Factors affecting toxic lead(II) ion bioremediation by *Fusarium equiseti* isolated from the mangrove soil environment of southeast Borneo

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ABSTRACT

Aims: Electronic waste (e-waste) is an inorganic pollutant which causes a serious environmental problem since it contains toxic heavy metals, which cannot be removed from contaminated sites easily. The use of biomaterials for removing heavy metals from contaminated soil and wastewater has emerged as a potential alternative method to the conventional techniques. The present study were aimed to isolate efficient lead tolerant fungi from mangrove soil environment and measure its capability for lead removal from aqueous solution.
Methodology and results: Lead tolerant fungal strains were isolated from soil samples using PDA (Potato Dextrose Agar) supplemented with varied concentrations of lead ions (100-500 mg/L). The most tolerant fungal strain was successfully isolated and identified molecularly as *Fusarium equiseti* KR706303. The isolated fungus was used for biosorption studies using Potato dextrose broth (PDB) supplemented with lead ions. The effects of pH, temperature, initial metal concentration, biomass dose and age, agitation and contact time to the Pb(II) removal efficiency were monitored in the study. The results showed that the optimal parameters for the removal of lead ions such as heavy metal concentration and pH were 300 mg/L, with a maximum Pb(II) adsorption of 97.9% observed at pH 4 and temperature of 30 °C during the batch biosorption experiments. The optimal parameters for biomass dose, agitation speed, contact time and biomass age were observed at 0.04 g, 150 rpm, 60 min and fifth day; respectively.
Conclusion, significance and impact of study: The observation in this study revealed that the biomass of the isolated *Fusarium equiseti KR706303* has the potential to be used as a biosorbent for heavy metal particularly Pb(II) removal from the contaminated sites. The technology is simple, efficient, cost effective and environmental friendly.

Keywords: fungi, e-waste, *Fusarium equiseti*, heavy metal, lead(II) removal.

INTRODUCTION

The electronic waste, or e-waste poses a global concern because it is one of the fastest growing solid waste streams in the world, growing at the rate of 3-5% per annum; approximately three times faster than any other individual waste streams. E-waste is made up of numerous substances including lead, cadmium, mercury, hexavalent chromium, and brominated flame retardants that are major threats to human health and the environment. (Dursun *et al*., 2003)

Although some of the e-wastes are recycled, the rest gets disposed into landfills such as agriculture fields and lakes (Ramasamy *et al*., 2011) leading to soil and water pollution. One of the most important environmental issues is the presence of heavy metal contamination in aqueous streams, arising from the discharge of metal-containing effluents into water bodies (Hawari and Mulligan, 2006; Ramasamy *et al*., 2011). According to Liu *et al.* (2008),

lead is non-biodegradable and concerns for ectotoxicity is increasing. World Health Organization (WHO) in 2007 reported lead as one of the more common air pollutants. Lead reaches the human body through drinking of affected water, inhaling polluted air particularly from automobiles, peeling of paints and through food chain via cereals, vegetables, fishes and meat.

The biosorption of heavy metals using fungal strains has been widely and intensively studied by researchers world-wide (Al-Kadeeb, 2007; Azila *et al*., 2008; Al-Fakih, 2011; Jaya *et al*., 2013). Fungi have been reported as an efficient economic source for the removal of toxic heavy metals from aqueous solution, because the fungal cell wall has different functional groups which are involved in metal binding, and that the fungi can be easily isolated from environment for metal biosorption purposes (Wang and Chen 2006; Iskandar *et al*., 2011). Fungal isolates

from *Aspergillus* and *Penicillium* genera were reported to be the most tolerant to lead and selenium and exhibited strong growth profiles (Jaya *et al.*, 2013). Al-Kadeeb, (2007) reported on the removal of lead and copper by *Fusarium poae* and others reporterd on the use of *Aspergillus niger* B-77 for the removal of Pb, Cd and Cu from aqueous solution (Kapoor *et al.*, 1999). Therefore, this study were aimed to isolate efficient lead tolerant fungi from mangrove soil environment and to measure its biomass capability for lead removal from aqueous solution.

MATERIALS AND METHODS

Sampling and isolation of fungal strains

Soil samples were collected from mangrove soil environment at different locations in Asajaya region in the southeast Borneo, Sarawak, Malaysia and stored at -20 °C. Lead solutions were prepared using $Pb(CH_3COO)_2 \cdot 3H_2O$ pellets (Merck, Germany). The pH of the working solution was adjusted to pH 5.0 using hydrochloric acid (3M HCl). Fresh dilutions were used for each biosorption experiments. Lead tolerant fungal strains were isolated from the soil samples using fungal medium (Potato Dextrose Agar), supplemented with lead ions (100-500 mg/L). Serial dilution techniques were performed to decrease the microbial load in the samples and a standard pour plate method was performed. The plates were then incubated at room temperature for 72 h. After incubation, different mascroscopic characterestics such as color, appearance of colony, shape of the fungal colonies from each plate were isolated and characterized for further use in the subsequent heavy metal removal studies. The concentration of lead ions added to the medium was determined and measured using atomic absorption spectrometer (Thermo Scientific iCE 3500, Japan).

Fungal characterization and identification

The fungal isolate that showed high growth rate and high tolerance towards lead was selected for further morphological characterization and molecular identification. The morphological appearances of the selected fungal isolates grown on PDA plates were characterized by visual observation and by micro-morphological techniques. As for molecular identification, fungal DNA was extracted according to the method of Cubero *et al.*, (1999). Extracted fungal DNA was then amplified using a universal primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.,* 1990) under the following condition: initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 7 min. PCR products of approximately 500 bp in molecular weight sizes was then purified and sequenced. Closely related sequences of the isolates were retrieved from the NCBI GenBank database. A neighbour-joining tree was constructed and the distances between sequences were calculated from the models of Jukes and Cantor (1969).

Preparation of of fungal biomass as biosorbent material

The malt, yeast, glucose, peptone (MYGP) media was used for the cultivation of isolated fungal strain, *Fusarium equiseti* KR706303 in Erlenmeyer flasks of 500 mL volume with 250 mL effective volume. The pH of the growth medium was maintained at 5.5 using 1M HCl and 1M NaOH. The flasks were closed with cotton plugs and covered with aluminium foil for autoclaving. After autoclaving, the media was cooled to 30 °C, and three mycelial plugs of 7 mm^2 in diameter was used as an inoculum and incubated in an orbital rotary shaker (Taitec, BR-43FL Japan) at 150 rpm and 28 °C. After 7 days of incubation, the biomass was harvested from the growth medium by centrifugation for 10 min at 10,000 rpm and then filter paper (90 mm size) was used for filteration for biomass collection. The residual growth medium was removed from the collected biomass through washing with plentiful amounts of distilled water. Then, the biomasses were drained, dried at 60 °C for 24 h, ground with a mortal pestle before metal biosorption experiments, and stored in a sealed bottle prior further use.

Evaluation of metal uptake capacity

In order to evaluate the metal uptake capacity and the percentage efficiency of the fungal strain, a mass balance equation (Equation 1) was used according to Akar and co-workers (2009):

$$q = V(Ci - Cf)/W)\qquad(1)$$

whereas the percentage biosorption of metal ion (Equation 2) was determined according to Sari and Tuzen (2009):

$$\text{Biosorption, \% } (= (Ci - Cf)/Ci \times 100)\qquad(2)$$

(Where Ci is.initial concentration of metal, Cf is. final concentration, V is reaction volume, W is biomass weight, and q being adsorbed lead)

Optimization of biosorption experiments

In each 100 mL of Erlenmeyer flask, a volume of 0.04 g of the powdered biosorbent of *Fusarium equiseti* UMAS A0 was incubated in 20 mL of lead(II) solution (50 mg/L) at 28 °C with shaking at 150 rpm. The biosorption experiment parameters were maintained throughout the experiments unless otherwise stated. After incubation, the fungal biomasses were harvested and the residual leads were measured using Atomic Absorption Spectrometer (AAS). For each experiment a blank, containing the metal

ions solution without any biosorbent and a control with distilled water (no metal ion added) and 0.04 g of biosorbent were prepared as well. The values presented in this study were means of three replicates and expressed as standard deviation (SD). The effects of different physical parameters on lead removal, such as, pH, temperature, initial metal concentration, agitation, biomass dose, and contact time were studied. The values of pH (2, 4, 5, 7 and 9), temperatures (20, 25, 30, 35 and 40 °C) and initial lead concentration (100, 200, 300, 400, and 500 mg/L), agitation rate (0, 25, 50, 75, 100, 125, 150, 175 and 200 rpm), biomass dosage (0.01, 0.02, 0.04, 0.06 and 0.08 g), biomass age (3, 4, 5, 6 and 7 days biomass old), and contact time (5, 10, 15, 30, 45, 60, 75 and 90 min) were varied, respectively.

Scanning electron microscopy (SEM)

The scanning electron microscopy (SEM), loaded and unloaded biomass of the fungal isolate with lead ions were treated with 6% glutaraldehyde, incubated overnight at 4 °C and washed 2-3 times with phosphate buffer. Dehydration was done with varied percentages of acetone. The samples were dried on CPD (critical point drying). The samples were mounted on a copper holder with a double stick tape followed by coating with a thin layer of gold under vacuum by Sputter coater. Then samples was viewed using Scanning Electron Microscope (JOEL JXA-840A SEM, Japan.)

RESULTS AND DISCUSSION

Isolation and identification of lead tolerant fungal strain

In isolation experiments, the number of fungal colonies in plates decreased with an increase in lead concentration. This was related to the toxic nature of lead as a heavy metal, and tolerant nature of the fungi. Introduction of metals in various forms into the environment can directly affect the microbial growth, activities and population thus producing numerous modifications of microbial communities (Doelman *et al.*, 1994). The isolated fungal strain, UMAS A0 showed a maximum lead tolerance of 300 mg/L. Morphological characterization of the isolated lead tolerant fungal strain by macro and micro-morphological techniques showed that, the fungal isolates UMAS A0 was *Fusarium* species. The morphology of the fungus was examined via colony surface, colony reverse and under electron microscope. Surface and reverse colony observations were done with regards to the colour of the mycelial mats and wooly nature of the mat (Figure 1).

Molecular identification of the fungal isolate further confirmed that these isolates was *Fusarium* species. A phylogenetic tree was constructed based of sequencing of the ITS1 and ITS4 regions, and it can be seen that the branches of the tree were short, indicating little divergence of the ITS sequences between the isolates (Figure 2). BLAST analysis of the ITS regions showed

that fungal isolate UMAS A0 have 98% sequence similarity to *Fusarium equiseti* (Accession number: FJ459976.1).

Effect of pH

Lead removal increases as pH increases in the metal solution up to pH 4 and decreases thereafter (Figure 3). Inhibition of lead (II) biosorption at low pH (less than 3) could be because of a nett positive charge density on metal binding sites due to high concentrations of protons in the solution. Protons compete effectively with the metal in binding to the functional groups. With an increase in pH, the negative charged density on the cell surfaces increases due to deprotonation of the metal binding sites and thus increases biosorption. At pH values higher than 7, metal ions might be precipitated because of the higher concentration of OH^- ions in the biosorption medium, therefore the metal biosorption is inhibited (Al-Fakih, 2011).

Effect of temperature

The removal of lead by the isolated *Fusarium equiseti* KR706303 appears to be energy dependent biosorption and regarded as endothermic reaction since it is affected by temperature (Ramasamy *et al.*, 2011). Maximum lead removal was observed at 30 °C (Figure 4). It increased with increased temperature while exothermic reaction decreased with increased temperature. This might be due to the physical damage towards the biosorbent expected at higher temperatures. The temperature of the biosorption medium could be important for energy dependent mechanisms in metal biosorption by microbial cells. Most of the time, biosorption is an exothermic process (Martins *et al.,* 2006), but also, there are some examples of endothermic biosorption that have been reported (Davis *et al.*, 2003; Ramasamy *et al.*, 2011). During the endothermic biosorption processes, as in the case of this study, the extent of biosorption processes increases with increasing temperature up to the optimal level. This effect may be due to either higher affinity of binding sites for metal or more binding sites on relevant cell mass (Guo *et al.*, 2002).

Effect of initial metal concentration

The initial metal concentration is an important parameter in biosorption technology, which influences the adsorption of metal to the biomass surface. The results in this study indicated that lead (II) biosorption was increased with the increasing lead (II) concentration of up to 300 mg/L by the isolated fungal strain (Figure 5). At lower initial concentrations, the ratio of initial number of metal ions to the available biosorption sites was low and higher biosorption efficiencies were obtained. In the case of higher initial concentrations, the available sites for biosorption became fewer and the saturation of the adsorption sites was observed. As a result the biosorption efficiencies decreased. This was obtained since initial

metal concentration provides a driving force to overcome mass transfer resistances between the biosorbent and the biosorption medium (Dursun, 2006). Similar results were reported for Pb(II) biosorption by *Pycnoporus sanguineus* (Azila *et al.*, 2008), and for Pb(II) and Cu(II) by *Aspergillus niger* (Dursun, 2006).
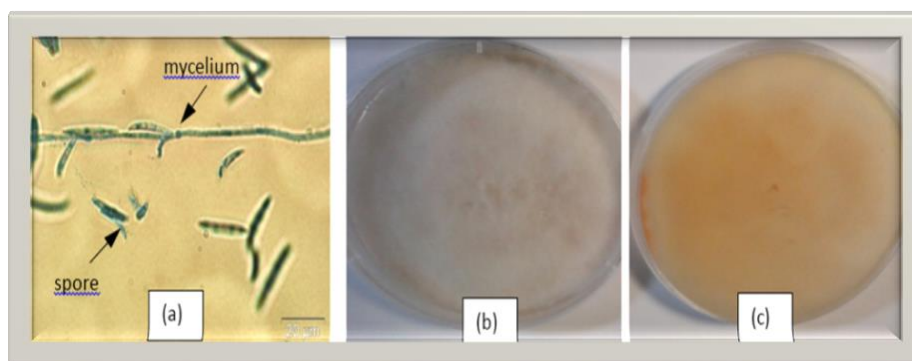

Figure 1: Fungal isolate, UMAS A0 grown on PDA at day 7, expected to be genus *Fusarium*. (a) A rapidly fungi producing characteristics sickle-shaped multi-septate microconidia (mag. 1000×). (b) Colony surface; colonies are rapidly growing woolly to cottony, flat, spread, white mycelium. (c) Reverse colony surface; white with pale brownish mycelium.
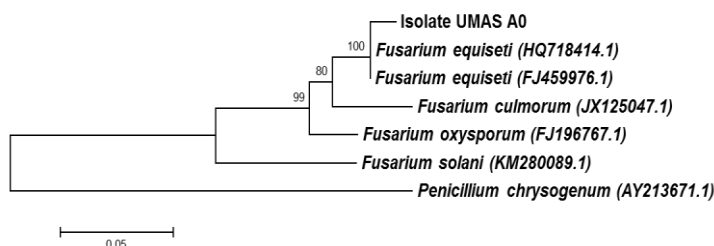

Figure 2: Neighbour-joining tree from ITS sequences showing the relationship between the isolated indigenous fungus UMAS A0 and other closely related Fusarium species retrieved from the GenBank (accession number). Bootstrap values >70 % (1000 replicates) are shown on the branches. Bar = 5 nucleotide substitutions per 100 nucleotides
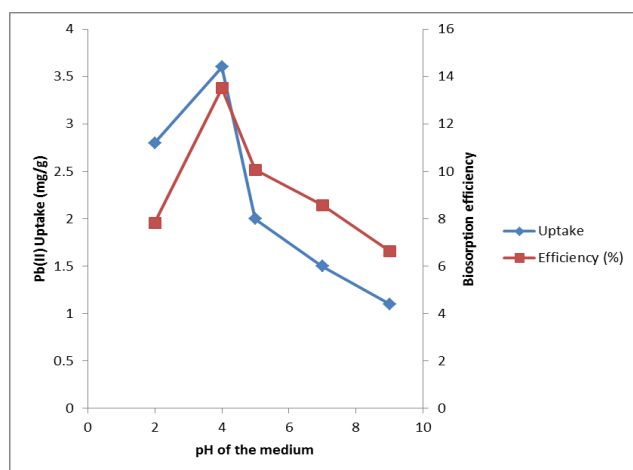

Figure 3: Lead (II) biosorption at different pH by *Fusarium equiseti* UMAS A0. Amount of dried biomass: 0.04 g; Suspension volume: 20 mL. The data is represented as mean values from three replicates.
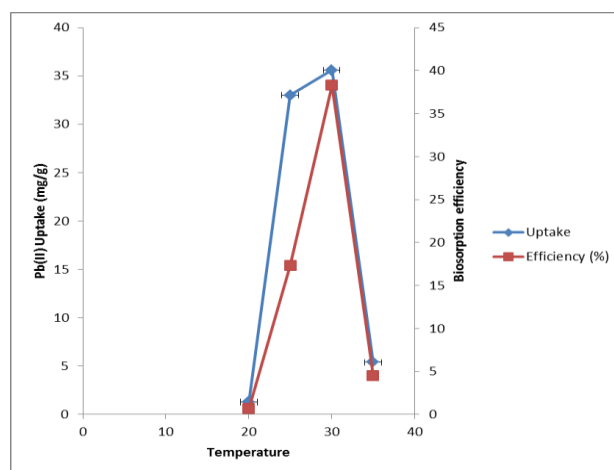

Figure 4: Lead (II) biosorption at different temperature by *Fusarium equiseti* UMAS A0. Amount of dried biomass: 0.04 g; Suspension volume: 20 mL. The data is represented as mean values from three replicates.

Effect of biomass dose

The size of biosorbent used in biosorption studies is an important parameter which determined the capability of potential biosorbent to remove heavy metal ions such as Pb(II) at a given initial dose. The results of this study indicated a substantial effect of the biomass size on the biosorption process. Generally, the amount of Pb(II) bioadsorbed per unit weight decreased with the increased amounts of biomass (Figure 6). Similar observations from previous studies had suggested decreased biosorption capacity at increased biosorbent dose to be influenced by electrostatic interaction and interference between binding sites (Tulani *et al.*, 2006), and a partial aggregation of biomass at higher biomass doses, which in turn results in a decrease in effective surface area available for the biosorption (Selatnia *et al.*, 2004; Karthikeyan *et al.*, 2007). Romera and co-workers (2007), also concluded that at higher biomass dose, biosorbent can exert a shell effect, which protect the active sites from being occupied by metal.
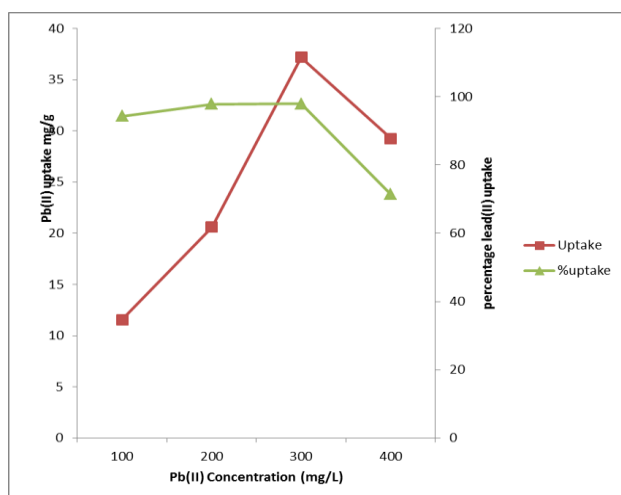
Effect of agitation speed

The agitation speed was highest at 150 rpm (Figure 7), similar to reports from previous studies. Cruz and co-workers (2004), reported that the biosorption of cadmium by *Sargassum* sp. was significantly affected by agitation speed and the maximum adsorption capacity was greater than 100 rpm. Cadmium (II) adsorption capacity by *Aspergillus niger* (Guo *et al.*, 2006) and chromium (VI) by *Rhizopus nigricans* (Bai and Abraham, 2001) were obtained at agitation speed of 120 rpm. Agitation provides the necessary contact between the metal ions in solution and the biomass binding sites, which in turn promotes effective transfer of metal ions to the biosorbent sites (Ahalya *et al.*, 2005). The results obtained is in agreement with reports of Parvathi and Nagendran (2007), that the highest biosorption capacity of lead(II) at an agitation speed of 150 rpm indicates least mass transfer resistance experienced by the system.
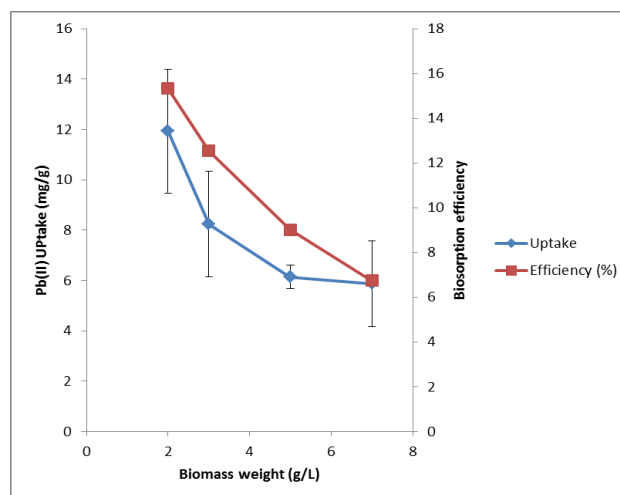

Figure 5: Lead (II) biosorption at different initial concentration by *Fusarium equiseti* UMAS A0. Amount of dried biomass: 0.04 g; Suspension volume: 20 mL. The data is represented as mean values from three replicates.

Figure 6: Lead(II) biosorption (mg g^{-1} d.wt) at different biomass dose and biosorption efficiency by *Fusarium equiseti* UMAS A0. Suspension volume: 20 mL. The data is represented as mean values of three replicates.

Effect of contact time

Pb(II) biosorption by the isolated *Fusarium equiseti* reached an equilibrium at approximately 60 min (Figure 8). Biosorption was rapid in the first 30 min of contact time, which suggests the active interaction of metals with functional groups on the surface of the biomass. The observed biosorption kinetics has significant practical importance in biosorption of heavy metals on a large scale, as it will facilitate smaller reactor volumes that ensures efficiency and cost effectiveness (Herrero *et al.*, 2005; Al-Fakih, 2011). In addition, Li *et al.,* (2008), reported a similar study on biosorption equilibrium of lead

and copper ions by biomass of *Penicillium simplicissimum*, which reached equilibrium at 60 min of contact time.

Biosorption processes depends on the availability of the functional groups on the cell surface and the nature of the metal ions (Engleand Kunz, 1995; Al-Fakih, 2011). That can only be done by futher identification and characterization of the available funtional groups on the fungal cell surface by employing titration and FTIR methods.
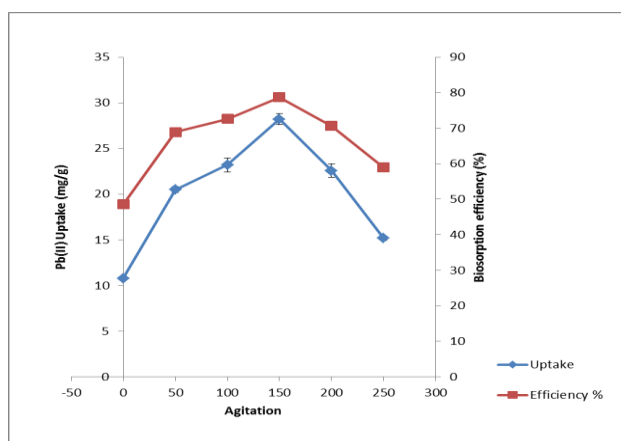
Figure 7: Lead (II) biosorption at different agitation by *Fusarium equiseti* UMAS A0. Amount of dried biomass: 0.04 g; Suspension volume: 20 mL. The data is represented as mean values from three replicates.

Effect of biomass age

The effect of biomass ages (ranging from 3-7 days) on the biosorption of lead (II) ions by the isolated fungal strains showed that younger cells had higher biosorption capacity than the older cells (Figure 9). It has been reported by Delgado *et al*. (1998), that in the biosorption of copper, cadmium and nickel by biomass of *Fusarium flocciferum*, older cultures showed a decrease in metal biosorption capacity. The observation in this study is also in agreement with the report of Al-Fakih, (2011), on biosorption of lead (II) and cobalt (II) ions by biomass of *Rhizopus oryzae* and *Saccharomyces cerevisiae*. During microbial growth, the cells at lag phase or early stage of growth have a higher biosorption capacity for metal ions than that of stationary phase (Kapoor and Viraraghavan, 1997). Also, the percentage of chitin and chitosan in the
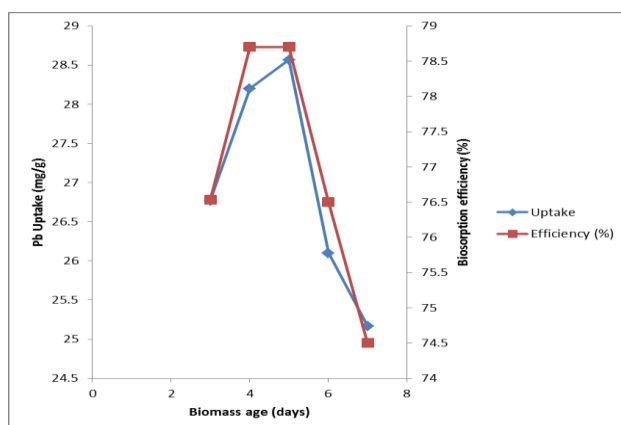

Figure 9: Lead (II) biosorption cultivated for different days by *Fusarium equiseti* UMAS A0. Amount of dried biomass: 0.04 g; suspension volume: 20 mL. The data is represented as mean values from three replicates.

Figure 8: Lead (II) biosorption at different contact time by *Fusarium equiseti* UMAS A0. Amount of dried biomass: 0.04 g; suspension volume: 20 mL. The data is represented as mean values from three replicates.
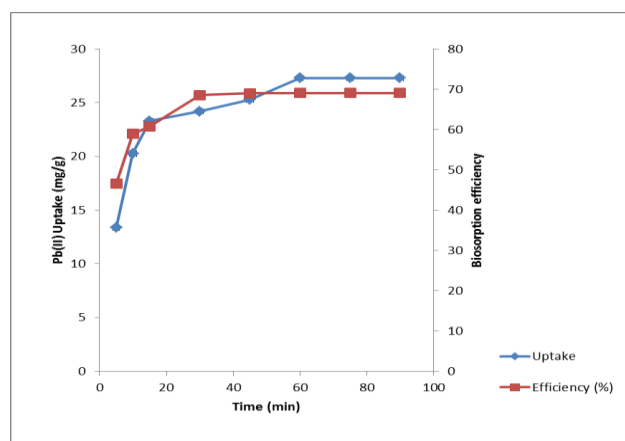
fungal cell wall varies with the culture age and growth conditions (Zhou and Banks, 1993; Gharieb, 2002; Al-Fakih, 2011).

Scanning electron microscopy (SEM)

The SEM micrographs was observed for *Fusarium equiseti* UMAS A0 loaded with lead ions and showed that the fungus can absorb lead from aqueous solutions and forms insoluble lead precipitates on the cell wall within the matrix of fungal mycelia (Figure 10). The finding is in agreement with the studies done by Anand and co-workers (2006). They reported that the electron microscopy and cell fractionation studies revealed that 70-80% of copper was present as a layer on the cell wall surface of *Trichoderma viride*.
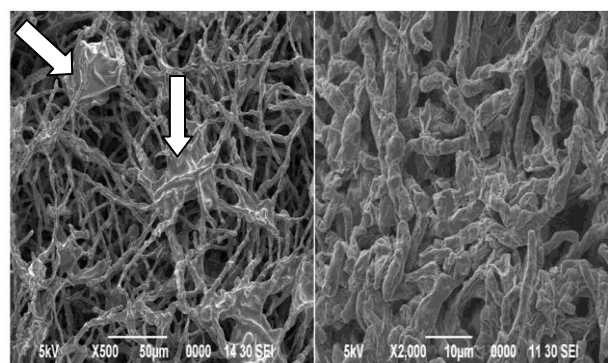

Figure 10: Scanning electron microscope (SEM) micrograph of *Fusarium equiseti* UMAS A0 with (A) and without (B) bioadsorbed lead (II) ions. Arrows indicated that Pb ion precipitated and deposited within fungal mycelia.

CONCLUSION

In this study, an indegenious lead-tolerant fungal strain was successfully isolated from mangrove soil environments, and its practicability of heavy metal removal from a simulated environment was measured at a laboratory scale basis. The isolated strain was successfully identified as *Fusarium equiseti*. Physical parameters such as pH, temperature, initial metal concentration, agitation, biomass age, contact time and biosorbent dose showed significant effects on lead biosorption by *Fusarium equiseti* UMAS A0 with maximum efficiency at pH 4.0, temperature of 30°C, initial concentration of 300 mg/L, agitation speed of 150 rpm, contact time of 60 min and biomass age of 5 days old. The study demonstrated that the newly isolated metal resistant *Fusarium equiseti* UMAS A0 from mangrove soil environments has the potential application for the lead removal from aqueous solution.

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