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Biosorption and proteomic analysis of an encapsulated endophytic heavy-metal resistant *Pestalotiopsis* sp.

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ABSTRACT

Aims: A study on biosorption ability using encapsulated endophytic fungi has been carried out to investigate its biosorption potential in removing heavy metals. Biosorption has emerged as an alternative bioremediation process to remove and sequester heavy metal ions from polluted water. An endophytic *Pestalotiopsis* sp. (isolated from *Nypa fruticans*) was found to be able to resist copper (Cu), chromium (Cr), lead (Pb) and zinc (Zn) up to 1,000 ppm and thus the aim of this study was to investigate the biosorption ability using encapsulated live and dead *Pestalotiopsis* sp. biomass (at pH 4-6) to remove heavy metals. Additionally, a proteomic study was conducted to investigate down- and up-regulation expression levels of proteins under the treatment of the heavy metals.
Methodology and results: Encapsulated live fungal biomass displayed higher efficiency in removing chromium at pH 5 and 6, while both encapsulated live and dead biomass were able to remove lead at pH 4 and 5 and copper at pH 5. Five (5) proteins of interest were identified via MALDI-ToF analysis. Among the proteins identified, multidrug resistance protein (MRP homolog) was up-regulated in the presence of lead.
Conclusion, significance and impact of study: The data obtained in this study provides an initial understanding of the biosorptive and defensive mechanisms of *Pestalotiopsis* sp. under heavy metal stress.

Keywords: Biosorption, heavy-metal resistant, proteomic, encapsulation, live and dead biomass

INTRODUCTION

In order to treat heavy metal pollution in aqueous solution, biosorption has been highlighted as an attractive alternative bioremediation process to solve this environmental issue. It acts by binding and sequestering metals through the use of biological biomass for the removal of metal ions from dilute aqueous solution (Skowronski *et al*., 2001). 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, and requires no additional nutrient with the possibility of metal recovery (Arıca *et al*., 2001; Kratochvil and Volesky, 1998).

Several types of biomass can be used and serve as potential biosorbents for the adsorption of heavy metal ions. Wang and Chen (2009) discussed three (3) main groups of potential biosorbents: bacteria (Gram positive and Gram negative), fungi (filamentous fungi and yeast) and algae. Microbes isolated from polluted environment have been studied as potential biosorbents (Iram *et al*., 2012) and it has been reported that those isolated microbes (*Sargassum natans*, *Bacillus subtilis*, *Aspergillus niger* and *Saccharomyces cerevisiae*) have successfully adapted and survived at the contaminated environment and were thus able to resist pollutants such as heavy metals. Several 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 leave and orange and banana peel (Tee and Khan, 1988; Cimino *et al*., 2000; Annadurai *et al*., 2002; Chuah *et al*., 2005; Al-Qodah, 2006; Hashem, 2006; Farooq *et al*., 2010). Farooq *et al*. (2010) also highlighted the use of wheat-based biosorbent such as straw and bran from wheat. However, the use of endophytic fungi as potential biosorbents has not been

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widely studied. Hence, in this present study, we aim to explore the potential of identified heavy-metal resistant endophytic fungus *Pestalotiopsis* sp. on the biosorption approach by undertaking investigation against various pH condition with encapsulated live and dead fungal biomass to explore and understand its biosorptive abilities. *Pestalotiopsis* sp. isolated from *Nypa fruticans* from wetlands of Sarawak has been identified to be able to resist heavy metal ions (Cu, Cr, Pb and Zn) up to 1000 ppm (Choo *et al*., 2015) which motivated our study to assess biosorptive abilities of this endophytic fungus. Members of the *Pestalotiopsis* have also shown potential to be used as biofuel feedstock (Sia *et al*., 2017) and been able to degrade polyurethane polymers (Bong *et al*., 2017).

Firstly, encapsulation of biomass can further enhance the biosorption process as free cells are not suitable in columns for providing high efficiency in removing metal ions (Veglio and Beolchini, 1997; Vijayaraghavan and Yun, 2008). This is due to the cells' low density and size which tend to plug the bed and result in large drops in pressure. Encapsulation of biomass has also been utilized in conventional reactor technology like the large packed and fluidized bed reactor systems (Gadd & White, 1993). Through encapsulation, biomass is converted into spherical shape and thus enabled to be used like the conventional adsorbents. There are several encapsulation techniques available for biomass including alginate, polyacrylamide, polyvinyl alcohol, polysulfone, silica gel, cellulose and glutaraldehyde (Park *et al.*, 2010) and the present study aims to encapsulate heavy-metal resistant *Pestalotiopsis* sp. (Choo *et al*., 2015) and further investigate the efficiency of dead and live encapsulated *Pestalotiopsis* sp. biomass on its biosorption abilities.

Secondly, biosorption process is mainly affected by pH, initial metal concentration, temperature, ionic strength, biomass concentration and size, and agitation speed (Park *et al*., 2010). pH is considered to be the most important parameter in the biosorption process as 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 (Park *et al*., 2010). As an example, several studies suggest that a pH of 4-6 is significantly more favorable for adsorption of lead ions (Yetis *et al*., 2000; Yan and Viraraghavan, 2003; Martínez *et al*., 2006; Jonglertjunya, 2008). 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 *et al*., 1992; Veglio and Beolchini, 1997). Therefore, in this study, we focus on investigating the potential effect of different pH conditions towards the biosorption efficiency of the encapsulated *Pestalotiopsis* sp.

Thirdly, several studies have shown that heavy metals affect the intracellular activities of cells (Ye *et al*., 1997). A study by Gardes and Bruns (1993) showed that heavy metals such as copper and cadmium do 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. 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 (Ye *et al*., 1997). Hence to further understand the biosorptive mechanism, biosorption experiments were coupled in this study with proteomic analysis to provide insights into the intracellular mechanisms of the heavy-metal resistant *Pestalotiopsis* sp.

MATERIALS AND METHODS

Encapsulation of fungal biomass into calcium alginate beads

The fungal culture *Pestalotiopsis* sp. (Choo *et al*., 2015) was obtained from the stock culture maintained on Potato Dextrose slants at pH 5.6. The fungal culture was cut aseptically and transferred onto sterile 100 mL of Potato Dextrose Broth (PDB) in a 250 mL conical flask and incubated for 5 days at 25 °C. 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 *et al*., 2001). Based on a study by Arıca *et al.* with modification, 2 g of alginate in 100 mL sterilized distilled water was used to prepare 2% alginate solution and mixed with 0.5 g live fungal biomass. A 5 mL 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 h at 4 °C to form beads and the beads were then filtered and rinsed thoroughly with sterile distilled water and air dried. For the preparation of encapsulated dead fungus, the fungal biomass was filtered and autoclaved at 121 °C for 15 min before being entrapped. The calcium alginate beads with encapsulated live and dead fungus were stored in 5mM of $CaCl_2$ solution at 4 °C until further use.

Determination of biosorption efficiency using encapsulated fungal culture under different pH condition

Biosorption of Cu, Cr, Pb, and Zn ions by encapsulated live and dead fungal biomass in calcium alginate beads from aqueous solution was investigated in biosorption-equilibrium experiments. The effects of the medium pH of Cu, Cr, Pb, and Zn ions on the biosorption rate and capacity were studied. The effect of pH on the biosorption rate of the encapsulated fungal beads was investigated in the pH range of 4-6 for Cr and Zn; pH of 4 and 5 for Cu and Pb (adjusted with HCl or NaOH at the beginning of the experiment) at 25 °C. Cu, Cr, Pb and Zn ions concentration in each solution was prepared in 1.0, 3.0 and 5.0 ppm with distilled water (10 mL).

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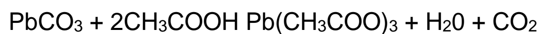
Based on several studies on the findings of the optimum pH condition, pH range of 4-6 seems to be a favorable pH condition for adsorption of lead ions (Yetis *et al*., 2000; Yan and Viraraghavan, 2003; Martínez *et al*., 2006; Jonglertjunya, 2008). Hence, the low pH range was selected in this study. 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 *et al*., 1995). 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.



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

Biosorption of Cu, Cr, Pb and Zn ions in aqueous solutions were studied in batch systems and measured after incubation period of approximately 30 min. 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 (Bayramoğlu *et al*., 2002). Thus, the experiment was designed to measure the removal efficiency after 30 min 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, Zn, Cu, and Pb ions were 357.9, 213.9, 324.8 and 217.0 nm respectively (Kojuncu *et al*., 2004). The instrument response was checked with 3.0 ppm of ion solution standard for each heavy metal. For each triplicates 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 and Hou, 2013):

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

Where,
Co = initial concentration (ppm)
Ct = concentration of metal ions (ppm) at time t (min)

Proteomics study

Fungal preparation and growth condition

Prior to protein extraction, heavy-metal resistant *Pestalotiopsis* sp. was cultured and incubated in Potato Dextrose Agar supplemented with 1000 ppm of Cr, Pb, Cu, and Zn respectively, as well as preparation of control samples for 3 days at room temperature. After 3 days, the fungus was inoculated into 100 mL Potato Dextrose Broth in a 250 mL 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 50 mL centrifuge tubes for subsequent proteomics studies.

Protein extraction

The mycelia of *Pestalotiopsis* sp. were harvested by filtration and centrifuged at 3,000*g* for 15 min 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, according to Sanger *et al*., (1977) with modification. 1 g of mycelia powder was added with 1.8 mL of cold TCA-2ME-acetone solution and was mixed and stored at -20 °C for 1 h. Then, the mixture was centrifuged for 10 min at 10,000g at 4 °C. Once centrifuged, the supernatant was discarded. The pellet obtained was resuspended in cold 1.8 mL rinsing solution (0.07% 2ME (v/v) in cold acetone) and stored at-20 °C for 1 h. After incubation, the resuspended pellet was centrifuged for 15 min at 10,000*g* and the supernatant was discarded twice. The pellet obtained was dried using SpeedVas for 30 min. The technical triplicates were pooled and the final pellet was then resuspended with the lysis buffer (8M urea solution; 4% (w/v) CHAPS; 2% (v/v) IPG buffer; 40 mM DTT) and vortexed for 1 min. After vortex, the mixture was centrifuged for 15 min at 10,000*g* 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).

SDS-PAGE two-dimensional analysis

For 2-DE analysis, the nonlinear IPG strips (11 cm, pH 3 - 10; Bio-Rad) were rehydrated for 18 hours with 50 µg mycelium protein in 250 µL of rehydration buffer (8 M urea; 4% (w/v) CHAPS; 2% (v/v) IPG buffer; 40 mM 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 following standard protocols based on Biored Handbook (IEF Protocol) with modifications: 500 V for 2 h, 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. Before running 2-D gel electrophoresis, the strips were thawed and alkylated (based on GE Healthcare 2D SDS PAGE Handbook) 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 (50 mM of Tris-HCL pH 8.8; 6 M

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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 min 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; 1.5 M 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; 1× running buffer; 200 µL bromophenol blue). The gel was allowed to polymerize for 1 h. After polymerised, the gel was run for 15 min at 10 mA and 20 mA for 3 h until the dye reached the bottom of the gel.

After 2-D gel electrophoresis, the gels were stained using the silver staining method (Kingsmore and 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 min. After fixation, the gel was sensitized in infiltrating solution (7.5% ethanol absolute; 3.4 g sodium acetate; 0.1 g sodium thiosulfate in 50 mL deionized water). The gel was then washed with deionized water 3 times for 5 min 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 min. 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 min. 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 min. The gel was then washed with deionized water for 3 times for 5 min each and was kept in distilled water prior to image capturing of gel.

The gel images were captured using the GS-800 Calibrated Densitometer. The images were 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.

Analysis by MALDI-ToF

Prior to MALDI-ToF analysis, the spots of interest were excised and placed it in a 1.5 mL centrifuge tube. The gel pieces were washed with 150 µL of 100 mM NH_4HCO_3 for 10 min. After removing the NH_4HCO_3 solution, destaining solution (0.045 g of 15mM $K_3Fe(CN)_6$, 0.079 g of 50 mM $Na_2S_2O_3$ in 10 mL water) was added and left for incubation for 15 min. The step was repeated twice. The protein was reduced by adding 150 µL of 10mM DTT in 100 mM NH_4HCO_3 and incubated for 30 min at 60 °C. The protein was then alkylated with 55mM IAA in 100 mM NH_4HCO_3 solution and incubated in the dark for 20 min. The gel pieces were washed with 50% ACN in 100 mM NH_4HCO_3 solution for 20 min for 3 times. The solution was removed and was added with 50 µL of 100% ACN and incubated for 15 min at room temperature. The pieces of gel were dried with speed vacuum for 15 min. 25 µL of 7 ng/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 min. 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 min. 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 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.

RESULTS AND DISCUSSION

The concentrations of heavy metal ions (Cu, Cr, Pb, and Zn) were measured using AAS after an incubation period of 30 min with (a) encapsulated live and (b) dead fungal *Pestalotiopsis* sp. biomass. Removal efficiencies of Cr, Pb and Cu ions by live and dead fungal biomass are presented in Table 1. Since only Cr, Pb and Cu displayed positive removal, biosorption of Zn will not be discussed in the following.

Biosorption of chromium ions with encapsulated Ca-alginate fungal biomass

Based on Table 1, encapsulated live *Pestalotiopsis* sp. biomass displayed the highest removal efficiency of Cr ions at pH 5 with 24.54% while the encapsulated dead fungal biomass at the similar pH removed 17.26% of the Cr ions in solution. Furthermore, a positive removal efficiency of 13.56% was reported by encapsulated live fungal biomass at pH 6, however the encapsulated dead fungal biomass demonstrated negative removal efficiency. On the other hand, encapsulated dead and live fungal biomass was not able to remove any Cr ions at pH 4. In this study, the live encapsulated Ca-alginate fungal biomass showed a slightly higher efficiency in removing Cr ions (Table 1) and this observation is supported by Doshi *et al.*, (2007) who found that live biomass of *Spirulina* sp. showed a higher efficiency than the dead biomass in adsorbing the Cr ions. This could be due to the fact that living cells could demonstrate more variety of metal accumulation mechanisms such as extracellular

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Table 1: The removal efficiency (%) of the encapsulated live and dead fungal biomass towards chromium ion at pH 4-6; lead and copper ions at pH 4 and 5.

Heavy metal ion	pH	Type of biomass	Removal efficiency (%)
Chromium	4	Live	NA
	4	Dead	NA
	5	Live	24.54
	5	Dead	17.26
	6	Live	13.56
	6	Dead	-1.27
Lead	4	Live	12.96
	4	Dead	45.88
	5	Live	6.057
	5	Dead	17.96
Copper	4	Live	NA
	4	Dead	NA
	5	Live	10.28
	5	Dead	14.50

complex formation and transport of metal ions (Mohapatra, 2011). Acidic conditions favour the binding of Chromium towards fungal biomass, with the hdroxyl group found to be the Chromium binding site within a pH range of 1-5 (Mohanty *et al.*, 2006).

A paired t-test was performed on the triplicate samples of encapsulated *Pestalotiopsis* sp. biomass tested at pH 5 which shows the highest Cr ions removal efficiency in this study. The statistical analysis reported that the effect of pH 5 showed significant impact on Cr ions removal by dead *Pestalotiopsis* sp. biomass ($t_{(2)}$ = 24.515, p <0.05) as shown in Table 2. This suggests that pH 5 provide a suitable medium for the removal of Cr ions by encapsulated dead *Pestalotiopsis* sp.

Fittingly, 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 *et al.*, 2008). At optimum sorption pH (acidic), the types of Cr 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 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 ions. Several studies have shown that biosorption was higher at even lower pH conditions (Bai and Abraham, 2001; Ozdemir *et al.*, 2004; Park *et al.*, 2005), however, our results did not confirm this trend for *Pestalotiopsis* sp. encapsulated in Ca-alginate beads. Instead, neither dead nor alive encapsulated biomass was able to remove Cr at pH 4.0 (Table 1). 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 *et al.*, (2005) in the use of *Mucor hiemalis.* As reported by Congeevaram *et al.* (2007) and Zhang *et al.* (2010), the removal of the Cr ions only showed rapid performance at pH 5, the same trend observed 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 ions. Besides the electrostatic binding of ions, a study undertaken by Park *et al.* (2005) has reported that the main mechanism of Cr removal was a redox reaction between Cr and the dead fungal biomass. This may explain the observed non-removal efficiency recorded in this study.

Biosorption of lead ions using encapsulated Ca-alginate fungal biomass

For the case of Pb ions removal, both encapsulated live and dead *Pestalotiopsis* sp. biomass recorded positive removal efficiency of Pb ions under pH 4 and 5. A paired t-test was carried out to study the impact of pH parameter had on Pb ions removal and a statistically significant impact of pH 5 on encapsulated dead *Pestalotiopsis* sp. was reported (t(2) = 12.865, p < 0.05 in this study (Table 2). The finding in this experiment correlates well with other studies which reported an optimum pH range of 4-6 for adsorption of Pb ions (Yetis *et al.*, 2000; Yan and Viraraghavan, 2003; Martínez *et al.*, 2006; Jonglertjunya, 2008). However, a reduction of removal efficiency beyond pH 6 has been reported due to a decrease in solubility and precipitation of the metal ions (Kwok and Higuchi, 1989). Hence, the study of pH 6 influence towards the adsorption of Pb in this study was removed as the Pb 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 *A. niger* strains (Zayed *et al.*, 1998; Zhu *et al.*, 1999). This clearly suggests that different types of biomass and fungal strains have a different affinity towards Pb ions and in this study, *Pestalotiopsis* sp. favours low pH conditions for adsorption of Pb ions. As mentioned, our results also showed that dead fungal biomass has a significantly higher removal efficiency (Table 2) and our results are supported by DeBusk *et al.*, (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 and 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 phytocheelatins (Cenis, 1992).

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Table 2: Paired t-test statistical analysis on the removal efficiency of chromium, lead, and zinc ions by live and dead *Pestalotiopsis* sp. biomass under pH condition 4-6.

Biosorption of heavy metals		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Cr_ph4_A_pre - Cr_ph4_A_post	-1.12247	.55183	.31860	-2.49329	.24836	-3.523	2	.072
Pair 2	Cr_ph4_D_pre - Cr_ph4_D_post	-1.14357	.17733	.10238	-1.58409	-.70304	-11.169	2	.008
Pair 3	Cr_ph5_A_pre - Cr_ph5_A_post	.44805	.05947	.04205	-.08625	.98235	10.655	1	.060
Pair 4	Cr_ph5_D_pre - Cr_ph5_D_post	.28460	.02011	.01161	.23465	.33455	24.515	2	.002
Pair 5	Cr_ph6_A_pre - Cr_ph6_A_post	.32247	.32259	.18625	-.47889	1.12382	1.731	2	.226
Pair 6	Cr_ph6_D_pre - Cr_ph6_D_post	-.02600	.06091	.03517	-.17731	.12531	-.739	2	.537
Pair 7	Pb_ph4_A_pre - Pb_ph4_A_post	.41170	1.50030	.86620	-3.31525	4.13865	.475	2	.681
Pair 8	Pb_ph4_D_pre - Pb_ph4_D_post	1.45287	.61530	.35524	-.07561	2.98135	4.090	2	.055
Pair 9	Pb_ph5_A_pre - Pb_ph5_A_post	2.00716	3.11547	1.79871	-5.73208	9.74640	1.116	2	.381
Pair 10	Pb_ph5_D_pre - Pb_ph5_D_post	.53177	.07159	.04133	.35392	.70961	12.865	2	.006
Pair 11	Cu_ph4_A_pre - Cu_ph4_A_post	-.28985	.08620	.06095	-1.06429	.48459	-4.756	1	.132
Pair 12	Cu_ph4_D_pre - Cu_ph4_D_post	-.02195	.26778	.18935	-2.42787	2.38397	-.116	1	.927
Pair 13	Cu_ph5_A_pre - Cu_ph5_A_post	.51050	.02206	.01560	.31228	.70872	32.724	1	.019
Pair 14	Cu_ph5_D_pre - Cu_ph5_D_post	.61993	.20012	.11554	.12281	1.11706	5.366	2	.033

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Biosorption of copper ions using encapsulated Ca-alginate fungal biomass

Based on the findings on Cu ions removal, the highest removal efficiency was reported by encapsulated dead *Pestalotiopsis* sp. biomass at pH 5, with a removal efficiency of 14.50%. A reduction of 4.22% removal efficiency was reported by the encapsulated live fungal biomass under the same pH condition. However, both the encapsulated live and dead fungal biomass were not able to remove Cu ions from the solution at pH 4 (Table 1). According to the paired t-test analysis, pH 5 showed a statistically significant impact on the removal of Cu ions by both encapsulated live *Pestalotiopsis* sp. ($t(1)$ = 32.724, $p < 0.05$) and dead biomass ($t(2)$ = 5.316, $p < 0.05$) as shown in Table 2. This suggests that low pH 5 condition enables *Pestalotiopsis* sp. of Cu ions efficiently, similarly as reported by Bayramoğlu, Bektaş and Arıca (2003) in which the encapsulated *Trametes versicolor* favours the acidic pH condition in the removal of Cu ions. However, the observed non-removal efficiency at pH 4 can possibly be further studied by investigating other suitable materials of encapsulation such as the carboxymethylcellulose (CMC) bead and polysulfone matrix which may further enhance the biosorption ability.

Efficiency removal of Cr, Pb, and Cu between live and dead fungal biomass at different pH levels

It is noted to highlight that there is a statistically significant difference among the efficiency removal of heavy metal ions (a) Cr (b) Pb and (c) Cu when treated with different pH levels by encapsulated live and dead *Pestalotiopsis* sp. biomass as determined by one-way ANOVA and encapsulated *Pestalotiopsis* sp. shows a significant removal efficiency of Cr ions in this study ($F(3,7)$ = 5.969, p = 0.024) as reported in Table 3. A Tukey post hoc test showed that the efficiency to remove Cr ions was statistically higher at level pH 5 by encapsulated dead *Pestalotiopsis* sp. biomass as compared to level pH 4 condition (0.2800 ± 0.2, p = 0.044) in this study.

Proteomic analysis

The identification of protein interest was done using MALDI-TOF (conducted at Agro-Biotechnology Institute, Kuala Lumpur). The five identified proteins of interest are summarized in Table 4. The identification of proteins is based on the UniProt database and the representative 2-DE gel of proteins from *Pestalotiopsis* sp. with treatment of Cr, Pb, Zn, and Cu are shown in Figure 1-4 respectively.

Based on the proteins of interest identified (Table 4), it is noteworthy to highlight that protein MRP homolog was among the newly induced proteins detected under the treatment of Pb in the case of *Pestalotiopsis* sp. 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. This

Table 3: ANOVA statistical analysis on the chromium, lead and copper metal ions removal efficiency between live and dead *Pestalotiopsis* sp. under pH condition 4 -6.

		Sum of Squares	df	Mean Square	F	Sig.
Cr	Between Groups	1.731	3	.577	5.969	.024
	Within Groups	.677	7	.097		
	Total	2.407	10			
Pb	Between Groups	2.526	3	.842	2.344	.149
	Within Groups	2.873	8	.359		
	Total	5.399	11			
Cu	Between Groups	.278	3	.093	5.438	.050
	Within Groups	.085	5	.017		
	Total	.363	8			

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, the finding of up-regulated MRP 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. Besides, the down-regulation of protein tryptophan synthase alpha chain under the treatment of both Pb and Zn could suggest that the metabolic pathways in the case of *Pestalotiops* sp. was inhibited. This is due to the fact that protein trytophan synthase alpha chain was observed to be down-regulated at a 2-fold against the control treatment in the treatment of Pb as well as the Zn treatment. This protein is involved in the aldol cleavage of indoleglycerol phosphate to indole and glyceraldehyde 3-phosphate. 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 *et al*., 1989). 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

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of cadmium. Taken together, the present study showing the down-regulation of the tryptophan synthase alpha chain under the treatment of heavy metal Pb and Zn 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. In addition, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (LytB) was found to be down-regulated at a 2.2 fold and 3.2 fold in the treatment of Pb and Zn, 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).

Table 4: The up-regulated and down-regulated protein spots of *Pestalotiopsis* sp. in response to the heavy metal treatment.

Spot no./ID	Accession no.	Protein ID	Putative function	Protein molecular weight	Protein P.I.
800	MRP_RICFE	Protein mnp homolog	ATP binding	35441	7.8
651	TRPA_ACAM1	Tryptophan synthase alpha chain	alpha subunit responsible for 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
932	HEM1_METB6	Glutamyl-tRNA reductase	catalyzes NADPH-dependent reduction of glutamyl-tRNA(Glu) to glutamate 1-semialdehyde (GSA)	46258	5.9
1173	SYV_AROAE	Valine--tRNA ligase	catalyzes 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

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 *et al*., 1993). The present study identifies LytB as a newly-expressed protein found upon Pb and Zn 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.

Another noteworthy finding to highlight is the detection of enzyme glutamyl-tRNA reductase during the treatment with Zn. The enzyme glutamyl-tRNA reductase (GluTR reductase) was reported to be down-regulated at a 3-fold and 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 C5 pathway of ALA synthesis (Schubert *et al*., 2002). This may give an indication that the gene from the plant may have incorporated into the endophytic fungi and this give rise to another interaction between plant and endophytes that can be further studied. Another protein detected is the Valine-tRNA ligase enzyme. The valine-tRNA ligase enzyme was found to be up regulated at a 2-fold against the control

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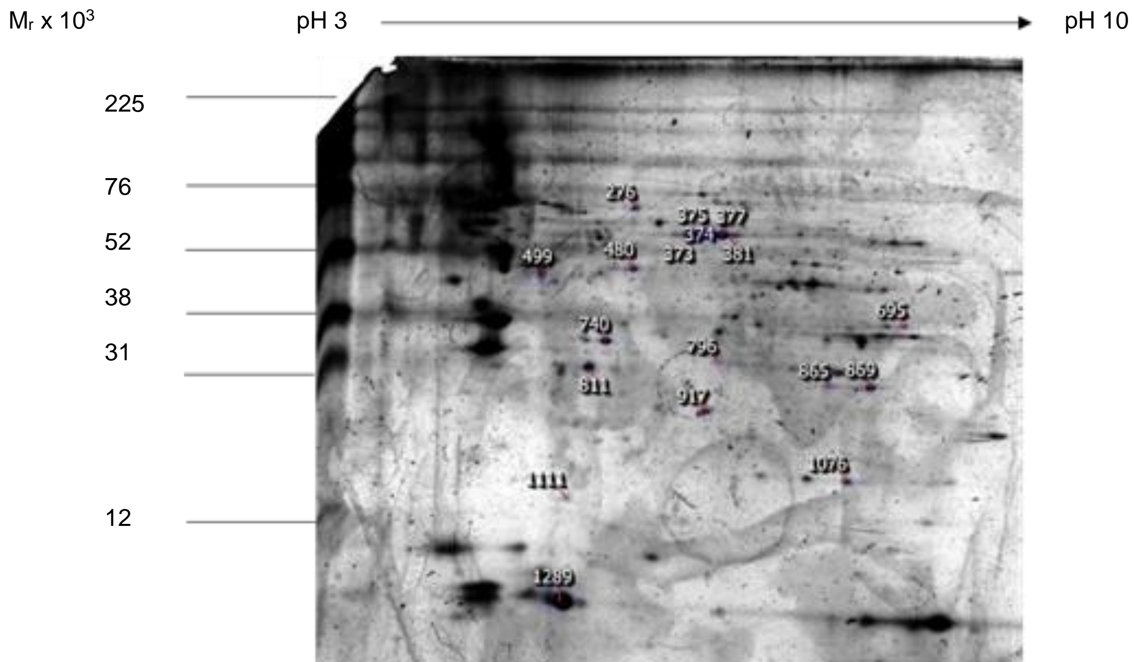

Figure 1: Representative of silver stained 2-DE gel of proteins from *Pestalotiopsis* sp. with treatment of Cr metal ion. Extracted proteins (50 µg) proteins were loaded on a 13 cm non-linear IPG strip (pH 3-10) for IEF, following electrophoresis of 12 % SDS-PAGE. The different expression spots present at the position of arrows are compared with the control.

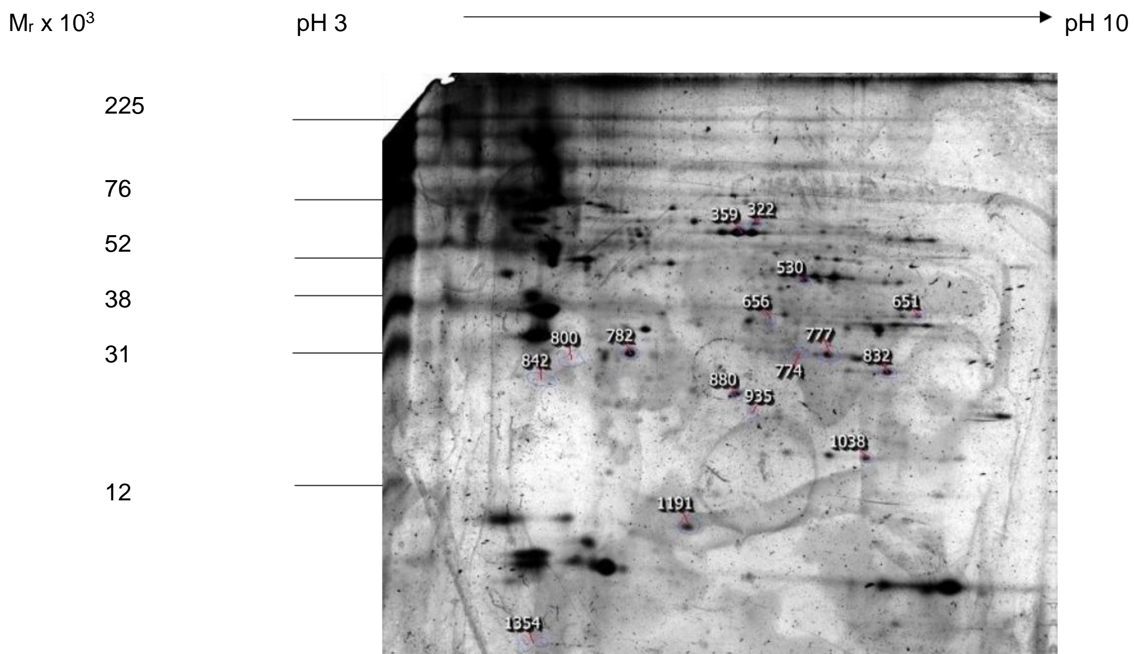

Figure 2: Representative of silver stained 2-DE gel of proteins from *Pestalotiopsis* sp. with treatment of Pb metal ion. Extracted proteins (50 µg) proteins were loaded on a 13 cm non-linear IPG strip (pH 3-10) for IEF, following electrophoresis of 12 % SDS-PAGE. The different expression spots present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

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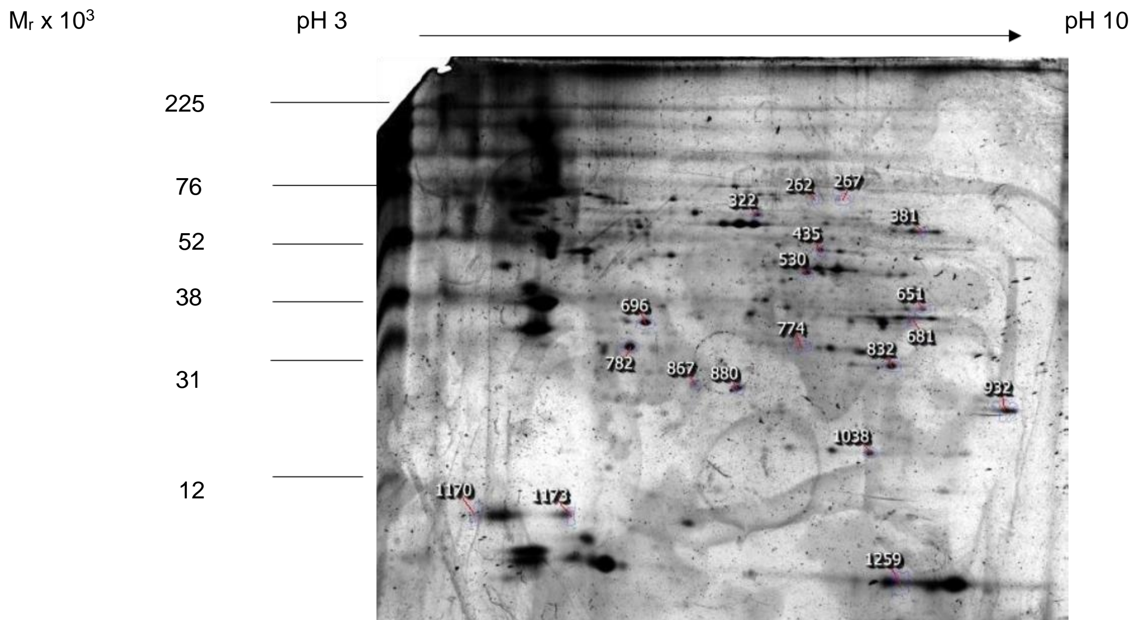

Figure 3: Representative of silver stained 2-DE gel of proteins from *Pestalotiopsis* sp. with treatment of Zn metal ion. Extracted proteins (50 µg) proteins were loaded on a 13 cm non-linear IPG strip (pH 3-10) for IEF, following electrophoresis of 12 % SDS-PAGE. The different expression spots present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

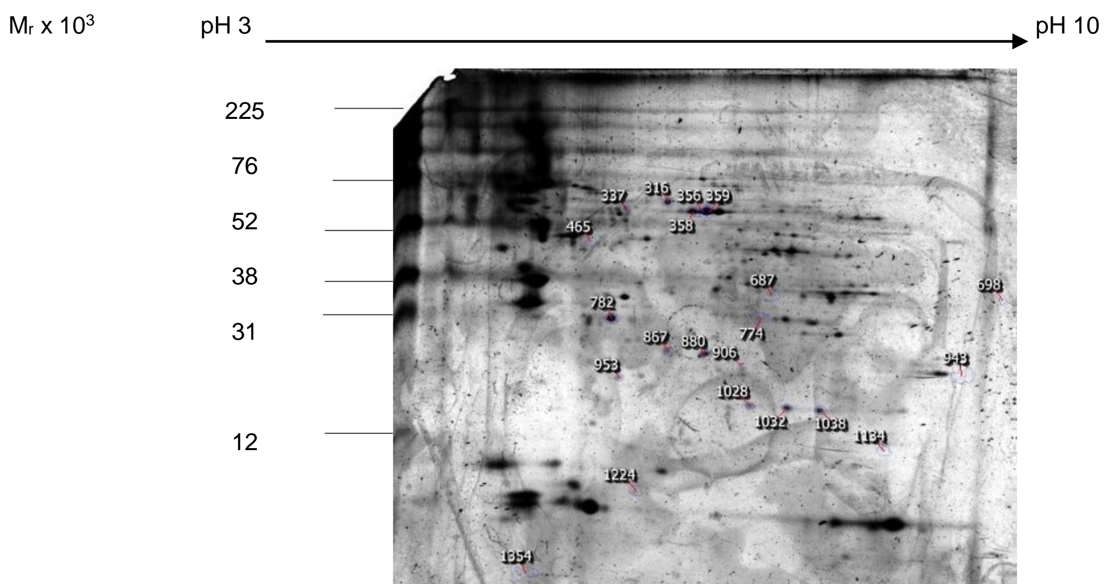

Figure 4: Representative of silver stained 2-DE gel of proteins from *Pestalotiopsis* sp. with treatment of Cu metal ion. Extracted proteins (50 µg) proteins were loaded on a 13 cm non-linear IPG strip (pH 3-10) for IEF, following electrophoresis of 12 % SDS-PAGE. The different expression spots present at the position of arrows are compared with the control.

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response to the presence of heavy metals. It seems that the enzyme may be contributing to the defense mechanism against Zn in *Pestalotiopsis* sp. due to the observed high up-regulation level.

CONCLUSION

Both encapsulated live and dead *Pestalotiopsis* sp. fungal biomass showed ability in sequestering and removing heavy metal ions from solution. The dead encapsulated *Pestalotiopsis* sp. fungal biomass shows significant higher efficiency in removing Cr metal ion as compared to removal of Pb, Cu and Zn ions at pH 5 and this may suggest that active metabolism such as the efflux mechanism of the cell may be shut off in dead cells which may promote higher affinity of metal accumulation within the cell membrane or cell body. The influence of pH in the biosorption process has shown that low pH (acidic) conditions favour the removal of metal ions by the heavy-metal resistant *Pestalotiopsis* sp. 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. This study also reports the down-regulation and up-regulation of selected proteins which provided an initial insight into the mechanisms involved in the biosorption and defense of heavy metal.

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