to another is by using the MEGA5 software. This software is one of the integrated

program tools that can carry out all four steps in a single environment with the use of single user interface which eliminates the need to interconvert the file formats from one program to another (Tamura et al. 2011).

2.7 Biosorption Technology

In order to treat heavy metal pollution owing to the rapid increase of toxic aqueous effluents being discharged into the environment, biosorption technology has been developed as a greener alternative technology to remove pollutants from industrial wastewaters.

Although the conventional methods of physicochemical and biological processes have been commonly employed, these processes are said to harbour several disadvantages. Application of conventional treatment processes such as the coagulation and chemical precipitation are considered more costly and less effective when the adsorbates or pollutants are presence in a low concentration range (Crini 2006; Vieira & Volesky 2010; Volesky 2007). Besides, it is reported that the activated carbons and ion-exchange resins used are costly and low in efficiency, and thus cause a limitation in their commercial application in the industry (Vijayaraghavan & Yun 2008). Therefore, it has driven the urge for researchers to develop an alternative technology to solve the limitations of conventional methods by using several various types of biomass to bind and concentrate selected ions or other molecules from aqueous solution (Volesky 2007), hence, the term ‘biosorption’ is coined.

The main advantages of the biosorption technology have reported to be less costly and offer high efficiency compared to the conventional methods. Besides, the technology can minimise biological or chemical sludges generated and it even has the ability to regenerate biosorbents and may yield the recovery of metal after the biosorption process (Volesky 2007). Numerous types of materials have been investigated to be used and applied as biosorbents in treating heavy metal pollution. The types of materials being studied include the living or nonliving biomass from various types of microorganisms, a number of natural and inorganic materials like ash and clay, and even synthetic materials to replace the physicochemical and biological processes of the conventional method (Ahmaruzzaman 2008; Babel & Kurniawan 2003; Bhatnagar & Minocha 2006).

Due to their affordable and abundant nature, the biomass type of biosorbents are reported to be the most attractive alternatives in replacing the effective commercial adsorbents like the exchange resins and activated carbons (Vijayaraghavan & Yun 2008). Therefore, the use of biosorbents in the removal of toxic pollutants and even the recovery of valuable resources from polluted wastewaters has been known to be one of the most attractive and recent technologies in the environmental or bioresource technology (Aksu 2005; Volesky 2007).

2.7.1 The History of Biosorption technology

The biosorption technology is pioneered by B.Volesky and M. Tsezos in 1980s by using biomass or biomolecules to remove pollutants through binding of selected ions to the biosorbents (Volesky 2007). In fact, it had been reported that the ability of living microbes to sequester metals from aqueous solutions had been studied as early as the 18^{th} and 19^{th} centuries (Modak & Natarajan 1995). However, the use of living or non-living microorganisms as adsorbents for removal and recovery of metals from aqueous solution has only been applied in the last 30 years (Park, Yun & Park 2010).

Several review papers have reported that the first quantitative study on metal biosorption was conducted to sequester copper by using fungal spores of *Tilletia tritici* and *Ustilago crameri* as biosorbents in 1902 by L. Hecke (McCallan & Miller 1958; Muraleedharan, Iyengar & Venkobachar 1991). Several years later in 1922, the study on the uptake of metals Ag, Cu, Ce and Hg by corn smut was investigated by F. Pichler and A. Wobler (Muraleedharan, Iyengar & Venkobachar 1991). The first ever removal of radioactive metals (plutonium-239) from contaminated domestic wastewater was reported by using activated sludge in 1949 by Ruchloft (Ruchhoft 1949). Subsequently, B. Volesky and M. Tsezos were granted the first to receive the patent on the biosorption technology for the removal of uranium or thorium ions from aqueous solution in 1982 (Volesky & Tsezos 1982). Hence, the biosorption technology has gradually been developed over the past several decades and it has inevitably garnered much interest and attention among the researchers and scientists to develop and enhance the technology to provide a better alternative in removing toxic effluents from industrial and polluted wastewaters.

2.7.2 Immobilization of Biomass

Despite the fact that the application of biosorbents has been proven to provide higher efficiency and biosorption capacity in removing pollutants compared to the conventional ion-exchange resins, the applications are reported to be slightly hindered by problems arising due to their physical characteristic of the biosorbents in industrial application (Vijayaraghavan & Yun 2008). Microbial biosorbents have several limitations as they are small in size with low density, poor mechanical strength and slightly rigid in nature. Thus, when used in column processes, it will have difficulty in separating the solid-liquid component, causing possible swelling or biomass and clogging problem, poor reuse/regeneration and development of high pressure drop can happen (Veglio & Beolchini 1997). In order to overcome these limitations, application of biomass immobilization can be applied.

Immobilization of biomass can solve these limitations by converting the biomass into spherical shape and thus enabled to be used like the conventional adsorbents. Through the immobilization of biomass, the size particle is ranged from 0.5 to 1.5mm, with good chemical and physical resistance and external porosity (Andres, Texier & Le Cloirec 2003). Besides, the immobilization of biomass is also needed in the use of conventional reactor technology like the large packed and fluidized bed reactors systems (Gadd & White 1993). However, it is interesting to highlight several exceptions in regards to the application of the immobilization technique. For example is the use of highly rigid biomass like seaweeds, and in such cases, the unprocessed form may be more favourable without being immobilized (Vijayaraghavan & Yun 2008). Apart from that, the flotation process used to separate the biomass from aqueous solution can also overcome the use of the immobilization technique (Kapoor & Viraraghavan 1995).

There are several techniques used for the immobilization of biomass. Generally, the techniques are divided into 3 categories. The first category is the immobilization of biomass on various inert support materials such as glass bead, polymers and even sand. The immobilization is conducted at the specific stages of the microbial biomass cultivation at an *in-situ* manner (Veglio & Beolchini 1997). The second category is the immobilization of the biomass especially the small particles of biomass within the polymeric matrix such as alginate, polyacrylamide, and polysulfone. This immobilization method can eliminate clogging problems, enable of high biomass

loading and control of suitable particle size, easy separation of liquid, and regeneration of biosorbent (Vijayaraghavan & Yun 2008). The third category of immobilization method is by cross-linking technique using chemical linkers such as formaldehyde and formaldehyde-urea mixtures. However, it is important to take into account the cost of both the types of immobilization used and the raw biomass in order minimize the overall cost of the commercial biosorbent produced and thus can be applied successfully in the industry.

2.8 Proteomics

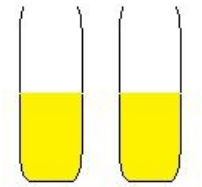
Proteomics involves the use of high-throughput techniques and protocols to enable large number of proteins to be analyzed in a short period of time (Washburn, Wolters & Yates 2001; Wolters, Washburn & Yates 2001) Proteomics studies have incorporated a large range of technologies, in which most of the studies utilize the conventional approach of two-dimensional electrophoresis (2-DE) coupled with Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-ToF) mass spectrometry (MS) (Carberry & Doyle 2007). Proteomic studies have been applied to understand the interaction between plant and fungus pathogenicity and virulence of the fungi (Bhadauria et al. 2010; Gao & Kolomiets 2009; González-Fernández, Prats & Jorrín-Novo 2010). Despite that, proteomic studies on heavy-metal resistant fungi are still limited and hence through this research, the response and mechanism of the fungi towards the heavy metal stress can be further understood.

The workflow of proteomic analysis according to González-Fernández, Prats and Jorrín-Novo (2010) includes these major steps: experimental design, sample preparation, protein extraction, protein separation via 2-DE, mass spectrometry (MS) analysis, protein identification and quantification, and statistical analysis of the data acquired (Figure 15). It is important to highlight that the first few steps of the proteomic workflow are crucial in the case of fungi as the choice of a suitable extraction protocol implemented is important in order to properly extract the protein and thus enabled to solubilise and identify the protein (Jorrín-Novo et al. 2009).

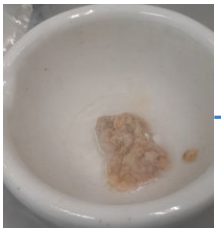
Two-dimensional electrophoresis separates the complex protein mixtures based on its charge (pI) and molecular mass. Subsequently, the protein spots are analyzed, excised and digested with proteolytic enzymes (trypsin). These peptide mixtures are then subjected to MS analysis and peptide mass fingerprints are then compared to

gene/protein sequence databases for protein identification (Resing & Ahn 2005). The principle of MS instruments include an ionisation source, a time of flight tube and an ion detector with different types of peptide ionization employed (MALDI or electrospray ionization (ESI)) (Carberry & Doyle 2007).

A variety of 2-DE staining methods are commonly used which include the colloidal Coomassie blue dyes, silver and fluorescent stains (Miller, Crawford & Gianazza 2006; Patton 2002; Wu et al. 2006). It is known that silver staining method is more sensitive than Coomassie blue stains and the former is more favourable to be used for low protein concentration analysis (Lanne & Panfilov 2005).

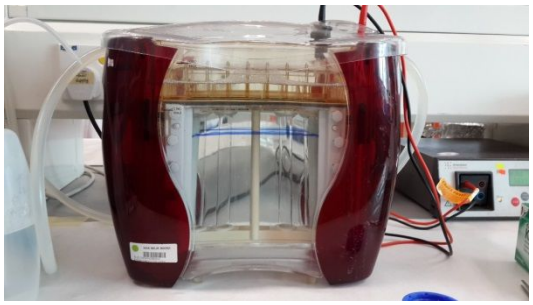


Samples

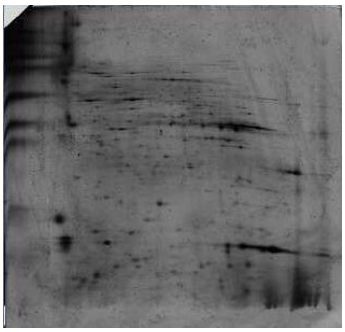


Protein extraction

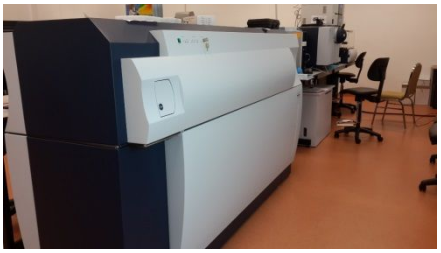
Protein separation via Isoelectric Focusing (IEF)



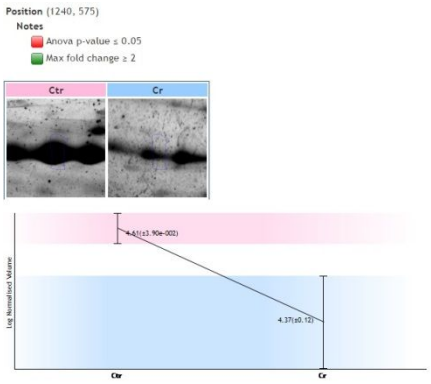
2-DE separation of protein



2D gels after silver staining



High-throughput protein identification by MALDI-ToF Mass Spectrometry



Studying differential protein expression level via PDQuest™

Figure 15.A schematic overview of the workflow in a fungal proteomic analysis.

Chapter 3

Heavy metal resistant endophytic fungi isolated from *Nypa fruticans* in Kuching Wetland National Park

Abstract

Heavy metal pollution is an environmental issue globally and the aim of this study was to isolate endophytic fungi from mangrove wetlands of Sarawak to assess and test their ability to grow in the presence of various heavy metals (copper (Cu), zinc (Zn), lead (Pb), and chromium (Cr)). Samples of *Nypa fruticans* were collected from 19 stations in the Kuching Wetland National Park (KWNP) for subsequent endophyte isolation. Ninety-three (93) isolates were obtained and assessed and the most resistant isolates (growing at concentrations up to 1000 ppm) were identified using fungal primers ITS 1 and ITS 4. All of the endophytic fungi identified to be closely related to *Pestalotiopsis* sp. and this is to our knowledge the first study reporting the ability of *Pestalotiopsis* sp.to grow at high concentrations of copper, lead, zinc and chromium. Our results highlight the potential of using endophytic fungi for the treatment of heavy metal pollution, for example as biosorbents.

Keywords: Heavy metals, Endophytic fungi, Kuching Wetlands National Park, *Nypa fruticans*

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3.1 INTRODUCTION

Heavy metals are released in many areas near urban complexes, metalliferous mines or major road systems due to the rapid growth of industrial activities and technological development (Alloway 1995). The current pattern of industrial activity has led to a continuous increase of heavy metal discharge into the environment including cadmium, lead, chromium, copper and nickel (Hemambika, Rani & Kannan 2011). This will eventually cause the soil, surface waters, ground water and sediments to be

contaminated and become toxic, and inevitably cause a significant threat to human health.

In Malaysia, agriculture, manufacturing industry, sewage and vehicle emissions have been the main contributors of heavy metal pollution. According to Shazili et al. (2006), studies have shown that the sediments of Juru River in Penang have been polluted with Pb (117 µg g^{-1} dry wt.) and Zn (483 µg g^{-1} dry wt.), while the Langat River in Negeri Sembilan is found to be highly polluted with Zn (71-374 µg g^{-1} dry wt.) and Cd (3.0 - 37.9µg g^{-1} dry wt.). According to Nriagu (1980), Pacyna (1986) and Nriagu and Pacyna (1988), Pb was emitted globally into water at 138 thousand metric tons, Zn was emitted at 226 thousand metric tons, while Cr and Cu were emitted at 142 and 112 thousand metric tons in the 1980s, respectively. This clearly shows that heavy metal pollution has been one of the major environmental problems not only locally but globally for the past years.

Several conventional physico-chemical approaches have been used for the removal and treatment of heavy metal pollution sites such as electrochemical treatment, ion exchange, precipitation, reverse osmosis, evaporation and sorption (Kadirvelu et al. 2002; Luo & Xiao 2010). However, these methods are reported to be uneconomical and ineffective and often result in incomplete metal removal, high reagent and energy requirements and generation of toxic sludge (Hemambika, Rani & Kannan 2011). Bioremediation on the other hand offers a promising and economical option to treat heavy metal in contaminated sites (Iskandar, Zainudin & Tan 2011). One of the processes, biosorption, involves the removal of metal ions by organisms through solid-liquid separation (Yang et al. 2012) and has received much attention due to several advantages such as high efficiency for removing heavy metal from diluted solutions (Cruz et al. 2004; Fan et al. 2008; Kapoor, Viraraghavan & Cullimore 1999). Studies have reported that both living and non-living biomass can be used as biosorbent and show a high potential in sequestering heavy metal ions (Arıca, Kaçar & Genç 2001b; Bayramoglu et al. 2002). Some biosorbents, however, display a narrow range in binding and collecting certain types of metals (Volesky & Holan 1995) and more studies are needed in order to discover novel biological resources that can act as biosorbents.

Wetlands are known for their high ion-exchange potential**.** In Peninsular Malaysia, a study has been conducted to assess the capacity of mangrove plants -which are within

the range of oil palm plantations, rubber and human settlements- to take up heavy metals (Nazli & Hashim 2010). This study reported that the mangrove species *Sonneratia caseolaris* exhibits tolerance towards heavy metals by storing Cu, Pb, Cr and Zn in its leaves without showing any sign of injury. Endophytes are ubiquitous in all plant species on earth and while there have been a considerable amount of studies on their ability to produce bioactive compounds (Guo et al. 2008; Kjer et al. 2010; Wiyakrutta et al. 2004), there are very few on the potential use of endophytes for the treatment and removal of heavy metals. Li et al. (2010) reported that endophytic fungi can be used for phytoremediation or bioremediation at contaminated sites and Yang et al. (2012) described the screening for potential biosorbents in endophytic fungi isolated from the marine environment and reported promising results.

3.1.1 Objectives

The aims of this study are to:

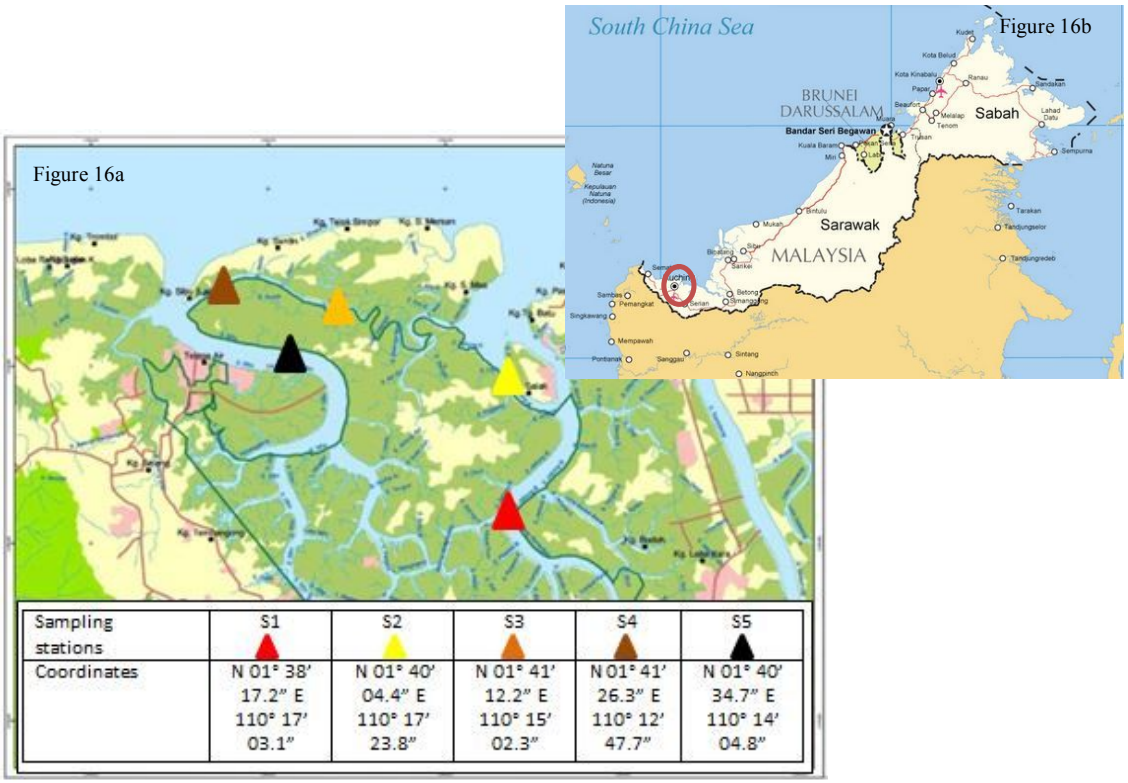
(i) screen and isolate endophytic fungi from *Nypa fruticans* collected in the Kuching Wetland National Park

(ii) assess their resistance to grow under elevated concentrations of Cu(II), Zn(II), Pb(II), and Cr(VI).

3.2 MATERIAL AND METHODS

3.2.1 Study site

Samples were collected on the 19th September 2013 in Kuching Wetlands National Park (KWNP), a Ramsar Convention sites. KWNP is an estuarine ecosystem located in western Sarawak and receives saltwater from the South China Sea and freshwater from two rivers, Sungai Salak and Sungai Sibu Laut. In total, 19 stations were sampled, however, only five stations are discussed as the most heavy metal resistant endophytes were found there (see Figure 16).



Sampling stations	S1	S2	S3	S4	S5
Coordinates	N 01° 38' 17.2" E 110° 17' 03.1"	N 01° 40' 04.4" E 110° 17' 23.8"	N 01° 41' 12.2" E 110° 15' 02.3"	N 01° 41' 26.3" E 110° 12' 47.7"	N 01° 40' 34.7" E 110° 14' 04.8"

Figure 16. Overview of sampling locations in Kuching Wetlands National Park, Sarawak. (a): map of Kuching Wetlands National Park, Sarawak, Malaysia in which *Nypa fruticans* were collected for isolation of endophytic fungi for screening against heavy metals. The five stations with the most heavy metal resistant endophytic fungi are indicated by coloured triangles and GPS coordinates provided. (b): The insert depicts the map of Malaysia and the sampling region is indicated by a red ring. Images/adapted from http://parochialsarawakian.wordpress.com/ and http://nationsonline.org.

3.2.2 *In-situ* parameters

Basic physical parameters such as pH, temperature, turbidity and salinity were measured and recorded *in-situ* at every station using an YSI Multiprobe. Triplicate measurements were undertaken to increase data accuracy. Water samples at each site were taken using a Van Dorn water sampler and brought back to the laboratory for analysis of nutrients and total suspended solids (TSS). Whole frond of *Nypa fruticans* from every station were cut into three (3) parts; upper, middle and lower and taken back to the laboratory for the isolation of endophytic fungi.

3.2.3 *Ex-situ* parameters

3.2.3.1 Nutrients analysis

All the bottles for nutrient analysis were acid washed prior to sampling. In order to minimise the use of water samples, samples used for this analysis were obtained from the filtered water of TSS analysis. Nutrients examined were Nitrate (NO_3), Ammonia (NH_3) and Orthophopshate (PO_4^{2-}) using Cadmium Reduction method, Salicylate method, and PhosVer 3 (Ascorbic Acid) method, respectively. Three (3) replicates were analysed for each station and nutrient using DR 2800 Spectrophotometer, according to Hach (2007).

3.2.3.2 Total Suspended Solids (TSS)

Glass fibre filter paper (GF/C, 47 mm diameter) were prepared pre-sampling by soaking in distilled water and wrapped with aluminium foil for drying overnight in the oven at 103 – 105 °C. The filter papers were set for three (3) replicates of each site and the procedure to determine TSS was according to Apha (2007).

3.2.4 Isolation of Endophytic fungi

Endophytic fungi were isolated from *Nypa fruticans,* which is one of the dominant and abundant species found in Kuching Wetlands National Park, Sarawak, Malaysia. Thus, it was chosen as the basis of our study. The leaves and stem of the *Nypa fruticans* were cut into smaller pieces and surface sterilized with 70% ethanol, 1% chlorox and rinsed

with sterilized water (El-Gendy et al. 2011) before placing them on Yeast Extract Glucose Chloramphenicol Agar (YGCA). Negative control plates were prepared by spreading the YGCA plates with the sterilized water used to rinse the *Nypa fruticans.* The plates were incubated at 25°C and observations were made daily. Once the growth of endophytic fungi was observed growing out from the parts of *Nypa fruticans*, the the isolates were transferred periodically onto fresh Potato Dextrose Agar (PDA) plates. The isolates were sub-cultured several times until pure cultures were obtained. Pure cultures of endophytic fungi were stored in slant PDA in universal bottles and used for screening against heavy metals.

3.2.5 Screening of fungal isolates against heavy metals

Fungal isolates were screened against Pb, Cu, Cr and Zn starting at concentration of 500ppm on PDA plates supplemented with heavy metals, using Zinc (II) Chloride ($ZnCl_2$), Lead (II) Carbonate ($PbCO_3$), Copper (II) Sulphate ($CuSO_4$), and Potassium Chromate (K_2CrO_4), respectively. For sterilization, PDA solutions were autoclaved and the heavy metals were filtered using a syringe with 0.22 µm syringe filter. The total of 93 isolates were transferred onto petri dishes containing PDA agar supplemented with 500 ppm concentration of specific heavy metals (Zn, Pb, Cu, and Cr) and allowed to grow for three (3) days at 25 °C. The growth of isolates was observed and all isolates that were able to grow in 500 ppm were selected for screening in 1000 ppm concentration of the heavy metals under the same conditions. Isolates that were able to grow at 1000 ppm were identified using morphological and molecular methods.

3.2.6 Identification of endophytic fungi isolate

3.2.6.1 Morphological Identification

After 3 days of incubation period on PDA, a sterile wire loop was used to transfer the isolates from the agar onto a microscope slide. Crystal violet was used to stain the endophytic fungi for easier and better visualization. The slides were then viewed under an inverted microscope and identification was based on fungal morphological keys (Jeewon et al. 2003; Maharachchikumbura et al. 2011).

3.2.6.2 Molecular identification

3.2.6.2a DNA extraction

Fungal DNA was extracted for DNA amplification using thermolysis method by Zhang et al. (2010b). Pure colonies of fungal isolates were added into 100µl of sterilized water in a 1.5ml microcentrifuge tube and centrifuged at 10,000rpm for 1 minute to homogenize it. After centrifugation, the supernatant was discarded and 100µl of the lysis buffer (50Mm potassium phosphate, 1mM EDTA, and 1% glycerol) was pipetted into the microcentrifuge tubes. The tubes were placed in a waterbath at 85°C for 30 minutes and used for DNA amplification.

3.2.7 DNA amplification

The DNA amplification method for fungal DNA was conducted according to Onn (2012). The fungal DNA was amplified using 0.6µl fungal primers Internal Transcribed Spacer (ITS) 1 (5'–TCC GTA GGT GAA CCT GCG G-3') and 4 (5'–TCC TCC GCT TAT TGA TAT GC-3'), 12.8µl deionized distilled water, 15µl of 2X MyTaqRedMix and 1µl of DNA template. The PCR conditions were set up as 95°C, 3 minutes (Initial Denaturation); 95°C, 3 seconds (Denaturation); 47°C, 30 seconds (Annealing); 75°C, 2 minutes (Elongation); 72°C, 5 minutes (Final Elongation) and 4°C (Cooling). The PCR tubes containing the MasterMix and DNA template were amplified using Bioer Little Genius Thermocycler. The PCR products were run in 1% agarose gel electrophoresis (AGE) using 1X TAE buffer at 90 V for 45 minutes and visualised under UV transluminator.

3.2.8 DNA Sequencing and Phylogenetic tree construction

The PCR products were sent to Beijing Genomic Institute (BGI), China for nucleotide sequencing. The sequences obtained were analyzed against the NCBI (USA) database (Zhang et al. 2000) and a phylogenetic tree was constructed from genetic distance and bootstrap values calculated using MEGA5 (Tamura et al. 2011) (see Figure 17).

3.3 RESULTS

3.3.1 *In-situ* and *ex-situ* parameters

At all stations, both surface and bottom water were collected and analysed. The *in-situ* (pH, salinity, temperature, and turbidity) and *ex-situ* parameters (Total suspended solids, Orthophosphate, Ammonia and Nitrate) of each sampling station are summarised in Table 1. Station 1 was located the furthest upstream and displayed the lowest turbidity at both surface and bottom, and the coldest bottom water compared to other stations (Table 1). Station 5 had the highest total suspended solids (TSS) readings for both water levels on average (0.1 mg/L at the surface and 0.123 mg/L at the bottom;Table 1) and was located near to Kampung Salak where terrestrial input may exert additional selective pressure on the endophytic fungi. Station 7 was located between two parts of land masses and displayed the most varied water parameters among all stations, for example an abrupt drop of salinity in the bottom waters (between stations 6 – 9; Table 1), as well as the highest concentration of orthophosphate of all bottom samples. Station 13 was near to Kampung Sibu Laut and had the most turbid waters (1561.07; Table 1). Station 15 was near to the most developed village in the area, Telaga Air, and had surprisingly low salinity around 10PSU despite receiving saltwater from the South China Sea (Table 1 and Figure 16). A possible explanation might have been low tide and increased freshwater input at the time of sampling.

Table 1. In-situ (pH, salinity, temperature, turbidity) and ex-situ (Total Suspended Solids, Orthophosphate, Ammonia, Nitrate) water parameters of sampling stations including their GPS position. S indicates Surface sample and B stands for Bottom sample.

Parameters	Station 1		Station 3		Station 5		Station 6		Station 7		Station 9		Station 11		Station 13		Station 15	
Layer	S	B	S	B	S	B	S	B	S	B	S	B	S	B	S	B	S	B
GPS	N 01° 38' 17.2" E 110° 17' 03.1"		N 01° 38' 51.3" E 110° 17' 51.2"		N 01° 40' 04.4" E 110° 17' 23.8"		N 01° 41' 00.1" E 110° 16' 21.8"		N 01° 41' 12.2" E 110° 15' 02.3"		N 01° 41' 19.2" E 110° 14' 20.6"		N 01° 41' 30.6" E 110° 13' 58.6"		N 01° 41' 26.3" E 110° 12' 47.7"		N 01° 40' 34.7" E 110° 14' 04.8"	
pH	8.10	8.04	8.00	8.02	8.14	8.14	8.16	8.18	8.19	8.15	8.24	8.25	8.30	8.28	8.44	8.44	8.58	8.60
Salinity (PSU)	7.00	11.00	12.00	14.00	14.00	14.00	14.00	22.00	14.00	14.00	11.00	8.00	13.00	8.00	18.00	19.00	9.00	19.00
Temperature (°C)	29.49	28.29	28.62	28.37	28.88	28.85	29.17	29.10	28.77	28.68	29.21	29.16	29.10	29.16	29.39	29.43	28.69	28.76
Turbidity (NTU)	833.70	935.13	1184.23	1214.83	1332.53	1354.17	1358.03	1372.93	1298.97	1403.27	1340.60	1363.37	1447.57	1491.97	1505.53	1561.07	1500.80	1553.07
Total suspended solids (mg/L)	0.022	0.033	0.022	0.071	0.100	0.123	0.092	0.116	0.032	0.056	0.033	0.049	0.046	0.017	0.033	0.055	0.115	0.034
Orthophosphate (mg/L)	0.057	0.057	0.087	0.057	0.063	0.033	0.070	0.057	0.117	0.330	0.067	0.040	0.040	0.037	0.070	0.070	0.033	0.040
Ammonia (mg/L)	0.013	0.070	0.080	0.020	0.030	0.013	0.017	0.040	0.033	0.013	0.013	0.023	0.017	0.033	0.070	0.010	0.077	0.013
Nitrate (mg/L)	0.967	0.487	0.010	0.680	1.003	0.850	0.463	0.987	1.023	1.043	0.980	0.660	0.660	0.050	0.873	0.857	0.777	1.063

3.3.2 Identification and fungal screening against heavy metals

A total of 342 endophytic fungal isolates have been successfully isolated from *Nypa fruticans* sp. collected from Kuching Wetland National Park.

From the 5 stations selected, 93 isolates were successfully isolated from *Nypa fruticans* sp. and were screened against copper, chromium, lead and zinc. From the preliminary screening**,** there were eight fungi which showed different resistance patterns against at least three of the four chosen heavy metals (Table 2).

Table 2. Summary of growth observed at 1000 ppm concentration of heavy metals (Pb, Zn, Cu, Cr) on day 3 (D3). + indicates growth, - no growth.

Isolate (Station of origin)	Heavy Metal			
	Lead (Pb)	Zinc (Zn)	Copper (Cu)	Chromium (Cr)
Isolate 2 (Station 7)	+	+	-	+
Isolate 3 (Station 7)	+	+	-	+
Isolate 4 (Station 15)	+	+	+	-
Isolate 5 (Station 5)	+	+	+	-
Isolate 6 (Station 13)	+	+	+	+
Isolate 7 (Station 5)	+	+	+	-
Isolate 8 (Station 5)	+	+	-	+

All seven fungal isolates were closely related to *Pestalotiopsis* sp. (Figure 2). Based on Xu et al. (2009), *Pestalotiopsis* sp. is reported to be an endophytic fungus and had been previously isolated from mangrove plants. Thus, the sequencing result correlates well with Xu et al. (2009) and proves that our isolates are endophytic fungi.

All seven isolates showed resistance against Pb and Zn upto 1000 ppm while only one isolate, ‘A6’ showed resistance against all four heavy metals, Pb, Cu, Cr and Zn at

1000ppm (Table 2). ‘A6’ was collected at station 13, which is near to a village and had the most turbid water (Table 2).

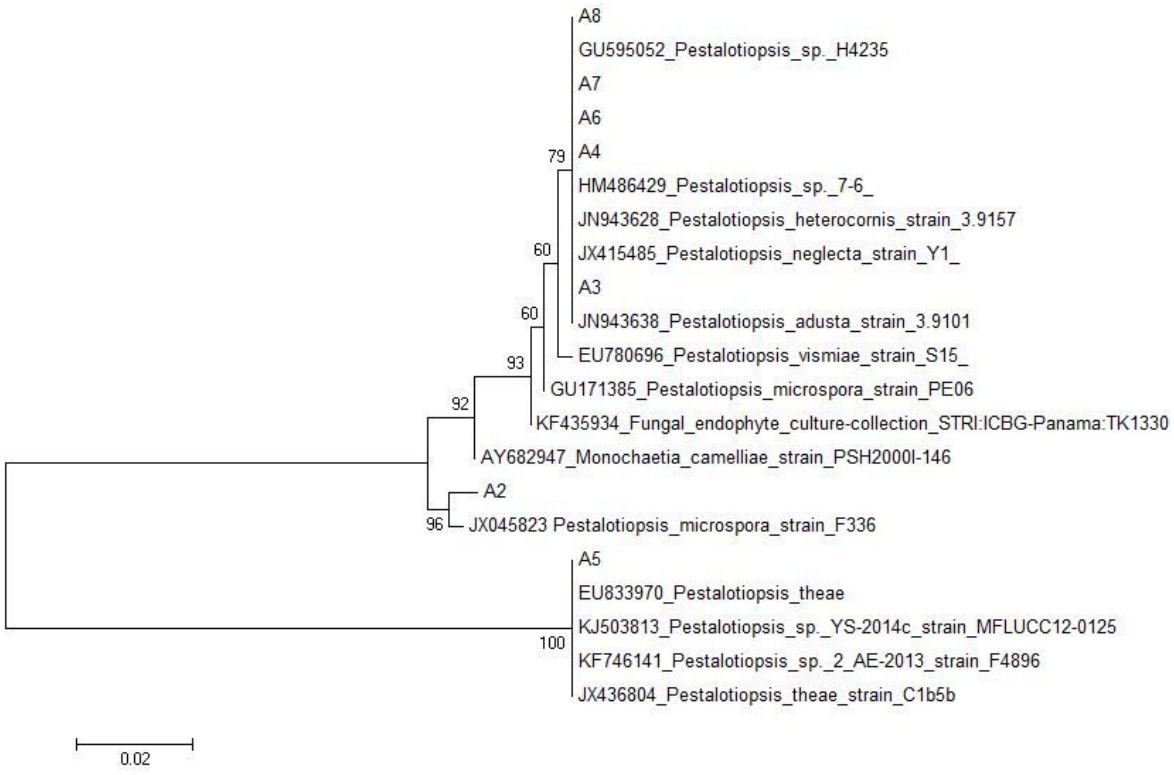


Figure 17. Phylogenetic tree representing fungal sequences found in *Nypa fruticans* sp. The phylogenetic tree was generated using Jukes-Cantor model and sequence distances were estimated by Neighbor-Joining method. Bookstrap values ≥50 are shown and the scale bar represents a difference of 0.02 substitution per site. Accession numbers for the reference sequences are indicated.

3.4 DISCUSSION

Microorganisms such as bacteria, fungi, algae and yeasts have been increasingly studied due to their metal sequestering property (Wang & Chen 2009b).

Based on previous studies, *Pestalotiopsis microspora* is known to produce taxol, an anticancer drug (Strobel et al. 1996), however, there is no report on the use of *Pestalotiopsis* species as heavy metal removal agents. Most studies have been undertaken on filamentous fungal strains and mostly members from the genera *Aspergillus*, *Fusarium*, *Humicola* and *Nannizzia* have been reported to possess resistance against heavy metals (Ezzouhri et al. 2009; Iram et al. 2013; Valix, Tang & Malik 2001). Recently, several studies have reported a similar trend among endophytic fungi being able to resist several heavy metals such as copper, zinc and cadmium (Deng et al. 2014; Hong, Park & Gadd 2010; Salvadori et al. 2013).

The preliminary screening of resistance among our endophytic isolates against heavy metal showed that the order of tolerance of our isolates for metals was Cr < Cu < Pb <Zn (Table 2). It can also be observed that as the concentration of the heavy metal increases, the growth of the isolates decreases (data not shown) which could be due to the fact that different heavy metals exhibit different toxicity to the different fungal isolates. According to Gupta et al. (2000), microbial biomasses are reported to bind to several heavy metals to different extents. Some biomasses can bind to most heavy metals with no specific priority, while others can be selective towards certain heavy metals (Volesky & Holan 1995). This can be due to the fact that various types of microorganisms have different cellular structures which contain several functional sites for ion metal binding (Wang & Chen 2009b).

Our preliminary results also revealed that various isolates which are of the same genera (*Pestalotiopsis*) and even matched to the same species *Pestalotiopsis microspora* did not exhibit the same tolerance towards the heavy metals. Isolate ‘A2’ which is closely matched with *Pestalotiopsis microspora* is able to resist lead, zinc and chromium while isolate ‘A7’ which is closely related to *Pestalotiopsis neglecta* is not able to resist chromium but copper instead. The difference of the metal tolerance may be due to the presence of various strategies of resistance mechanism exhibited by the fungi (Iram et al. 2013). Fungal cell walls are typically composed of the polysaccharides chitin and

cellulose and these constituents of the cell wall possess functional groups such as amino, carboxyl, hydroxyl and sulphate which have high metal binding capacities and believed to have a significant potential for metal binding (Davis, Volesky & Mucci 2003).

Our preliminary screening results reported that *Pestalotiopsis* sp. has a higher resistance potential towards copper compared to bacterium *Pantoea* sp.(Ozdemir et al. 2004) in which the bacterial isolate was able to only resist below 200 ppm of copper. However, higher heavy metal resistance of fungi towards chromium (up to 10,000 ppm) was reported by Congeevaram et al. (2007) and Faryal et al. (2007) reported *Aspergillus niger* strains that were able to resist lead up to 7000 ppm. Both studies isolated fungi from heavy metal contaminated environments, suggesting that the stresses of the environment towards the microorganisms play a major role in causing the fungi to resist heavy metals and adapt in order to survive. Endophytic fungi in this study, which were isolated from a mangrove environment, are also capable of resisting heavy metals up to high concentrations. One possible explanation could be that the nearby villages put external pressures on them; however, more detailed studies are needed to ascertain the cause for the observed high multi-metal resistance among our fungal isolates. Further studies are also needed to understand the mechanisms that enable our fungi to grow in heavy metal concentrations up to 1000 ppm.

3.5 CONCLUSION

Our preliminary findings indicate that endophytic fungi from wetlands of Sarawak can grow at high heavy metal concentrations (up to 1000 ppm). This is to our knowledge the first report on their heavy metal tolerance and eight isolates (all related to *Pestalotiopsis*) displayed tolerance against a suite of heavy metals (Cu, Cr, Zn, and Pb) and have high potential to be screened as potential biosorbents. Further investigations are however needed to assess their biosorption capabilities and determine for example the proteins involved in the biosorption process, the most efficient contact time and optimum growth conditions.

3.6 Acknowledgments

The authors would like to thank the Sarawak Forestry Department for their kind permission to conduct research at the Kuching Wetlands National Park (Permit No. NCCD,907.4.4(Jld.9)-77 and Park Permit No 142/2013). We also thank the Sarawak Biodiversity Centre for their kind permission to conduct research (Permit No. SBC-RA-0092-MM).This project was financially supported by MyBrain15 sponsorship programme by the Malaysian Ministry of Education, and Postgraduate Fee Discount Scheme by Swinburne University of Technology, Sarawak. The research leading to these results has received funding from Swinburne Sarawak under grant agreement no. SSRG/FECS/2013/2/4.

Chapter 4

Biosorption and Proteomic analysis on the heavy-metal resistant *Pestalotiopsis* sp. fungus

4.1 BIOSORPTION

Biosorption can be defined as a bioremediation process in which a certain type of biomass is used to bind and sequester metals for the removal of metal ions from dilute aqueous solution (Skowronski, Pirszel & Pawlik-Skowronska 2001). The biosorption approach was pioneered by Volesky's group from McGill University (Bapat, Kundu & Wangikar 2003) in the hope of removing heavy metals through the use of biological organisms. According to Volesky (2001), biosorption is a passive process of metal uptake in which the metals are sequestered using (microbial) biomass such as bacteria, fungi, algae, and even waste material. The process rates and efficiencies depend on the type of biomass used, mixture of the solution, type of biomass preparation and the chemico-physical environment.

Biosorption process involves 2 phases, the solid phase (sorbent phase, biological material) and liquid phase (solvent) which contain the metal ions to be sorbed (Arıca, Kaçar & Genç 2001a). Due to the metal binding capabilities of the biological materials, the metal ions are attracted and removed from the polluted water (Bayramoğlu, Bektaş & Arıca 2003). The microbial biomass is mainly composed of protein, carbohydrates, and phenolic compounds, since they contain functional groups such as carboxyl, hydroxyl and amines, which are able to attach to the metal ions (Kogej & Pavko 2001). According to Gadd (1994), all biological materials can be used as biosorbent materials for metals, except for mobile alkali metal cations like Na^+ and K^+, and this can be an important passive process for living and dead biomass (Ilhan et al. 2004).

Biosorption process occurs generally through 2 different modes. The first mode is the passive uptake of metal ions which involves surface binding of metal ions to cell walls and extracellular material. This process is independent of the cells' metabolic activity. The second mode of uptake is dependent on the cell metabolism as metal ions are transferred into the cell across the cell wall membrane. It is also known as active uptake or bioaccumulation (Çabuk et al. 2005). The passive uptake of the metal ions occurs

through ion-exchange, complexation and physical adsorption which allow the interaction between metal ions and functional groups of the cells such as carboxylate, hydroxyl, sulfate, phosphate and amino group. It is also highlighted that the different composition of the functional groups contributes to the various affinities of the sorption cells towards the metal ions. However, for the active uptake of the metal ions, the cell metabolism of the living cells plays an important role but the mechanism is complicated and yet to be fully understood.

There are numerous advantages of biosorption over conventional treatments which include low cost, high efficiency in removing pollutants from dilute solution, minimization of chemical and biological sludge, regeneration of biosorbent, no additional nutrient required and the possibility of metal recovery (Arıca, Kaçar & Genç 2001a; Kratochvil & Volesky 1998). Numerous studies have been undertaken to search for potential biological materials in the hope to develop desirable biosorbents with a wide range of metal affinities that can remove a variety of heavy metal cations from diluted waste water.

4.1.1 Factors affecting biosorption

Biosorption process is mainly affected by pH, initial metal concentration, temperature, ionic strength, biomass concentration and size, and agitation speed (Park, Yun & Park 2010). According to Park, Yun & Park (2010), pH is considered to be the most important parameter in the biosorption process. The difference in pH affects the activity of functional groups on the cell wall of the biomass, the solution chemistry of the metals and the competition of the metallic ions.

Biomass concentration also affects the process as the lower the concentration, the higher the interference for binding sites. However, temperature does not seem to influence the biosorption process in the range of 20 - 35°C (Aksu, Sag & Kutsal 1992; Veglio & Beolchini 1997). The factors affecting the biosorption process is summarized in Table 3.

Table 3. Factors affecting the biosorption process are categorized and the effects on biosorbent/pollutants are reported.

Factors affecting Biosorption	Effects on biosorbent /pollutants
pH	increase of pH increases the cationic metals uptake

	but reduces the anionic metals uptake
ionic strength	increase of ionic strength reduces the metal uptake by competing with binding sites of biosorbents
biosorbent concentration	increase biosorbent concentration increases metal removal efficiency until reaches equilibrium point
Biosorbent saiz	reduce biosorbent saiz increases surface area for biosorption
Initial heavy metal concentration	increase initial concentration reduces heavy metal removal efficiency as resistance of biosorbent against heavy metal increases
Agitation speed	increase agitation speed increases metal removal by minimizing mass transfer resistant but may damage the physical structure of biosorbent

4.1.2 Biosorbents

Wang and Chen (2009b) discussed three (3) main groups of potential biosorbents: bacteria (Gram positive and Gram negative), fungi (filamentous fungi and yeasts) and algae. Various algae (marine green microalgae, fresh water green microalgae, marine red macroalgae and marine brown macroalgae) have been highlighted as biosorbents by Lesmana et al. (2009). Microbes isolated from polluted environment have been studied as potential biosorbents (Iram et al. 2012) and it has been reported that those isolated microbes have successfully adapted and survived at the contaminated environment and were thus able to resist pollutants such as heavy metals. Among those biosorbents are marine algae (*Sargassum natans*), bacteria (*Bacillus subtilis*), fungi (*Aspergillus niger*), and yeast (*Saccharomyces cerevisiae*).

Besides, the utilization of agricultural waste as potential biosorbents has been reviewed by Sud, Mahajan and Kaur (2008) as it has reported that agricultural waste which contains cellulose shows potential in heavy metal removal. Numerous studies have been undertaken on agricultural waste materials such as activated sludge, rice husk, egg shell,

peat moss, hazelnut shell, sunflower and cotton stalk, waste tea leaves and orange and banana peel (Al-Qodah 2006; Annadurai, Juang & Lee 2002; Chuah et al. 2005; Cimino, Passerini & Toscano 2000; Farooq et al. 2010; Hashem 2006; Tee & Khan 1988). Farooq et al. (2010) also highlighted the use of wheat-based biosorbent such as straw and bran from wheat.

However, investigation on the use of endophytic fungi as potential biosorbents has not been widely studied and thus this present study aims to explore the potential of fungi, particularly endophytic fungi, in removing heavy metal ions from aqueous solution.

4.1.3 Mechanism of fungal biosorption

Fungal cell wall plays the key role in heavy metal adsorption as it contains several functional groups where coordination complexes with metals can be formed (Sağ 2001). The amino groups of chitin (R_2-NH) and chitosan (R-NH_2) are reported to bind heavy metal ions in the fungal cell and its associated proteins harbour carboxylate groups with pKa 4 to 5, while the phosphate groups which are mainly found in the glycoproteins are believed to play an integral role in the biosorption process as they display a negative charge above pH 3 (Sağ 2001; Tobin, Cooper & Neufeld 1984). Besides, glutamate, cysteine and aspartate are also believed to be responsible in the metal chelation process (Brady et al. 1994; Delgado, Anselmo & Novais 1998; Huang, Huang & Morehart 1991).

Several studies had been done on the investigation of the fungal biomass in removing heavy metals ion in the hope to understand and elucidate the mechanisms and biosorption sites being involved. Kapoor and Viraraghavan (1997b) studied the role played by the several functional groups of *Aspergillus niger*'s cell wall in adsorbing lead, copper and cadmium and postulated that both amine and carboxyl groups played a central role in removing the heavy metal ions. However, it was also found from Kapoor and Viraghavan's study that the phosphate groups did not play an integral role in the biosorption process of the metals studied, while reporting that the biosorption process occurs as a result of ion-exchange process as it was found that the potassium, calcium and magnesium ions have been released from the fungi.

Hence, the investigation of the removal of Cu(II), Cr(VI), Pb(II) and Zn(II) ions in this study can add to a further understanding of the biosorptive mechanisms involved in the fungal cell by comparing the trend shown by *Pestalotiopsis* sp. and other reported heavy-metal resistant fungal studies, coupled with proteomic studies being done.

4.1.4 Immobilization of Biomass

Numerous studies have reported that free cells are not suitable in columns for providing high efficiency in removing metal ions. This is due to the cells' low density and size which tend to plug the bed and result in large drops in pressure. There are several immobilization techniques available for biomass including alginate, polyacrylamide, polyvinyl alcohol, polysulfone, silica gel, cellulose and glutaraldehyde (Park, Yun & Park 2010).

It is important to select a suitable immobilization technique to prepare commercial biosorbents which retain the ability of the microbial biomass to adsorb metals during the continuous treatment process. The immobilization of the biomass material can help to develop the right size, mechanical strength, rigidity, and porosity necessary for use in application. Immobilization is one of the key techniques and thus the alginate immobilization technique is used as it is a simple and rapid technique and has been widely used in numerous encapsulation studies (Mehta & Gaur 2005).

4.1.5 Properties of Calcium Alginate beads

In a study done by Rodríguez-Lázaro, Cook and Hernández (2013), the SEM micrographs of the fungus immobilized on calcium alginate beads displayed a uniform growth of the fungus on the bead surfaces, indicating that immobilization of fungal biomass offers an additional advantage in increasing and enhancing the adsorption of metal ions compared to free fungal biomass.

Alginate is more favourable over other immobilization material based on several advantages, such as its biodegradability, hydrophilic properties, presence of carboxylic groups which may enhance the affinity towards the metal ions in the adsorption process, and it is not toxic (Rodríguez-Lázaro, Cook & Hernández 2013). Besides, alginate is found to have mechanical stability and low density, which makes it suitable for various biotechnological applications. Apart from that, the Ca-alginate beads were found to be stable under a pH range of 3.0 – 7.0, which applies to the pH range designed in this

study. In addition, the degradation of alginate by microorganisms in nature is considered safe and does not cause major pollution to the environment, and can also serve as a greener alternative to replace polymers of petroleum origin (Felsenstein & Felenstein 2004). Moreover, the heavy metal adsorption is also believed to be influenced by the presence of other cations, like Ca^{2+}, Mg^{2+} and K^{+} ions, and those cations could not be adsorbed by the fungal biomass (Ilhan et al. 2004). Hence, the use of immobilized fungal biomass on Ca-alginate beads can serve as an enhancement for the adsorption of heavy metal in the presence of the cations in an adsorption medium or industrial wastewater.

4.1.6 Application of biosorption

As a promising alternative solution for heavy metal pollution in wastewater, several biological biomass have been developed and commercialised for removal of metal ions in dilute solution (Table 4) (Park, Yun & Park 2010). Biosorption can also be used in purification and recovery of rare proteins, steroids, pharmaceuticals and drugs (Volesky 2007). Pilot installations and a few commercial scale units have been constructed in the USA and Canada (Tsezos 2001). According to Tsezos (2001), there are limitations in the process such as the difficulty in obtaining a reliable supply of cheap raw biomass and regeneration and reuse of the biomass, and the negative effects of co-existing ions on biosorptive capacity. Through continued research to overcome the limiting factors of the biosorption process, the situation is likely to progress and change in the near future with biosorption technology becoming more beneficial and attractive. However, with the present knowledge, the commercialised products summarised in Table 4 have not been reported to be used by any industrial facility.

Table 4.Various examples and description of biological resources which have been commercialized for removal of metal ions.

Commercialised Products	Remarks
Bio-Fix	developed by using biomass from several sources like cyanobacterium (Spirulina), yeast, algae and plant (*Lemma* sp. and *Sphagnum* sp.) by being immobilized in porous polypropylene bead with the

	particle saiz of 0.5-2.5mm
AlgaSorb™	developed using fresh water alga, *Chlorella vulgaris* by being immobilized on silica
AMT-BIOCLAIM™ (MRA)	was developed using bacterial biomass *Bacillus* sp. by being immobilized with polyethyleneimine and glutaraldehyde
B.V. SorbexBiosorbent	manufactured from a variety of sources include algae *Sargassum natans, Ascophyllum nodosum, Halimedaopuntia, Palmyra pamada, Chondrus crispus, and Chorella vulgaris*
RAHCO Bio-beads	prepared from a variety of sources including peat moss by being immobilized within an organic polymer
Tsezos-Baird- Shemilt	developed using *Rhizopus arrhizus* and immobilized with the particle size of 1mm

4.1.7 The Future of Biosorption

There are two trends for future biosorption development for metal removal, one trend is to use hybrid technology especially using living cells for pollutant removal, and another trend is to develop good potential biosorbents that act as ion exchange resin.

Several elements have to be taken into consideration such as the sources and types of biosorbent. Maintenance and production expenses cost will have to be considered if the biomass needs to be specifically cultured.

Immobilization of biomaterials and the optimization of the parameters of biosorption process and physicochemical conditions, as well as the ability to reuse and recycle, need to be improved for optimal efficiency of metal ions removal. The mechanism of the metal-microbe interactions should be further studied with great efforts.

Besides, further study is also needed on the biosorption technology. Eventhough several biosorbents have been patented and commercialised (Table 4), there have been no reports on their use in the industrial facility. Thus, numerous studies have to be done, such as the selection of bioreactor used to yield maximum biosorption process and developing mechanistic models for biosorption application (Park, Yun & Park 2010). With continued research progress and studies, better understanding of the biosorption mechanism will be achieved to develop the biosorption technology and will become more beneficial and attractive. Cost and expenses of isolating the microorganisms have to be considered given that large amounts of microorganism are needed for large-scale fermentation in order to supply for the industry.

4.1.8 Proteomics studies

4.1.8.1 The effects of heavy metals towards extracellular enzymes of fungal cells

Generally, heavy metals are inhibitors of enzymatic reactions. Heavy metals such as copper and cadmium are reported to not only bind to aromatic amino acid residues in enzyme molecules, but also induce oxidative stress related to the production of reactive oxygen, such as hydroxyl and or superoxide radicals, and eventually cause oxidative damage of proteins (Gardes & Bruns 1993). The enzymes which are released into the extracellular environment are often faced with high concentrations of heavy metals, due to the fact that they are not protected by the cell-associated metal detoxification mechanisms. Thus, the heavy metal ions which enter the cell can also influence the production of extracellular enzymes on the levels of transcriptional and translational regulation (Ye et al. 1997).

However, low concentrations of heavy metals are still needed for the development of ligninolytic enzyme systems. For example, the activity of lignin peroxidase and Mn-peroxidase of *Phanerochaete chrysoporium* was reported to be increased with the addition of Zn (0.006-18µM) and Cu (0.0004-1.2µM) into the metal-free synthetic cultivation medium (White et al. 1990). In addition, Cu, which is the cofactor of the enzyme laccase, was found to be responsible for lignin degradation. The addition of Cu helped to increase the production of all isoenzymes of laccase produced by the fungus *Pleurotus ostreatus* while Metal Responsive Elements (MRE) were found in the promoters of *Pleurotus ostreatus* laccase genespoxc and poxa1b. These MREs interact

with the Cu-responsive factors and the organization of the MREs site is said to be similar to metallothionein genes (Sannia et al. 2001).On the other hand, other heavy metals like Zn, Cd, Hg and Ag were not found to be enhancing the production of laccase (Embong et al. 2008). While it is said that addition of copper increases the activity of the produced extracellular enzyme, it is interesting to highlight that extracts of different age cultures exhibited different sensitivity to copper addition (Ye et al. 1997). Apart from that, the toxic effect of other nonessential metals such as Pb^{2+} and Ag^{+} is reported to inhibit Acryl alcohol oxidase in *Pleurotus Eryngii* (Bridge & Spooner 2001). Moreover, pyranose oxidase in *Trametes versicolor* is reported to be inhibited by Ag^{+}, Co^{2+}, Cu^{2+} and Ni^{2+}(Heinonsalo, Jørgensen & Sen 2001).

In addition, heavy metals also interfere with the carbon and energy supplying system of cellulases and hemicellulases. In the case of *Phanerochaete chrysosporium,* cellulase activity was inhibited in the presence of 50-150 ppm of Cd, Cu, Pb, Mn, Ni, and Co, while the cellulase activity was completely inhibited at a higher concentration at 150-300 ppm of Mn and 300 ppm of Co and Cd (Wirsel et al. 2001). Cadmium was found to increase the endocellulase activity significantly in the case of *Pleurotus ostreatus* on straw, while the β-glucosidase activity was strongly inhibited by Hg^{2+}, Fe^{3+} and Cu^{2+} in *Trametes gibbosa* (Viaud, Pasquier & Brygoo 2000). Furthermore, Cu, Pb, Hg and Sn were found to inhibit the enzyme β-glucosidase in the brown-rot fungi *Gloeophyllum sepiarium* and *Gleophyllum trabeum* (Coen 2009).

4.2 Objectives of the study

Hence, the aims of this study are to:

(a) determine the effect of pH on the adsorption of Cu (II), Pb (II), Cr (VI) and Zn (II) by immobilized living and dead fungal biomass

(b) study the expression levels of heavy-metal resistant fungal protein under the treatment of the heavy metals

4.3 MATERIAL AND METHODS

4.3.1 Biosorption preparation for fungal immobilization

The fungal culture (*Pestalotiopsis* sp.; Choo et. al, accepted for OSJ publication manuscript ID: OSJO-D-14-00054R1) was obtained from the stock culture maintained on Potato Dextrose slants at pH 5. The fungal culture was cut aseptically and transferred into sterile 100ml of Potato Dextrose Broth (PDB) in a 250ml conical flask and was incubated for 5 days at 25°C in an incubator. After incubation period, the fungal biomass with spherical clumps were harvested and filtered from the growth medium. The clump biomass was homogenized with a commercial blender to destroy cell aggregates (Arıca, Kaçar & Genç 2001a).

4.3.2 The immobilization of fungal biomass into calcium alginate beads

Based on a study by Arıca, Kaçar and Genç (2001a) with modification, 2g of alginate in 100ml sterilized distilled water was used to prepare 2% alginate solution and was mixed with 0.5g fungal biomass. A 5ml hypodermic syringe was used to dispense the alginate fungal biomass mixture into 3% $CaCl_2$. The mixture was stirred to prevent aggregation of the fungal calcium alginate beads. The mixture was kept for 2 hours at 4°C to form beads and the beads were then filtered and rinsed thoroughly with sterile distilled water and air dried. The filtered calcium alginate beads contained the immobilized live fungus. For the preparation of immobilized dead fungus, similar immobilization technique was used. The difference was that fungal biomass was autoclaved at 121°C for 15 minutes after filtration. The calcium alginate beads with immobilized live and dead fungus were stored in 5mM of $CaCl_2$ solution at 4°C until further use.

4.3.3 Determination of biosorption efficiency using immobilized fungal culture under different pH condition

Biosorption of Cu(II), Cr(VI), Pb(II), and Zn(II) ions on calcium alginates beads on both immobilized live and dead fungal biomass from aqueous solution was investigated in biosorption-equilibrium experiments. The effects of the medium pH of Cu(II), Cr(VI), Pb(II), and Zn(II) ions on the biosorption rate and capacity were studied. The effect of pH on the biosorption rate of the alginate beads and both immobilized fungal preparations was investigated in the pH range of 4-6 for Cr(VI) and Zn(II) and pH of 4

and 5 for Cu (II) and Pb(II) (adjusted with HCl or NaOH at the beginning of the experiment) at 25 °C. Cu(II), Cr(VI), Pb(II) and Zn(II) ions concentration in each solution was prepared in 1.0, 3.0 and 5.0 ppm with distilled water (10ml).

Based on several studies on the findings of the optimum pH condition, pH range of 4 – 6 seems to be a favourable pH condition for adsorption of lead ions (Jonglertjunya 2008; Martínez et al. 2006; Yan & Viraraghavan 2003; Yetis et al. 2000). Hence, the low pH range was selected in this study. However, the lead ions adsorption above pH 6 was not included as the lead acetate ion was observed to precipitate at pH 6. Lead carbonate that was used in this study could not be dissolved in water. Thus, acetic acid was used to react with the lead carbonate for making it soluble in water. This attributes to the fact that lead carbonate is insoluble in neutral water and will only dissolve in acids or alkaline water (Carr, Spangenberg & Chronley 2001). Hence, in order to dissolve lead carbonate in water for this study, acetic acid was used to dissolve lead carbonate to produce lead acetate. Acetic acid will dissolve lead carbonate by the following reaction to produce soluble lead acetate, water and carbon dioxide.

$$PbCO_3 + 2CH_3COOH \longrightarrow Pb(CH_3COO)_3 + H_20 + CO_2$$

4.3.4 Analytical procedure

Biosorption of Cu(II), Cr(VI), Pb(II) and Zn(II) ions from aqueous solutions were studied in batch systems and measured after incubation period of approximately 30 minutes. Biosorption process is a rapid process and it happens rapidly as 85% of the process is reported to take place in the first half an hour (Bayramoglu et al. 2002). Thus, the experiment is designed to measure the removal efficiency after 30 minutes of immobilized calcium alginate beads incubation with the metal ions.

The concentrations of the ions in these phases were measured using Atomic Absorption Spectrophotometer (AAS), Thermo Fisher Scientific iCE 3000 Series AA. The working wavelength for Cr(VI), Zn(II), Cu(II), and Pb(II) ions were 357.9, 213.9, 324.8 and 217.0 nm respectively. The instrument response was checked with 3.0ppm of ion solution standard for each heavy metal. For each set of data, standard statistical methods were used to determine the mean values and standard deviations. The removal

efficiency, R, of metal ions adsorbed per unit on both alginate entrapped live and dead fungal biomass was obtained by using the following expression (Hao & Hou 2013):

$$R = \frac{Co - Ct}{Ct} \times 100\%$$

Co = initial concentration (ppm)

Ct = concentration of metal ions (ppm) at time t (min)

4.3.5 Proteomics study

4.3.5.1 Fungal preparation and growth condition

Pestalotiopsis sp. mycelium which was isolated from *Nypa fruticans* sp. (Choo et. al., OSJ publication manuscript ID: OSJO-D-14-00054R1) was used in this study. Prior to protein extraction, *Pestalotiopsis* sp. was cultured and incubated in Potato Dextrose Agar for 3 days at room temperature. After 3 days, the fungus was inoculated into 100ml Potato Dextrose Broth in a 250ml Erlenmeyer flask for 4 weeks at room temperature. After the incubation, fungal biomass obtained was filtered using Whatman no 5 filter paper. The filtered fungal biomass was then kept in 50ml centrifuge tubes in 4°C and transported to Agro-Biotechnology Institute (ABI), Kuala Lumpur for proteomic studies via air transportation. Proteomic studies was conducted at ABI for approximately six weeks.

4.3.5.2 Protein extraction

The mycelia of *Pestalotiopsis* sp. were harvested by filtration and centrifuged at 3,000g for 15 minutes at 4 °C. After centrifugation, the broth was discarded and miliQ water was added. The steps were repeated twice to obtain the fungal biomass. The fungal biomass was frozen in liquid nitrogen to lyophilise the cells. The mycelia were grounded using a mortar and pestle in liquid nitrogen to obtain dry mycelia and kept at -80 °C.

The dried mycelia powder (1 g) was subjected to the TCA/acetone method of protein extraction, according to Sanger, Nicklen and Coulson (1977) with modification. 1g of mycelia powder was added with 1.8ml of cold TCA-2ME-acetone solution and was mixed and stored at -20 °C for 1 hour. Then, the mixture was centrifuged for 10 minutes at 10,000g at 4 °C. Once centrifuged, the supernatant was discarded. The pellet obtained was resuspended in cold 1.8ml rinsing solution (0.07 % 2ME (v/v) in cold acetone) and stored at -20 °Cfor 1 hour. After incubation, the resuspended pellet was centrifuged for 15 minutes at 10,000g and the supernatant was discarded twice. The pellet obtained was dried using SpeedVas for 30 minutes. The technical triplicates were pooled and the final pellet was then resuspended with lysis buffer (8M urea solution; 4% (w/v) CHAPS; 2% (v/v) IPG buffer; 40 mM DTT) and vortexed for 1 minute. After vortexed, the mixture was centrifuged for 15 minutes at 10,000g and the supernatant was collected twice. The protein content was quantified using the Bradford method with BSA as the standard maker Rizzo and Buck (2012).

4.3.5.3 SDS-PAGE Two-dimensional analysis

For 2-DE analysis, the nonlinear IPG strips (11 cm, pH 3 - 10; Bio-Rad) was rehydrated for 18 hours with 50 µg mycelium protein in 250µl of rehydration buffer (8M urea; 4% (w/v) CHAPS; 2% (v/v) IPG buffer; 40mM DTT; 0.002% of 1% bromophenol blue solution). The IEF strips were loaded onto the Bio-Rad Protean IEF Cell system, and the Isoelectric Focusing (IEF) was performed according to Ewing et al. (1998) with modifications: 500 V for 2 hours, 1,000 V for 1 h, 8,000 V for 1 h, and finally focused on 29,000 Vh at 8,000 V. The strips were run at 50 mA. Triplicates IEF strips were run for each heavy-metal resistant mycelia protein. The strips were then stored at -20 C prior to 2-D gel electrophoresis.

Prior to 2-D gel electrophoresis, the strips were thawed and alkylated according to Ewing and Green (1998) with SDS Equilibration 1 solution (50 mM of Tris-HCL pH 8.8; 6 M urea; 30% (v/v) of 37% glycerol; 2% (w/v) of SDS; 0.002% (w/v) of bromophenol blue; 50 mg DTT) and SDS Equilibration 2 solution (50mM of Tris-HCL pH 8.8; 6 M urea; 30% (v/v) of 37% glycerol; 2% (w/v) of SDS; 0.002% (w/v) of bromophenol blue; 125 mg IAA). The strips were alkylated for 15 minutes in each SDS equilibration solution.

For the 2-D gel electrophoresis, the SE 600 Ruby Standard Dual Cooled Vertical Unit system was used according to the Mini-PROTEAN Tetra Cell Instruction, Bio-Rad. The SDS-PAGE was performed using precast 12% resolving gel (30% acrylamide/bis solution; 1.5M Tris-HCL pH 8.8; 10% (w/v) SDS; 10% (w/v) APS; 0.05% TEMED and deionized water). The 10X electrode running buffer, pH 8.3 (0.25M Tris-Base; 1.92M glycine; 1% (w/v) SDS; deionized water) was prepared for the gel electrophoresis. Once the IEF strip was placed in the SE 600 Ruby system with the BSA protein marker, the strips were overlay and sealed with agarose sealing solution (0.5 g agarose; 1X running buffer; 200 µl bromophenol blue). The gel was allowed to polymerize for 1 hour. After polymerised, the gel was run for 15 minutes at 10 mA and 20 mA for 3 hours until the dye reached the bottom of the gel.

4.3.5.4 Gel staining

After 2-D gel electrophoresis, the gels were stained using the silver staining method (Kingsmore & Saunders 2011) with modification. The gel was stained in fixation solution (50% ethanol absolute; 2.5% acetic acid absolute in 50 ml deionized water) for 30 minutes. After fixation, the gel was sensitized in infiltrating solution (7.5% ethanol absolute; 3.4g sodium acetate; 0.1g sodium thiosulfate in 50ml deionized water). The gel was then washed with deionized water 3 times for 5 minutes each. After washing, silver solution (0.1% silver nitrate and 10 µl formaldehyde in 50 ml deionized water) was added to the gel for 20 minutes. After discarding the silver solution, the staining solution (1.25 g sodium carbonate and 5 µl formaldehyde in 50 ml deionized water) was added to the gel with gentle agitation until protein spots appeared. Protein spots appeared approximately after 10 minutes. After protein spots appeared on the gel, the gel was added with stop solution (0.73 g EDTA.$Na._2H_20$ in 50 ml deionized water) for 10 minutes. The gel was then washed with deionized water for 3 times for 5 minutes each and was kept in distilled water prior to image capturing of gel.

4.3.5.5 Image capture and analysis

The gel images were captured using the GS-800 Calibrated Densitometer. The images were then analysed using the PDQuest^{TM} software (Bio-Rad) by using 2-fold over background as criteria for detecting the presence or absence of the protein spots on the heavy-metal resistant protein and control gel. Manual alignment of the gel was conducted to increase the reliability of the matching protein spots which were present or

absent in all triplicates of the heavy-metal resistant protein gel against the control gel with Anova p-value of < 0.05.

4.3.5.6 Analysis by MALDI-ToF

Prior to MALDI-ToF analysis, the spots of interest were excised and placed it in a 1.5ml centrifuge tube. The gel pieces were washed with 150 µl of 100mM NH_4HCO_3 for 10 minutes. After removing the NH_4HCO_3 solution, destaining solution (0.045g of 15mM $K_3Fe(CN)_6$, 0.079 g of 50mM $Na_2S_2O_3$ in 10 ml water) was added and left for incubation for 15 minutes andthe step was repeated twice. The protein was reduced by adding 150 µl of 10mM DTT in 100mM NH_4HCO_3 and incubated for 30 minutes at 60°C. The protein was then alkylated with 55mM IAA in 100mM NH_4HCO_3 solution and incubated in the dark for 20 minutes. The gel pieces were washed with 50% ACN in 100mM NH_4HCO_3 solution for 20 minutes for 3 times. The solution was removed and was added with 50 µl of 100% ACN and incubated for 15 minutes at room temperature. The pieces of gel were dried with speed vacuum for 15 minutes. 25 µl of 7ng/µl of trypsin solution was added and incubated overnight at 25°C. After overnight incubation, the 25 µl of 50% ACN was added and the solution was incubated for 15 minutes. The solution was then collected into a new tube (tube A) and second extraction of protein was performed with 100% ACN in the tube containing the gel pieces and incubated for 15 minutes. The solution was then transferred into the new tube (tube A). The solution in the tube was dried using speed vacuum. The dried protein was kept at -80 °C and was reconstituted by adding in 5 µl of 1.0% formic acid in water prior to MALDI-ToF analysis. The protein identification was undertaken by Agro-Biotechnology Institute, Kuala Lumpur via MALDI-ToF as described in Li et al. (2013) with modifications. The generated data files were then sequenced with similarity searches using MASCOT NCBInr database (http://www.matrixscience.com) with default parameters based on peptide mass fingerprinting PMF and MS/MS spectra.

4.4 RESULTS

The concentrations of heavy metal ions (Cu(II), Cr(VI), Pb(II), and Zn(II)) were measured using AAS after an incubation period of 30 minutes with (a) immobilized live and (b) dead fungal *Pestalotiopsis* sp. biomass. The mean values and the standard deviations are presented in Tables 5 (Cr), 7 (Pb), and 9 (Cu). Removal efficiencies of live and dead fungal biomass are presented in Tables 6 (Cr), 8 (Pb), and 10 (Cu). For heavy metal ion Zn(II), the removal efficiency of the immobilized live and dead fungal biomass was found to be negative under all conditions (data not included).

4.4.1 Removal efficiency of Cr (VI) by immobilized live and dead fungal biomass

The mean values and standard deviation of Cr(VI) ion concentrations at pH 4, 5, and 6 after 30 minutes incubation with the immobilized live and dead *Pestalotiopsis* sp. biomass are presented in table 5. The mean values were in the range of 1.36 – 4 ppm after incubation; the lowest mean value of concentration was recorded at pH 5 for immobilized dead fungal biomass while the highest was recorded at pH 4 for live immobilized fungal biomass.

Table 5. Mean values and standard deviation of heavy metal Cr(VI) ion concentrations (ppm) at pH 4, 5 and 6 after incubation with calcium alginate immobilized live and dead fungal biomass. The concentration of the metal ion solution was analysed using AAS.

pH	Types of biomass	Mean values (ppm)	Standard deviation
4	Live	4.046	0.497
4	Dead	2.941	0.4901
5	Live	1.378	0.0391
5	Dead	1.364	0.008
6	Live	2.060	0.062
6	Dead	2.078	0.061

Based on table 6, immobilized live fungal biomass displayed the highest removal efficiency at pH 5 with 24.54% while the immobilized dead fungal biomass at the same pH removed 17.26% of the Cr(VI) ions in solution. Besides, the immobilized live fungal biomass showed positive removal efficiency of 13.56% at pH 6. On the other hand, immobilized dead fungal biomass was not able to remove any Cr(VI) ions at pH 4 and pH 6 (Table 6).

Table 6.The removal efficiency (%) of the immobilized live and dead fungal biomass towards the Cr(VI) ion at pH 4 and 5.

Heavy metal ion	pH	Type of biomass	Removal efficiency (%)
Chromium	4	Live	-38.57
	4	Dead	-14.05
	5	Live	24.54
	5	Dead	17.26
	6	Live	13.56
	6	Dead	-1.27

4.4.2 Removal efficiency of Pb (II) ions by immobilized live and dead fungal biomass

The mean values and standard deviation of Pb(II) ion concentration (ppm) at pH 4 and 5 was recorded in table 7 after incubation with immobilized live and dead fungal biomass. Based on the table, the lowest mean value was recorded at pH 5 for immobilized live biomass with 0.75 ppm, while the highest mean value was recorded at 2.76 ppm for immobilized live fungal biomass at pH 4.

Table 7. Mean values and standard deviation of heavy metal Pb(II) ion concentrations (ppm) at pH 4 and 5 after incubation with calcium alginate immobilized live and dead fungal biomass. The concentration of metal ion was analysed using AAS.

pH	Types of biomass	Mean values (ppm)	Standard deviation
4	Live	2.766	0.795
4	Dead	1.714	0.754
5	Live	0.751	0.893
5	Dead	2.429	0.278

The removal efficiency of the immobilized live and dead fungal biomass towards Pb(II) ions was recorded in table 8. Based on the table, both immobilized live and dead fungal biomass recorded positive removal efficiency under pH 4 and 5. The highest removal efficiency of Pb(II) ions was recorded by immobilized dead fungal biomass at pH 4 with 45.88 % removal. The pH parameter had a significant impact on the removal, with both dead and live biomass displaying higher removal efficiencies at pH 4 (Table 8).

Table 8. The removal efficiency (%) of the immobilized live and dead fungal biomass towards the Pb(II) ions at pH 4 and 5.

Heavy metal ion	pH	Type of biomass	Removal efficiency (%)
Lead	4	Live	12.96
	4	Dead	45.88
	5	Live	6.057
	5	Dead	17.96

4.4.3 Removal efficiency of Cu (II) ions by immobilized live and dead fungal biomass

The mean values and standard deviation of the Cu(II) concentration in ppm and the removal efficiency of the Cu(II) ions by the immobilized Ca-alginate live and dead *Pestalotiopsis* sp. biomass were recorded in table 10. For the biosorption of Cu(II) ions, the mean values were in the range of 2.89ppm to 3.82ppm (Table 9).

Table 9. Mean values and standard deviation of heavy metal Cu(II) ion concentrations at pH 4 and 5 after incubation with calcium alginate immobilized live and dead fungal biomass.

pH	Types of biomass	Mean values (ppm)	Standard deviation
4	Live	3.821	0.033
4	Dead	2.890	0.229
5	Live	3.450	0.141
5	Dead	3.632	0.408

The removal efficiency of the Cu(II) ions by the immobilized live and dead fungal biomass was recorded in table 10. The highest removal efficiency was reported by immobilized dead fungal biomass at pH 5, with a removal efficiency of 14.50%. A reduction of 4.22 % removal efficiency was reported by the immobilized live fungal biomass under the same pH condition. However, both the immobilized live and dead fungal biomass were not able to remove Cu(II) ions from the solution at pH 4 (Table 10).

Table 10. The removal efficiency (%) of the immobilized live and dead fungal biomass towards the Cu(II) ions at pH 4 and 5.

Heavy metal ion	pH	Type of biomass	Removal efficiency (%)
Copper	4	Live	-11.17
	4	Dead	-3.310

5	Live	10.28
5	Dead	14.50

4.4.4 Proteomics analysis

The representative 2-DE gel of proteins from *Pestalotiopsis* sp. with treatment of Cr(VI) (Figure 18); Pb(II) (Figure 19); Zn(II) (Figure 20); and Cu(II) (Figure 21) are shown below.

The presence and absence of 18 protein spots were numbered and detected on the Cr(VI) treated gel which were found to be up-regulated/down-regulated with max fold change of < 2, ANOVA p-value <0.02 against the control gel (Figure 18). For the 2-DE of proteins with treatment of Pb(II) resistant *Pestalotiopsis* sp., 16 protein spots were detected (Figure 19) with max fold of > 2.0, Anova p-value <0.05 against the control gel, and19 protein spots were detected (Figure 20) for the treatment of *Pestalotiopsis* sp. with Zn(II) resistance while 21 protein spots were detected for the treatment of Cu(II) resistant *Pestalotiopsis* sp. (Figure 21) with max fold > 2.0, Anova p-value <0.05.

Out of the 74 protein spots detected, 10 proteins spots were observed to show expression under the treatment of two or more heavy metals. The results are summarized in Table 11.

Table 11. The summarised of the various protein spots detected on the silver stained SDS-PAGE polyacrylamide gel at different treatment of heavy metals.

Protein	**Treatment of heavy metals**			
spot number	**Cr**	**Pb**	**Zn**	**Cu**
322		+	+	
359		+		+
651	+	+	+	
774		+	+	+
782	+	+	+	+

880	+	+	+	+
1038	+	+	+	+
1354		+		+
530		+	+	
696	+		+	

Based on table 11, two similar protein expression spots were detected in each treatment of Pb(II) and Zn(II) (#322 and #530); and Pb(II) and Cu(II) (#359 and #1354). Protein spot (#651) showed protein expression under the treatment of Cr(IV), Pb(II) and Zn(II) while protein spot (#774) showed protein expression under the treatment of Pb(II), Zn(II) and Cu(II). Moreover, there are 3 protein spots detected under the treatment of all the heavy metals (#782; #880; #1038). One protein spot (#696) was found to be expressed under the treatment of Cr(VI) and Cu(II).

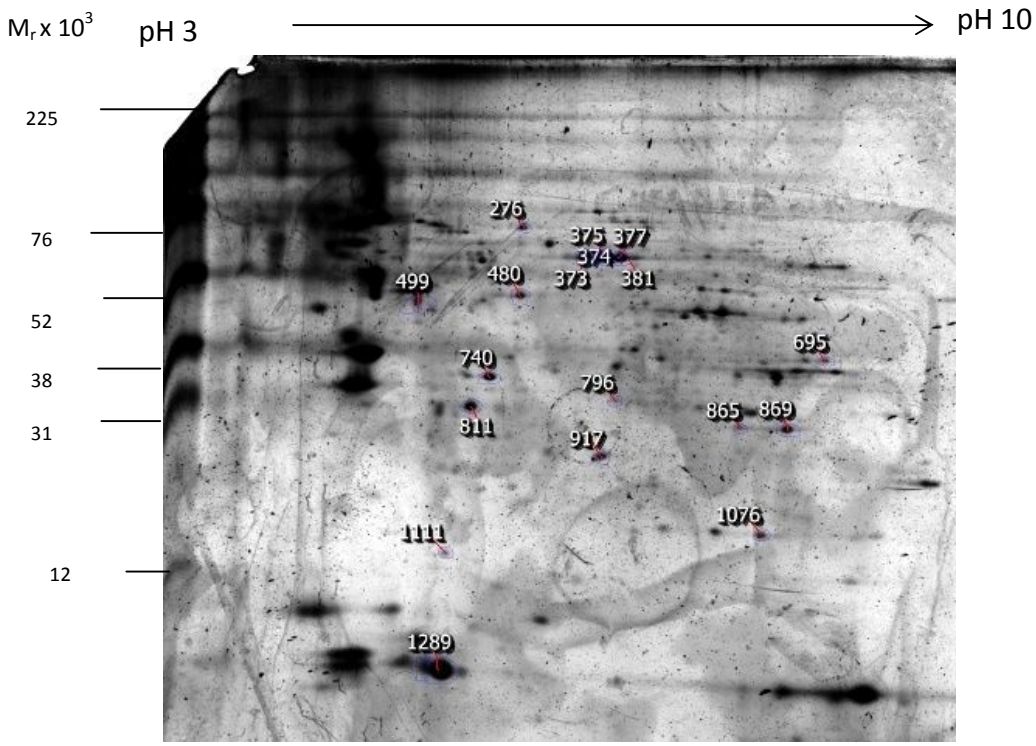


Figure 18.Representative of silver stained 2-DE gel of proteins from *Pestalotiopsis* sp. with treatment of Cr(VI). Extracted proteins (50 µg) proteins were loaded on a 13 cm non-linear IPG strip (pH 3 – 10) for IEF, following electrophoresis of 12 % SDS-PAGE. The different expression spots present at the position of arrows are compared with the control.

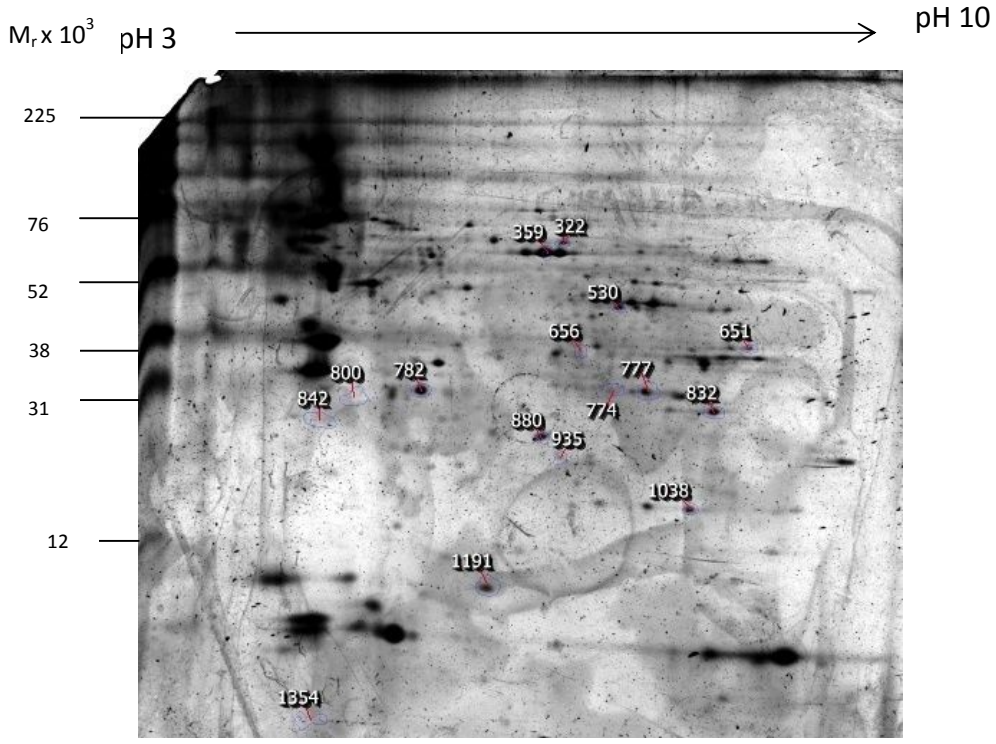


Figure 19.Representative of silver stained 2-DE gel of proteins from *Pestalotiopsis* sp. with treatment of Pb(II). Extracted proteins (50 µg) proteins were loaded on a 13 cm non-linear IPG strip (pH 3 – 10) for IEF, following electrophoresis of 12 % SDS-PAGE. The different expression spots present at the position of arrows are compared with the control.

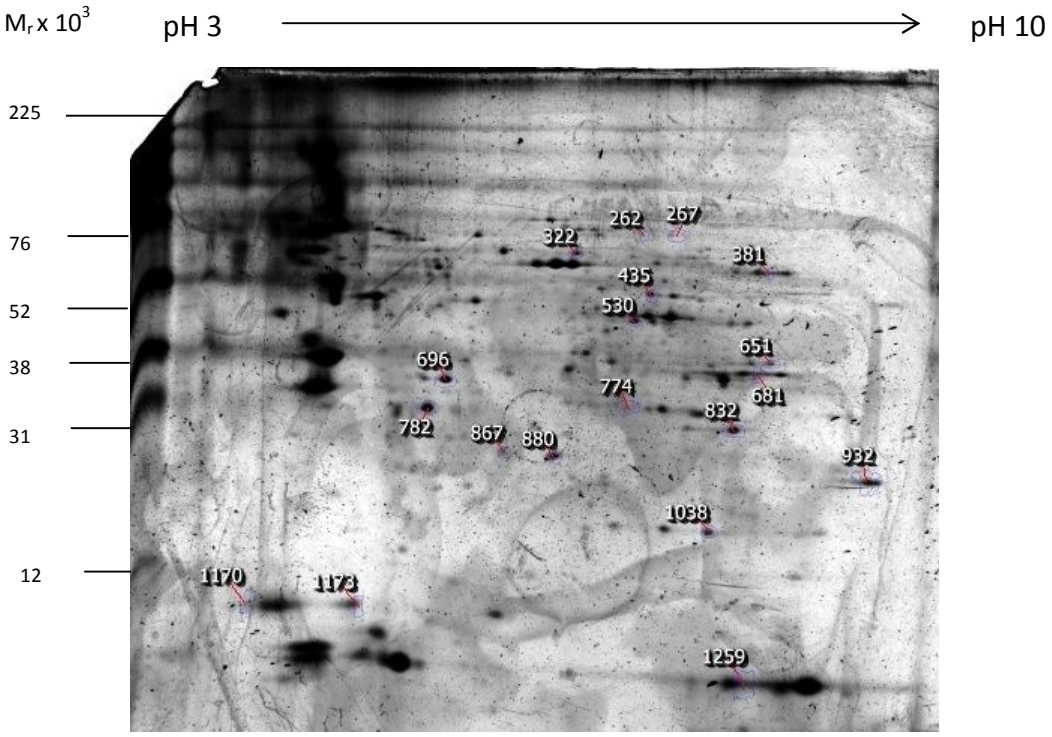


Figure 20.Representative of silver stained 2-DE gel of proteins from *Pestalotiopsis* sp. with treatment of Zn(II). Extracted proteins (50 µg) proteins were loaded on a 13 cm non-linear IPG strip (pH 3 – 10) for IEF, following electrophoresis of 12 % SDS-PAGE. The different expression spots present at the position of arrows are compared with the control.

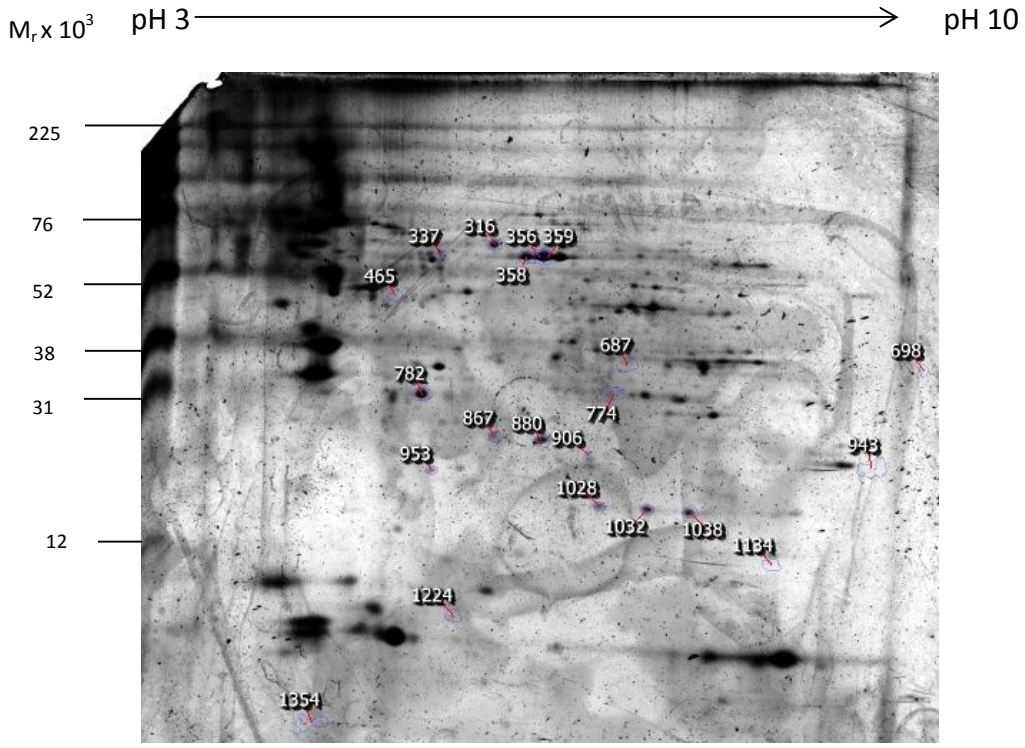


Figure 21. Representative of silver stained 2-DE gel of proteins from *Pestalotiopsis* sp. with treatment of Cu(II). Extracted proteins (50 µg) proteins were loaded on a 13 cm non-linear IPG strip (pH 3 – 10) for IEF, following electrophoresis of 12 % SDS-PAGE. The different expression spots present at the position of arrows are compared with the control.

4.4.5 Identification of protein of interest

The identification of the protein interest was done using MALDI-TOF (conducted at Agro-Biotechnology Institute, Kuala Lumpur). Out of the 10 protein spots in Table 11 that were presence under the treatment of two or more heavy metals, only two protein spots were successfully identified. Hence, altogether nine proteins of interest were successfully identified and the protein accession number and function are summarized in Table 12. The identification of protein is based on the UniProt database.

Table 12. The up-regulated and down-regulated protein spots in response to Pb(II), Zn(II), Cr(VI) and Cu(II).

Spot no./ID	Accession no.	Protein ID	Putative function	Protein molecular weight	Protein P.I.
807	MNMA_ERYLH	tRNA-specific 2-thiouridylase MnmA	catalyzes the 2-thiolation of uridine at the wobble position (U34) of tRNA, leading to the formation of s2U34	40878	5.1
800	MRP_RICFE	Protein mnp homolog	ATP binding	35441	7.8
651	TRPA_ACAM1	Tryptophan synthase alpha chain	the alpha subunit is responsible for the aldol cleavage of indoleglycerol phosphate to indole and glyceraldehyde 3-phosphate	28835	4.8
530	ISPH_ALCBS	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	converts 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate into isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP)	34523	5.1
1032	YHFE_BACSU	Putative aminopeptidaseYhfE	binds 2 divalent metal cations per subunit	49568	5.9
932	HEM1_METB6	Glutamyl-tRNA reductase	catalyzes the NADPH-dependent reduction of glutamyl-tRNA(Glu) to glutamate 1-semialdehyde (GSA)	46258	5.9

1173	SYV_AROAE	Valine--tRNA ligase	catalyzes the attachment of valine to tRNA(Val). As ValRS can inadvertently accommodate and process structurally similar amino acids such as threonine, to avoid such errors, it has a "posttransfer" editing activity that hydrolyzes mischarged Thr-tRNA(Val) in a tRNA-dependent manner	107751	5.5

4.5 Discussion

Several studies have reported the use of immobilized fungal biomass for biosorption of heavy metals as a promising alternative solution to remove heavy metals from aqueous solutions (Arıca et al. 2004; Arıca, Kaçar & Genç 2001a; Bayramoğlu, Bektaş & Arıca 2003). In this experiment, fungal biomass was immobilized using calcium alginate (3% sodium chloride, 2% calcium alginate) as it is believed that immobilization enables a more consistent and more rapid removal of metal ions (Bayramoğlu, Bektaş & Arıca 2003). Besides, impact of pH was investigated in this study as different pH will influence the ionization state of the functional groups found on the fungal cell wall (carboxylate, phosphate, and amino group) and the solubility of the metal ions (Deng, Ye & Wong 2004). Since only Cr(VI), Pb(II) and Cu(II) displayed positive removal, Zn(II) will not be discussed in the following.

4.5.1 Biosorption of Cr (VI) with immobilized Ca-alginate fungal biomass

Metal ion biosorption by immobilized fungal biomass was influenced by the types of biomass (alive or dead) and the pH (Table 3). The live immobilized Ca-alginate fungal biomass shows a slightly higher efficiency in removing Cr(VI) ions in this study (Table 6), which is supported by Doshi, Ray and Kothari (2007) who found that live biomass of *Spirulina* sp. showed a higher efficiency than the dead biomass in adsorbing the cadmium ions. This could be due to the fact that living cells could demonstrate more

variety of metal accumulation mechanisms such as extracellular complex formation and transport of metal ions (Mohapatra 2011).

Acidic conditions favour the binding of Cr(IV) towards fungal biomass, with the hydroxyl group found to be the chromium binding site within a pH range of 1 – 5 (Mohanty et al. 2006). Fittingly, in this experiment, the removal efficiency is the highest at pH 5 (24.54% removal efficiency for live biomass; Table 6). Similar results were also reported by Lesage et al. (2007) using *Aspergillus niger* at pH 5.0.

The fungal biomass contains chitin and chitosan, which contain COOH and $–NH_2$ groups that are responsible for the binding of metal ions (Seo, Yu & DeLaune 2008). At optimum sorption pH (acidic), the types of Cr(VI) in the solution are mainly chromic acid, dichromate ion, trichromate and tetrachromate ions. In acidic condition, the surface of the sorbent is protonated and causes a stronger attraction towards the negatively charged Cr(VI) ions. As the pH increases, the concentration of OH- increases and this eventually results in a negative charged surface of the sorbent, in turn limiting the adsorption of the negatively charged Cr(VI) ions.

Several studies have shown that biosorption was higher at even lower pH conditions (Bai & Abraham 2001; Ozdemir et al. 2004; Park et al. 2005), however, our results did not confirm this trend for *Pestalotiopsis* sp. immobilized in Ca-alginate beads. Instead, neither dead nor alive immobilized biomass was able to remove Cr at pH 4.0 (Table 6). This finding could be attributed to the chemical change of *Pestalotiopsis* sp. due to the hydrolytic activity at higher acid concentrations as reported by Tewari, Vasudevan and Guha (2005) in the use of *Mucor hiemalis*. Furthermore several studies found that different fungal biomass used will exhibit different optimum Cr(VI) removal efficiencies although all experiments were carried under acidic conditions. As reported by Congeevaram et al. (2007) and Zhang et al. (2010b), the removal of the Cr(VI) ions only showed rapid performance at pH 5, the same trend as the *Pestalotiopsis* sp. used in this experiment. Different adsorbents used will exhibit different adsorption efficiencies and it is important to study and determine the optimum pH for the various adsorbents towards the removal of Cr(VI) ions.

Besides the electrostatic binding of ions, a study undertaken by Park et al. (2005) has reported that the main mechanism of Cr(VI) removal was a redox reaction between Cr(VI) and the dead fungal biomass. It is known that Cr(VI) bound on the biomass can

be eluted in the form of Cr(III) through reduction under very strong acidic conditions (Kratochvil, Pimentel & Volesky 1998). Thus, Park et al. (2005) suggested two mechanisms which could have been involved in the adsorption of the Cr(VI) ions. The Cr(VI) ions could have been reduced to Cr(III) ions by contact with the electron donor (biomass) in the solution and the second mechanism may involve the following 3 steps:

(1) Cr(VI) ions bind to the chitin and chitosan present in the fungal cell wall,
(2) Cr(VI) ion is reduced to Cr(III) ion by functional groups that have lower reduction value
(3) the release of Cr(III) into the solution by electronic repulsion between the positively charged groups and the ion.

Based on these results, it could be that the Cr(VI) ions in the solution might have also been reduced to Cr(III) and further studies should be carried out in the future to determine their valency state.

In a future study, the amount of biomass can also be optimised as other studies have reported optimum biomass loading of 8% (Spohr et al. 1998). However, it should also be highlighted that the adsorption of Cr (VI) will likely decrease with an increase of biomass due to the fact that the surface area of the bead is expected to be reduced (Vanhoutte et al. 1995).

4.5.2 Biosorption of Pb (II) ions using immobilized Ca-alginate fungal biomass

Based on the results shown in table 8, both dead and live biomass were able to remove Pb(II) at pH 4 and 5. The finding in this experiment correlates well with other studies which reported an optimum pH range of 4 – 6 for adsorption of Pb(II) ions (Jonglertjunya 2008; Martínez et al. 2006; Yan & Viraraghavan 2003; Yetis et al. 2000). A reduction of removal efficiency beyond pH 6 has been reported due to a decrease in solubility and precipitation of the metal ions (Kwok & Higuchi 1989). Hence, the study of pH 6 influence towards the adsorption of Pb(II) in this study was removed as the Pb(II) ions also showed precipitation at pH 6. On the other hand, there are several studies that report on a higher pH (alkaline conditions) enhancing the

removal of lead ions by using *Moringa oleifera* pods and *Aspergillus niger* strains (Zayed, Gowthaman & Terry 1998; Zhu et al. 1999). This clearly suggests again that different types of biomass and fungal strains have a different affinity towards Pb(II) ions and in this study, *Pestalotiopsis* sp. favours low pH conditions for adsorption of Pb(II) ions.

Our results also showed that dead fungal biomass has a higher removal efficiency compared to live biomass at both pH 4 and 5 (Table 8). Although the difference was moderate (P=0.5), the finding does show a noticeable effect in the use of immobilized fungal biomass compared to free biomass. Our results are supported by DeBusk, Peterson and Reddy (1995) who observed a similar trend in their study. The increase of adsorption for metal ions by dead biomass may be due to the fact that dead fungal biomass has a higher surface area when the cell is ruptured. Besides, dead biomass is not influenced as much by external factors such as the difference of pH as compared to live biomass (Modak & Natarajan 1995). In addition, among the possible mechanisms of metal binding towards the fungal biomass, is the intracellular complexation with proteins such as metallothioneins and phytochelatins (Cenis 1992).

The lower uptake capacity by free fungal biomass could be attributed to the fact that the fungal hyphal biomass aggregates in the form of pellets and thus reduced its exposed surface area for adsorption, eventually limiting the metal ions accessibility to the binding sites (Iqbal & Edyvean 2004). Hence, the immobilization of biomass clearly brings an enhancement to the adsorption of heavy metal ions in aqueous solution and should be recommended and widely used in order to enhance in the biosorption process.

4.5.3. Biosorption of Cu(II) ions using immobilized Ca-alginate fungal biomass

The immobilized Ca-alginate *Pestalotiopsis* sp. fungal biomass was reported to show positive removal efficiency of the metal Cu(II) ions at pH 5, while negative removal efficiency was demonstrated pH 4 condition. A comparatively similar trend was reported by Bayramoğlu, Bektaş and Arıca (2003) in which the immobilized *Trametes versicolor* favours the acidic pH condition in the removal of Cu(II) ions. The negative removal efficiency reported can possibly be further studied by investigating other

suitable materials of immobilization such as the carboxymethylcellulose (CMC) bead and polysulfone matrix which may further enhance the biosorption ability.

4.5.4 Proteomic analysis

Based on Table 11, it is observed that 10 protein spots were found to be presence under the treatment of two or more heavy metals. This may suggest that similar proteins could play a role in the defence mechanism under the different heavy metal stress response.

Based on the UniProt database, the protein spot no. 867 - detected under the treatment of Zn(II)- was found to be tRNA-specific 2-thiouridylase MnmA. It functions as a catalyst and is responsible in catalyzing the 2-thiolation or uridine at wobble position of tRNA, and leads to the formation of 2-thiouridine (s2U34), a modification of the modified nucleoside 5-methyalamino-methyl-2-thiouridine (Ikeuchi et al. 2006; Kambampati & Lauhon 2003). The protein was previously identified in *Erythrobacter litoralis* (strain HTCC2594). According to Kambampati & Lauhon (2003), the s^2 group of the s^2U34 protein stabilizes the anticodon structure and hence confers the ribosome binding ability to tRNA and improves the reading frame maintenance. In the present work, the tRNA-specific 2-thiouridylase was found to be down-regulated with 3-fold against the control by using the PDQuest^{TM} analysis software. The down-regulation of the protein in *Pestalotiopsis* sp. in response to zinc stress suggests that the ribosomal binding regulation to tRNA is affected which in turn affects the translational process. In the present study, tRNA-specific 2-thiouridylase MnmA was detected as a newly expressed protein upon zinc exposure and thus provides a new evidence for its role in heavy metal stress response.

Besides, the protein spot no. 800 was identified as protein MRP homolog, which is also among the newly induced proteins detected under the treatment of Pb(II). This protein was previously identified in *Rickettsia felis* (strain ATCC VR-1525 / URRWXCal2). The protein MRP homolog was found to be one of the multidrug resistance proteins (Borst et al. 1999). In this study, the protein MRP homolog was observed to be up-regulated at a 3.6 fold against the control treatment. The multidrug resistance-associated protein 1 (MRP1) is reported to be among the members of the ATP-binding cassette (ABC) transporter family that is involved in the efflux processes (Keppler & Konig 1997). This suggests that the protein MRP homolog acts as a resistance mechanism of the fungi towards the heavy metal by pumping extra metals out of the cells. Other

studies have reported the same mechanism in which the role of the MRP1 in transfection experiments of the cDNA encoding human MRP1 into human tumour cells have shown that the efflux pump ability does not only mediate outwardly transport of anticancer drugs, but may also react with genotoxic heavy metals like antimony and arsenite (Cole et al. 1994). Besides, the overexpression of the efflux pump MRP1 was also demonstrated in GLC4/Sb30 cells at both the mRNA and protein level (Vernhet et al. 1999). Thus, our finding that the MRP is up-regulated in the presence of lead exposure highlights its essential role in responding to metal toxicity. Multidrug resistance proteins could possibly be used in genetically modified microbes to confer higher resistance towards heavy metal and to enhance bioremediation.

In addition, the protein tryptophan synthase alpha chain (protein spot no. 651) was identified under the treatment of Pb(II) and Zn(II). The protein trytophan synthase alpha chain was observed to be down-regulated at a 2-fold against the control treatment in the treatment of Pb(II) as well as the Zn(II) treatment. This protein is involved in the aldol cleavage of indoleglycerol phosphate to indole and glyceraldehyde 3-phosphate. Indole is known to act as an intracellular signal in microorganisms. The role of indole includes spore formation, plasmid stability, drug resistance, biofilm formation and virulence (Lee and Lee (2010) while glyceraldehyde 3-phosphate is an intermediate in various central metabolic pathways and produced in the pentose phosphate pathway (Cronín, Nolan & Voorheis 1989). This clearly shows that the presence of Pb(II) and Zn(II) inhibit the metabolic pathways in the case of *Pestalotiopsis* sp. and also interfere with the function of indole. The tryptophan synthase alpha chain was found to be the newly-expressed protein under the influence of Pb (II) and Zn (II) ions in *Pestalotiopsis* sp. This effect may not be that harmful though. A study by Hsiao et al. (2008) demonstrated that increased tryptophan (Trp) levels lead to the *Arabidopsis thaliana* plant becoming less accessible to cadmium due to a decreased cadmium transport and subsequent reduction in the accumulation of cadmium. Taken together, the present study shows the down-regulation of the tryptophan synthase alpha chain under the treatment of heavy metal Pb(II) and Zn(II) indicates that the protein is affected in response to heavy metal stress in the fungal communities, but, with the overexpression of the tryptophan protein, it may facilitate in high tolerance towards heavy metal and hence provide new horizons in metabolic engineering for Trp mechanisms towards heavy metal response.

The protein spot no. 530 was identified as 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (LytB) (Table 14) and was found to be down-regulated at a 2.2 fold and 3.2 fold in the treatment of Pb (II) and Zn (II), respectively. 4-hydroxy-3-methylbut-2-enyl diphosphate reductase is the last enzyme for isoprenoid biosynthesis which catalyzes the conversion of 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate into isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) in the methylerythritol 4-phosphate pathway (MEP) (Wolff et al. 2003). The protein is absent in mammalian systems and the pathway was genetically validated in pathogenic organisms such as the *Plasmodium falciparum* and *Mycobacterium tuberculosis* (Hale et al. 2012). Due to its absence in the mammalian pathway, the enzymes found in the MEP pathway provide novel targets for the development of antimicrobial chemotherapeutics and herbicides. Besides, the enzyme was also identified as one of the components in penicillin tolerance (Gustafson, Kaul & Ishiguro 1993). The present study identifies LytB as a newly-expressed protein found upon Pb(II) and Zn(II) exposure. Hence, the down-regulation of the protein implies that the enzyme is metal-sensitive and gives a new line of evidence that the biosynthesis of isoprenoid can be affected due to the sensitivity of the enzyme in response to heavy metal stress.

The enzyme glutamyl-tRNA reductase which was found in the treatment of Zn(II) in this study was found to be protein no. 932. The enzyme glutamyl-tRNA reductase (GluTR reductase) was reported to be down-regulated at a 3-fold and the enzyme is responsible to catalyze the NADPH-dependent reduction of glutamyl-tRNA(Glu) to glutamate 1-semialdehyde (GSA), the key regulatory enzyme of tetrapyrrole biosynthesis pathway which was previously identified in *Arabidopsis thaliana*(Czarnecki et al. 2011). A similar trend has been observed in a study by Moser et al. (1999) who reported that the presence of heavy metal compounds such as $PbCl_2$, $PtCl_4$, $KdPCl_4$, and Zn(II) inhibit the GluTR reductase enzyme in *Methanopyrus kandleri*. It is interesting to note that the enzyme is generally found in plant, algae and most bacteria involved in the C_5 pathway of ALA synthesis. This may give an indication that the gene from the plant may have incorporated into the endophytic fungi and this gives rise to another interaction between plant and endophytes that can be further studied.

The putative aminopeptidase encoded by the YhfE gene which was previously identified in *Bacillus subtilis* (strain 168) was found to be protein no. 1032. The

expression of this enzyme was found to be down-regulated at a 3.4 fold in the treatment of Cu(II) in this study. Based on the UniProt database, the role of this enzyme is to bind 2 divalent metal cations per subunit and it is found that this enzyme is a newly-expressed protein under the treatment of Cu(II) metal. However, the study on the aminopeptidase YhfE seems to be very limited and hence the data found from this study may contribute to a new line of evidence indicating its role or effect in the presence of Cu(II) in fungi. The protein spot no. 1173 which was found in the treatment of Zn(II) is identified as Valine-tRNA ligase enzyme and is previously identified in *Aromatoleum aromaticum* (strain EbN1). The valine-tRNA ligase enzyme was found to be up-regulated at a 2-fold against the control treatment. The enzyme acts as a catalyst as it catalyzes the valine attachment to tRNA(Val)and tRNA(Val) is involved in the reaction of L-Valine and ATP in the production of AMP, diphosphate and L-valyl-tRNA (val). To date, there is near to noreport about the involvement of the Valine-tRNA ligase enzyme in response to the presence of heavy metals. It seems that the enzyme may be contributing to the defence mechanism against Zn(II) in *Pestalotiopsis* sp. due to the observed high up-regulation level.

4.6 Conclusion

Both immobilized live and dead *Pestalotiopsis* sp. fungal biomass showed ability in sequestering and removing heavy metal ions from solution. The live immobilized fungal biomass shows higher efficiency in removing the Cr(VI) metal ions, as the living cells may exhibit a wider variety of metal accumulation through transport and extracellular complex formation in and on the cells. However, there is only statistically significant difference between the immobilized live and dead fungal biomass for the removal of Pb(II). The influence of pH in the biosorption process has shown that low pH (acidic) condition favours the removal of metal ions by the fungi *Pestalotiopsis* sp. However, it is interesting to note that different types of biomass and fungal strains may exhibit different affinity towards the heavy metal ions and hence pH study is crucial in the biosorption processes in order to determine the optimum condition for metal removal. Besides, the calcium alginate immobilization technique has successfully provided a more uniform manner for fungal biomass to adsorb and remove metal ions and thus should be widely used to enhance the biosorption processes. The aim to investigate and

study the intracellular mechanisms involved in the biosorption process of *Pestalotiopsis* sp. has provided several new evidences in regards to the newly-expressed proteins found in response to the heavy metal stress condition in this study. The down-regulation and up-regulation of the identified proteins have given a better understanding of the biosorptive and defence mechanisms involved.

Chapter 5

Summary and Future Work

This study has successfully (i) isolated endophytic fungi from Nipah palm from wetlands in Sarawak, (ii) screened for the most heavy-metal resistant endophytic fungi as potential biosorbent, (iii) determined the effect of pH on the adsorption of Cu(II), Cr(VI), Pb(II) and Zn(II), and (iv) studied the protein expression levels under the treatment of heavy metals.

The present study reports for the first time that the endophytic fungus *Pestalotiopsis* sp. can potentially be used as a biosorbent to remove heavy metal pollution from aqueous solutions. Numerous studies have reported the investigation of filamentous fungal strains as potential heavy metal removal agents but near to none has reported the application of endophytic fungi. The findings not only give new knowledge of one of the many capabilities of endophytic fungi, but also offer a new insight into the interaction between plant and fungi that is yet to be fully understood. Apart from that, the present study has explored the uniqueness and diversity of the wetlands environment and its ecosystem which has successfully led to the discovery of endophytic fungi with unique metabolic strategies. Our findings clearly show that endophytic fungi isolated from the wetlands are capable of resisting high heavy metal concentrations.

Furthermore, it is noteworthy to highlight the importance of studying the influence of pH in the biosorption processes. This is due to the fact that low or high pH condition can lead to changes in the chemical structure of the fungal cell due to the functional groups, which play an integral role in binding the heavy metal ions. In the case of *Pestalotiopsis* sp., it favours the low pH (acidic) condition in removing the heavy metal ions and this could be due to the fact that the chitin and phosphate groups found on the fungal cell wall harbour negative charges and thus make the cell wall a potent ion-

exchange resin for heavy metal adsorption. Besides, the application of immobilization technique in encapsulating the fungal biomass has demonstrated a more consistent and rapid removal of metal ions. Thus, immobilization techniques should be widely used to enhance the removal of metal ions.

The use of live and dead *Pestalotiopsis* sp. fungal biomass in the investigation of biosorption process in the present study has contributed to a new knowledge in understanding the biosorptive mechanism, particularly the intracellular process of the cell. The study has shown that both live and dead fungal biomasses have the ability in sequestering metal ions. However, both live and dead biomasses have their own advantages and disadvantages. It is known that the use of live biomass can provide a wider variety of mechanisms involving with the transport of metal ions into the cells and the extracellular complex formation with the metal ions. On the other hand, the use of dead biomass is capable of eliminating external factors such as the nutrients and the culture maintenance needed and also the toxicity problem towards the cells. Coupled with the proteomic analysis of the *Pestalotiopsis* sp. fungus in response to heavy metal stress, it was found that newly-expressed proteins have been identified and thus provide a new understanding of the biosorptive mechanisms involved. It is noteworthy that the multi-drug resistance protein (MRP homolog) has been identified and seems to play a key role in the defence mechanism of *Pestalotiopsis* sp. towards the heavy metals.

5.1 Future work

Continual research and investigation is crucial to develop the biosorption process as an attractive tool to be applied in the industrial scale in hope to treat and remove heavy metals from polluted wastewater. On the laboratory scale, a more comprehensive study on the culturing parameters such as pH, contact time, and temperature influencing the biosorption process is needed in order to determine an optimum condition. This will improve our understanding on the condition needed for the fungal biomass in order to achieve maximum removal efficiency of the heavy metal ions.

Apart from that, different types of immobilization materials can be explored to further determine its optimum removal efficiency as it is known that there are numerous types of materials that can be used such as the polyacrylamide, glass bead and formaldehyde immobilization techniques. Besides, the attractive feature of a biomass not only depends on its biosorptive capacity, but also the ease to regenerate and reuse in addition of

suitable chemicals. Hence, the fungal biomass can also be assessed for its ability to desorb and adsorb the heavy metal ions in hope to reuse the biomass which is highly important for industrial application.

Moreover, in order to have a better picture of the biosorption mechanisms in response to the heavy metals, more proteins can be identified through proteomic studies to have a complete mapping of the pathway involved. This can provide a useful insight in understanding the role of each protein involved in the fungi which in turn provides a better understanding of the intracellular processes of the heavy metal adsorption that is yet to be fully understood.

Another aspect to look into is the industrial application. Unlike laboratory solutions, industrial effluents contain numerous types of pollutants to be removed. Thus, it is necessary to study the simultaneous removal of many co-existing pollutants. Different types of biomass can be studied in order to develop a potential 'combo' biosorbent which can remove the various pollutants found in the wastewaters (Modak & Natarajan 1995). Thus, ongoing research on the biosorption study is needed to further develop its application in hope to treat heavy metal pollution.

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