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BIOSORPTION OF LEAD (II) ION USING *Penicillium citrinum* KR706304 ISOLATED FROM THE MANGROVE SOIL ENVIRONMENT OF SOUTHEAST BORNEO.

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ABSTRACT

The use of biological entities (fungi, bacteria, and algae) is considered to be a potential cost-effective and environmental friendly technique for heavy metals pollution sequestration. The present study aimed to isolate efficient lead tolerant fungi from mangrove soil environment and measure its capability for lead removal from aqueous solution. Lead tolerant fungal strains were isolated from soil samples using MEA (malt extract agar) amended with varied concentrations of lead ions (100-500 mg l^{-1}). The most tolerant fungal strain was successfully isolated and identified molecularly as *Penicillium citrinum* KR706304 The isolated fungus was used for biosorption studies using malt extract broth (MEB) amended 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 lead removal was optimal at concentration of 400 mg l^{-1}, maximum adsorption of 329±33.4 mg g^{-1} was observed at pH 7 and temperature of 30 ^{o}C during the batch biosorption experiments. The optimal parameters for biomass dose, agitation speed, contact time and biomass age were 0.04 g l^{-1} , 150 rpm, 60 min and fifth day; respectively.

The study revealed that the isolated *Penicillium citrinum* KR706304 has the potential to be used as a biosorbent for heavy metals particularly Pb(II) removal from the contaminated sites.

Keywords: Fungi, Heavy metals, Lead(II) removal, *Penicillium citrinum.*

INTRODUCTION

Bioremediation is an alternative green technology comprising of various processes involved in the decontamination of environments already polluted by contaminants into its original status. The processes include, biosorption, bioaccummulation and bioaugmentaton (Abdulqawi, 2011). Biosorption is one of the processes of bioremediation in which metal ion adsorption by biomass occurs as a result of interactions with functional groups native proteins, lipids, and carbohydrates that make up the cell wall (Chen *et al*., 2007; Sana *et al.,* 2015). The advantage of biosorption over conventional treatment methods is the low cost, environmental friendliness, and also often offers metal recovery and biomass regeneration (Volesky, 2007; Muñoz *et al.,* 2015). The heavy metal ion biosorption by fungi is based mainly on two mechanisms: covalent bonding with functional groups including carboxyl, hydroxyl, phosphate, amino, sulphydryl, and the result of physico-chemical interactions directed by adsorption phenomena (Say *et al*., 2011).

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 (Abdulqawi, 2011). Lead is known for its high environmental impact and toxicity (Mason *et al.,* 2014), and along with mercury and cadmium, is considered one of "the big three" heavy metals in contaminated effluents (Volesky, 2007; Muñoz *et al.,* 2015). The European Directive 2008/105/CE includes lead and its compounds in the list of priority substances in the environment quality standards; establishing a concentration of 0.0072 mg l^{-1} as the maximum permissible in the case of surface water (Muñoz *et al.,* 2015).

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). Among microbial biosorbents, fungal biomass are widely used in biosorption technology (Gupta *et al*., 2002; Zulkarnain *et al.,* 2015). Previous reports by Li *et al.* (2008) showed the potentiality of *Penicillium simplicissimum* for Pb(II) and Cu(II) while *Penicillium purpurogenum* exhibits selectivity for Pb(II) over metals such as Cd(II), Hg(II) and As(II) (Say *et al.,* 2003). In order to improve biosorption effectiveness, the identification of additional fungal strains with high metal sorption capacity is essential (Volesky, 2007; Kumar *et al.,* 2014; Muñoz *et al.,* 2015). Therefore, this study is aimed at isolating efficient lead tolerant fungi from mangrove soil environment and measure its capability for lead removal from aqueous solution.

MATERIALS AND METHODS

Sampling and Isolation of Fungal Strain

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 lead acetate {$Pb(CH_3COO)_2.3H_2O$} (Merck, Germany). The pH of the working solution was adjusted to pH 5.0 using hydrochloric acid (3 M HCl). Fresh dilutions were used for each biosorption experiments. Lead tolerant fungal strains were isolated from the soil samples using fungal medium (malt extract agar) amended with lead ions (100-500 mg l^{-1}).

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 morphological colonies from each plate were isolated and characterized for further use in subsequent heavy metal removal studies. The concentration of lead ions added to the medium was determined and measured using atomic absorption spectrometer (AAS) (Thermo Scientific iCE 3500, Japan).

Preparation of Fungal Biomass as Biosorbent Material

Malt extract broth was used for the cultivation of isolated fungal strain, *Penicillium citrinum* KR706304 in Erlenmeyer flasks of 500 ml volume with 250 ml effective volume. The pH of the growth medium was maintained at 5.5 using 1 M HCl and 1 M 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 mycelia plugs of 7 mm in diameter was used as an inoculum and incubated in an orbital rotary shaker (Taitec, BR-43FL Japan) at 150 rpm and 30 °C. After 7 days of incubation, the biomass was harvested from the growth medium by centrifugation for 10 min at 10,000 rpm and filter paper (90 mm size) was used for filtration for biomass collection. The residual growth medium was removed from the collected biomass through washing with distilled water. Then, the biomass was drained, dried at 60 °C for 24 h, ground with a mortar and pestle before metal biosorption experiments, and stored at room temperature in a sealed bottle prior further use.

Evaluation of Metal Uptake Capacity

In order to evaluate the metal adsorption capacity and the percentage efficiency of the fungal strain, a mass balance equation (Equation 1) was used according to Akar *et al.* (2009):

$$q = V(Ci - Cf)/W$$

Where,

q= the adsorbed metal (mg g^{-1})

V= the volume of metal solution (l)

C*i*= initial metal concentration (mg l^{-1})

C*f*= final/ residual concentration (mg l^{-1})

W= amount of biomass (mg l^{-1})

The percentage biosorption of metal ion was determined using the methods of Sari and Tuzen (2009):

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

Optimization of Biosorption Experiments

In each 100 ml of Erlenmeyer flask, a volume of 0.04 g of the powdered biosorbent of *Penicillium*

citrinum KR706304 was incubated for 3 h in 20 ml of lead (II) solution (50 mg l^{-1}) at 30 °C with shaking at 150 rpm. The biosorption experiment parameters were maintained throughout the experiments unless otherwise stated. After incubation, the fungal biomass was harvested and the residual lead was 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 set up. The effects of different physical parameters on lead removal, such as pH, temperature, initial metal concentration, agitation, biomass dose, and contact time were studied.

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 lead holder with a double stick tape followed by coating with a thin layer of gold under vacuum by Sputter coater. Then samples were viewed using scanning electron microscope (JOEL JXA-840A SEM, Japan).

Statistical Analysis

The values presented in the study were means of three replicates and expressed as means ± standard deviation (SD). The Microsoft Office Excel (2010) was employed to calculate the standard deviation where needed. Results were analysed statistically amongst and between mean of data samples with variance analysis at 95% level of confidence.

RESULTS AND DISCUSSION

Isolation and Identification of Lead Tolerant Fungal Strain.

In isolation experiments, the number of fungal colonies in plates reduced 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. The isolated fungal strain showed a maximum lead tolerance of 400 mg l^{-1}. Morphological characterization of the isolated lead tolerant fungal strain was then done using conventional light microscopy. This was done using Lacto phenol cotton blue mounting with the pure culture of the isolated strain (Figure 1). Molecular identification of the fungal isolate further confirmed that these isolates were *Penicillium* species. A phylogenetic tree was constructed based on 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).

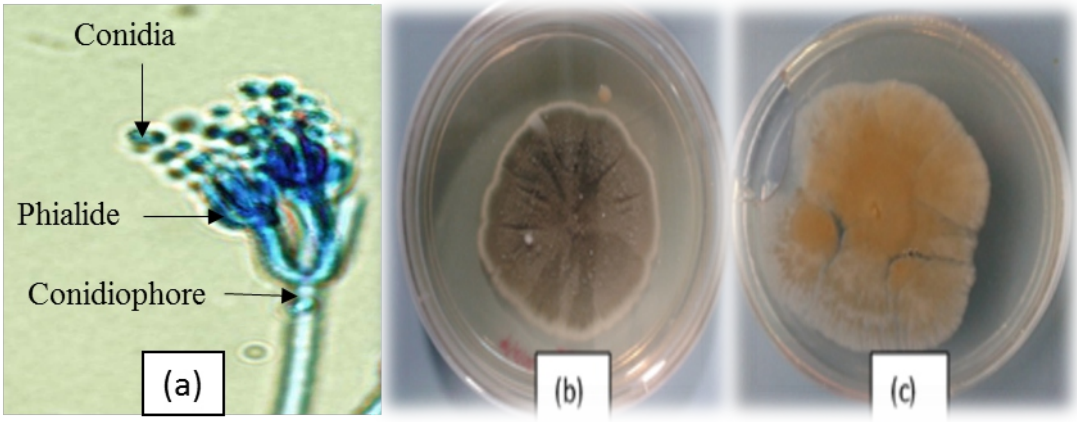

Figure 1: UMAS B2 grown on PDA after day 21, expected to be genus *Penicillium*. (a) Mass of conidia formed at the tip of a single long, flask-shaped phialide, septate hyphae (mag. 1000x). (b) Colony surface; colonies were slow growing with a dense greenish mycelial mat. Conidiation were noted on the greenish floccules, which later grew into white tufts. No pigmentation occurred. (c) Reverse colony surface; white with pale brownish mycelium, the brownish colour is the colour of the growth medium. UMAS B2 was the code for the isolate before molecular identification of the isolate.

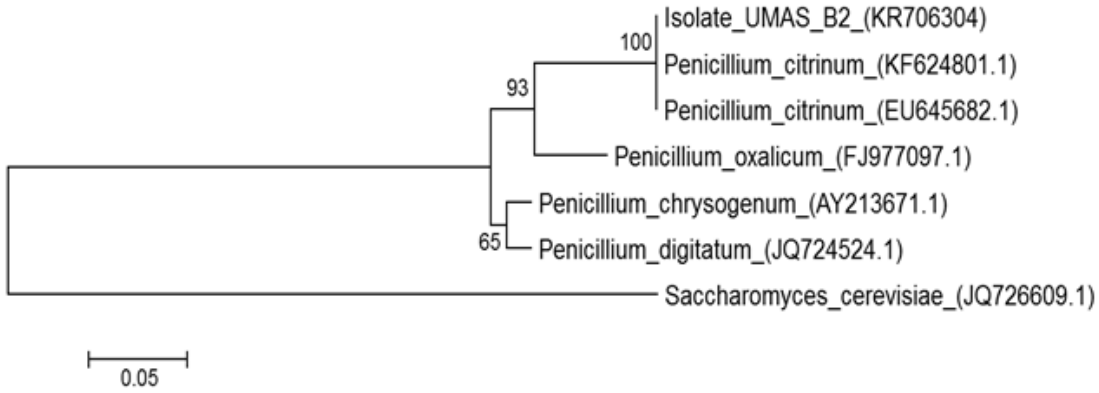

Figure 2: Neighbour-joining tree from ITS sequences showing the relationship between the isolated indigenous fungus UMAS B2 and other closely related *Penicillium* species retrieved from the GenBank (accession number). Bootstrap values >70% (1000 replicates) are shown on the branches. Bar = 5 nucleotide substitution per 100 nucleotides

Effect of pH on Lead Biosorption

Lead removal increases as pH increases in the metal solution up to pH 7 and decreases thereafter (Figure 3). Baysal *et al.* (2009) found that the biosorption of lead (II) by *Candida albicans* was maximal at pH 5, whereas it was at pH 7 by *Kluyveromyces marxianus* immobilized in alginate beads (Subhashini *et al.,* 2013).

Inhibition of lead (II) biosorption at low pH (less than 3) could be because of a net positive charge density on metal binding sites due to high concentrations of protons in the solution (Abdulqawi, 2011). In acidic medium, the fungal cell wall becomes highly protonated due to excess H^+ ions that binds to functional groups (-OH, -NH_2, -NH, and –C=O) (Bennett *et al.*, 2013; De Sotto *et al.*, 2015).

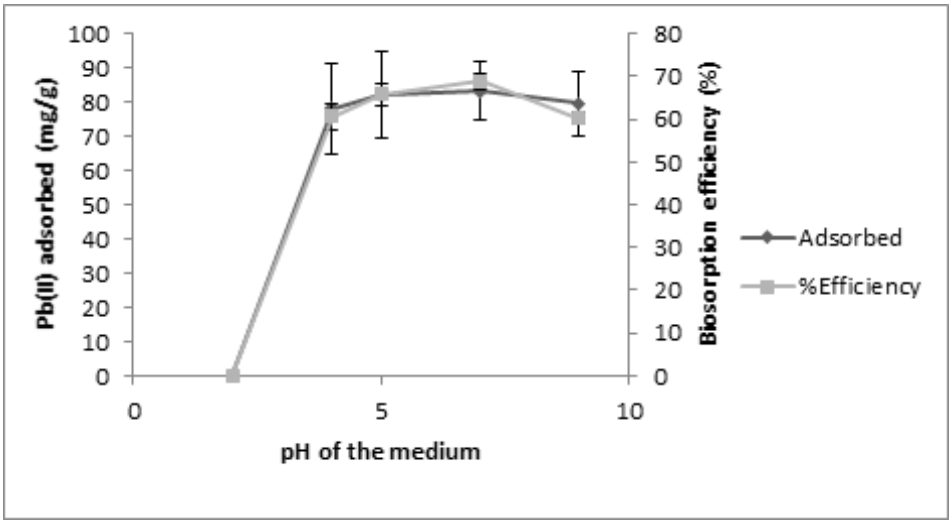

Figure 3: Lead (II) biosorption (mg g^{-1} d.wt) and biosorption efficiency by *Penicillium citrinum* KR706304 at different pH values. Amount of dried biomass: 0.04 g; initial metal concentration (C_i): 50 mg l^{-1}; suspension volume: 20 ml; temperature: 30 °C; agitation speed: 150 rpm. The data are the mean values of 3 replicates, and the bars indicate the standard deviation of the mean.

Effect of Temperature on Lead Biosorption

The removal of lead by the isolated *Penicillium citrinum* KR706304 appears to be energy dependent biosorption, because it is affected by temperature. Although, the effects was not statistically significant ($p > 0.05$). Maximum lead removal was observed at 30 °C (Figure 4). 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.*, 2006).

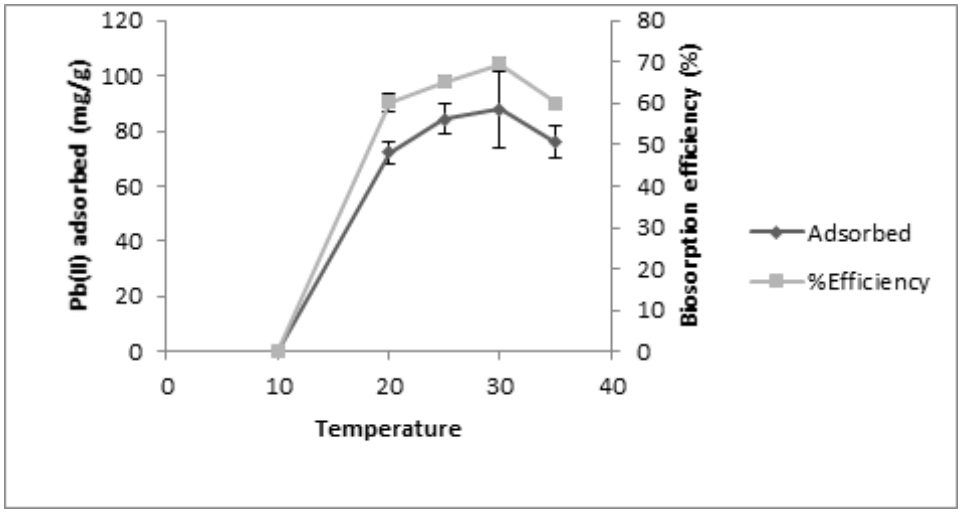

Figure 4: Lead (II) biosorption (mg g^{-1} d.wt) and biosorption efficiency by *Penicillium citrinum* KR706304 at different temperature. Initial metal concentration (C_i): 50 mg l^{-1}; suspension volume: 20 ml; pH: 7; Amount of dried biomass: 0.04 g; agitation speed: 150 rpm. The data are the mean values of 3 replicates, and the bars indicate the standard deviation of the mean.

Effect of Initial Metal Concentration on Lead Biosorption

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 increasing lead(II) concentration of up to 400 mg l^{-1} 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; Wahab *et al.*, 2015). Similar results were reported for lead(II) biosorption by *Pycnoporus sanguineus* (Azila *et al.*, 2008), and for lead(II) and Cu(II) by *Aspergillus niger* (Dursun, 2006).

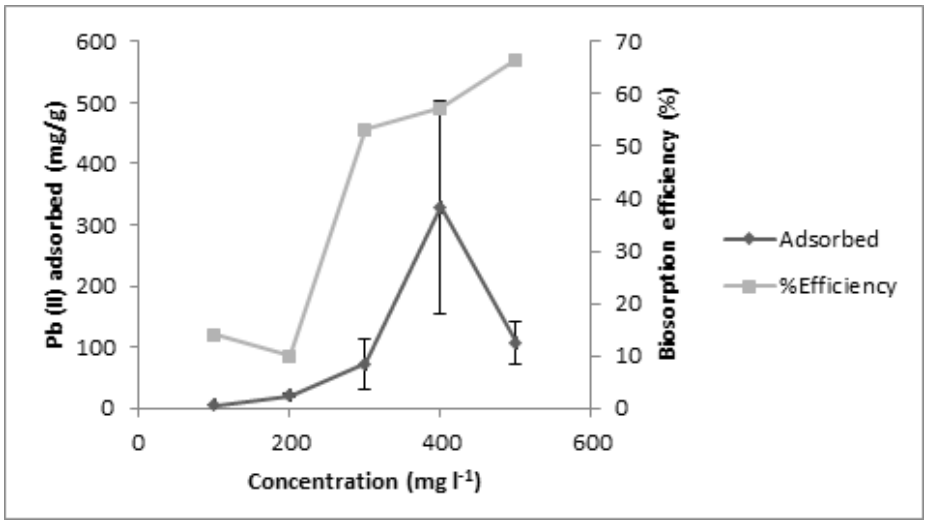

Figure 5: Lead (II) biosorption (mg g^{-1} d.wt) and biosorption efficiency by *Penicillium citrinum* KR706304 at different initial concentration.Suspension volume: 20 ml; temperature: 30 °C; pH: 7; agitation speed: 150 rpm. The data are the mean values of 3 replicates, and the bars indicate the standard deviation of the mean.

Effect of Biomass Dose on Lead Biosorption

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 lead(II) at a given initial dose. The results of this study indicated a significant effect of the biomass size on the biosorption process. Generally, the amount of lead(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 (Karthikeyan *et al*., 2007). Romera *et al*. (2007) also, concluded that at higher biomass dose, biosorbent can exert a shell effect, which block the active sites from being occupied by metal.

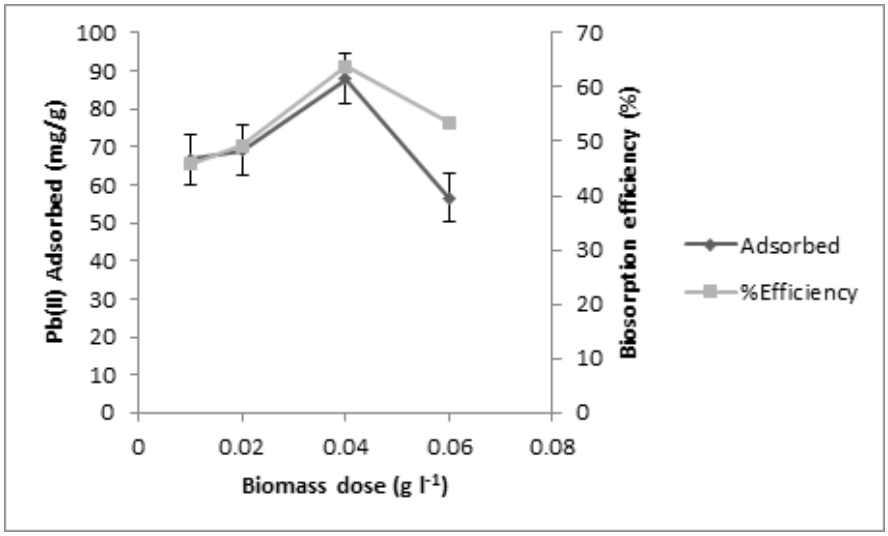

Fig. 6: Lead (II) biosorption (mg g^{-1} d.wt) and biosorption efficiency by *Penicillium citrinum* KR706304 at different biomass dose. Initial metal concentration (C_i): 50 mg l^{-1}; suspension volume: 20 ml; temperature: 30 °C; pH: 7; agitation speed: 150 rpm. The data are the mean values of 3 replicates, and the bars indicate the standard deviation of the mean.

Effect of Agitation Speed on Lead Biosorption

The agitation speed was highest at 150 rpm (Figure 7), similar to reports from previous studies, Cruz *et al.* (2004), reported that the biosorption of cadmium by *Sargassum* sp. was significantly affected by agitation speed and the maximum adsorption capacity was greater at 100 rpm. Cadmium(II) adsorption capacity by *Aspergillus niger* 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), and Abdulqawi, 2011, that the highest biosorption capacity of lead(II) at an agitation speed of 150 rpm indicates least mass transfer resistance experienced by the system.

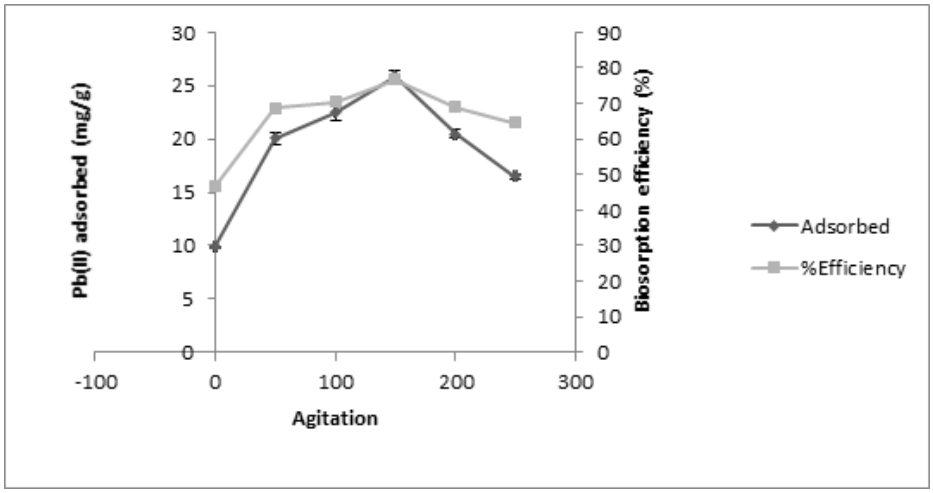

Figure 7: Lead(II) biosorption (mg g^{-1} d.wt) and biosorption efficiency by *Penicillium citrinum* KR706304 at different agitation speed. Amount of dried biomass: 0.04 g; initial metal concentration (C_i): 50 mg l^{-1}; suspension volume: 20 ml; temperature: 30 °C; pH: 7. The data are the mean values of 3 replicates, and the bars indicate the standard deviation of the mean.

Effect of Contact Time on Lead Biosorption

Pb(II) biosorption by the isolated *Penicillium citrinum* KR706304 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; Abdulqawi, 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 functional groups on the cell surface and the nature of the metal ions (Engle and Kunz, 1995; Abdulqawi, 2011). It takes place in two stages; passive uptake which takes place immediately, and active uptake which takes place slowly (Goyal *et al.*, 2003). The physical sorption is relatively a fast adsorption step. The rapid increase in the biosorption capacity during the initial stages of lead(II) biosorption observed in this study may be a physical adsorption, which can be categorized as extracellular sorption or surface binding. The chemical sorption or intracellular sorption is more of a metabolic process than the physical sorption (Lopez *et al.*, 1995; Wahab *et al.*, 2015).

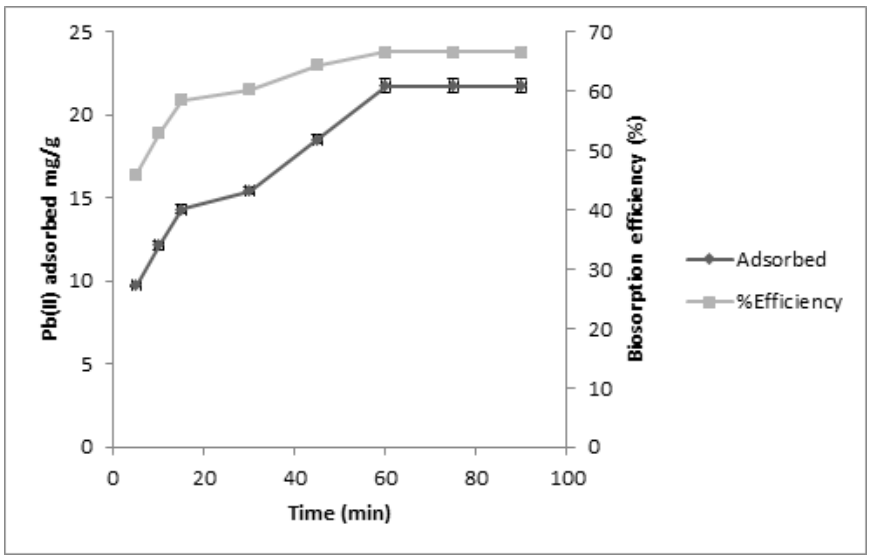

Figure 8: Lead(II) biosorption (mg g^{-1} d.wt) and biosorption efficiency by *Penicillium citrinum* KR706304 at different time intervals. Amount of dried biomass: 0.04 g; initial metal concentration (C_i): 50 mg l^{-1}; suspension volume: 20 ml; temperature: 30 °C; pH: 7; agitation speed: 150 rpm. The data are the mean values of 3 replicates, and the bars indicate the standard deviation of the mean.

Effect of Biomass Age on Lead Biosorption

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 Abdulqawi (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 were more active and possesses higher biosorption capacity for metal ions than that of stationary phase (Kapoor and Viraraghavan, 1997). Also, the percentage of chitin and chitosan in the fungal cell wall varies with the culture age and growth conditions (Zhou and Banks, 1993; Gharieb, 2002; Abdulqawi, 2011).

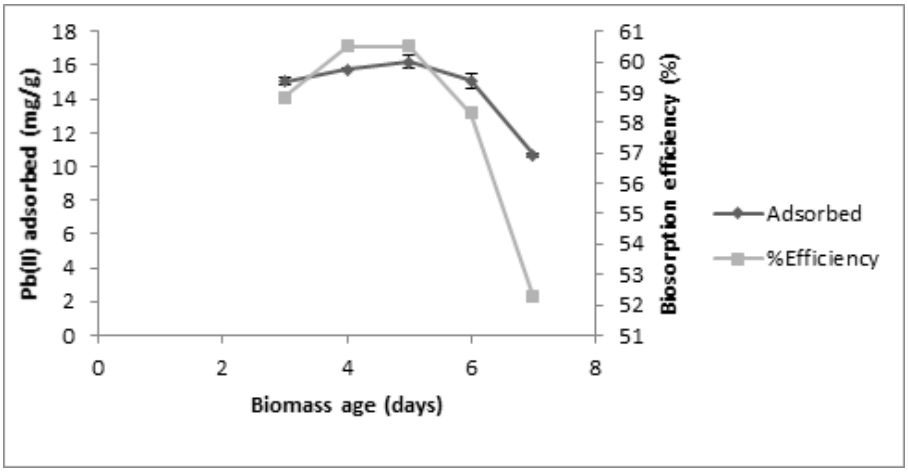

Figure 9: Lead(II) biosorption (mg g^{-1} d.wt) and biosorption efficiency by *Penicillium citrinum* KR706304 biomass cultivated at different growth periods. Amount of dried biomass: 0.04 g; initial metal concentration (C_i): 50 mg l^{-1}; suspension volume: 20 ml; temperature: 30 °C; pH: 7; agitation speed: 150 rpm. The data are the mean values of 3 replicates, and the bars indicate the standard deviation of the mean.

Scanning Electron Microscopy (SEM)

The SEM micrographs observed for *Penicillium citrinum* KR706304 loaded with lead ions showed that the fungus could absorb lead from aqueous solutions to form insoluble lead precipitates within the matrix of fungal mycelia (Figure 10). The fungal mycelial morphologies of the outer surfaces were closely merged together in samples unloaded with heavy metals, while the outer surface network of the treated fungal mycelium became more porous and flexible, than in control. Natarajan *et al*. (2010), reported similar observations, and concluded that the physical strength of the treated mycelium was weaker under metal stress. The highly porous surface observed in the treated fungal mycelium favours the diffusion of metal ions into the cell, thereby leading to higher adsorption capacity (Zulkarnain *et al*., 2015).

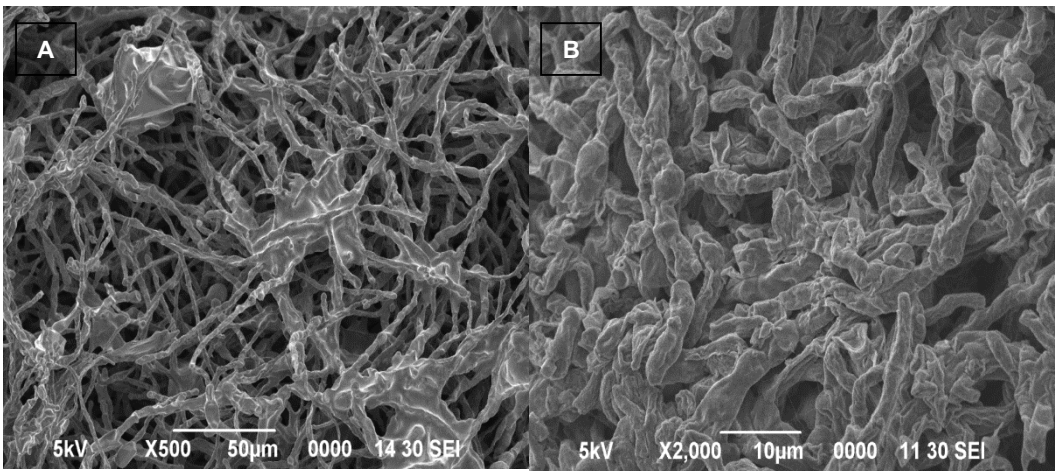

Figure 10: SEM micrographs of *Penicillium citrinum* KR706304 loaded with lead (A), and unloaded with metal (B).

CONCLUSION

The study demonstrated that the newly isolated metal resistant *Penicillium citrinum* KR706304 from mangrove soil environments has the potential application for the lead removal from aqueous solution.

Conflict of interest

There is no conflict of interest in this research work.

ACKNOWLEDGEMENT

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