Biosorption of Copper (II) ions in Aqueous Solution using Biomass of *Fusarium equiseti* KR706303 isolated from Mangrove soil environment

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Abstract: Bioremediation is an alternative green technology for the removal of heavy metals pollution because of their toxicity to the environment and public health. Mangrove is an extreme environment which acts as natural filters of water flowing into the sea, and retain heavy metals and sediments in the roots and soil substrate. The present study aimed to isolate efficient copper tolerant fungi from mangrove soil environment and measure its potentiality for copper removal from aqueous solution. 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) amended with copper ions. The effects of physical parameters on copper (II) ions biosorption were monitored. The results showed that the optimal parameters for the removal of copper ions such as heavy metal concentration and pH were 30 mg L^{-1}, with a maximum Cu(II) adsorption of 8.5mg/g observed at pH 9 and temperature of 30°C during the batch biosorption experiments. The optimal parameters for biomass weight, agitation speed, contact time and biomass age were observed at 0.04 g/L, 150 rpm, 60 min and fifth day; respectively. The observation in this study reveals that the biomass of the isolated *F. equiseti KR706303* has the potential to be used as a biosorbent for heavy metal particularly Cu(II) removal from the contaminated sites. The technology is simple, efficient, cost effective and environmental friendly.

Keywords: Biosorption, Fungi, *Fusarium equiseti,* mangrove,copper(II) removal

INTRODUCTION

Rapid urbanization and industrialization have led to increased heavy metals pollution in the environment (Shatarupa and Tapan, 2014). According to the World Health Organization (WHO, 2004; Al-Fakih, 2011), the metals of most immediate concern are cadmium, chromium, iron, copper, lead, arsenic, mercury, aluminium, nickel and zinc. The presence of such metals in aquatic environments causes severe damage to aquatic life, killing microorganisms during water purification process. Copper is an essential micronutrient to living organisms, involved in biochemistry processes such as detoxification and oxidation (Tsivkovskii *et al*., 2003), it is also known to be one of the heavy metals that is toxic to living organisms. Most copper containing compounds were commonly used as anti-microbial agents as well as feed additives. This is responsible for the elevated levels of copper as could be seen in waters and soils (Jain, 1990). Consequently, large volumes of wastewaters and wastes from electronic containing copper results from industry (Subbaiah *et al*., 2011).

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Heavy metals are non-biodegradable in nature and their accumulation in the environment and body of living organisms may lead to a variety of diseases and disorders (Mohammad et al., 2013). Bioremediation provides an effective and in situ alternative of cleaning up heavy metals from contaminated soils (Ahluwalia and Goyal, 2007; Zafar *et al*., 2007).

Conventional physico-chemical elimination methods of heavy metal from aquatic environment as well as from soil such as electrochemical treatment, ion exchange, precipitation, osmosis, evaporation and sorption (Gupta and Rastogi, 2008) of heavy metal from waste stream are either much expensive as well as not much efficient (Wang and Chen, 2009; Shatarupa and Tapan, 2014). Biosorption is a new technique and less expensive method for heavy metals removal from wastewaters and soils such as copper (II) ions, even in low concentration (Sari and Tuzen, 2009; Wahab *et al*., 2015).

Among biomaterials used in biosorption technology, fungal biomass has been recognized for the removal of heavy metals and radionuclides from polluted environment (Kavamura and Esposito, 2010).

This is due to the fact that fungal cell walls mainly consist of polysaccharides, protein and lipids, with different functional groups that are available for metals bonding. Fungal also often exhibit excellent tolerance toward metals and other adverse conditions such as low pH (Al-Fakih, 2011). This study is aimed to isolate efficient copper tolerant fungi from mangrove soil environment and to measure its potentiality for copper 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. Copper solutions were prepared using $Cu(NO_3)_2$ 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. Copper tolerant fungal strains were isolated from the soil samples using fungal medium (Potato Dextrose Agar), supplemented with copper ions (10-100 mg/L). Serial dilution techniques were performed to decrease the microbial load in the samples and a standard pour plate method was done.

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 copper ions added to the medium was determined and measured using atomic absorption spectrometer (Thermo Scientific iCE 3500, Japan)

Preparation of fungal biomass as biosorbent material

Potato Dextrose Broth (PDB) 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 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 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 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 (mgg^{-1})

V= the volume of metal solution (L)

Ci= initial metal concentration (mgL^{-1})

Cf= final/ residual concentration (mgL^{-1})

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

The percentage biosorption of metal ion was determined using Sari and Tuzen, 2009;

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 *Fusarium equiseti* KR706303 was incubated in 20 mL of copper (II) solution (50 mg/L) at 30 °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 copper 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 copper 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 copper concentration (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg/L), agitation rate (0, 25, 50, 75, 100, 125, 150, 175, 200 and 250 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.

2.5 Scanning electron microscopy (SEM)

The scanning electron microscopy (SEM), loaded and unloaded biomass of the fungal isolate with copper 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).

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 ststistically amongst and between mean of data samples with variance analysis at 95% level of confidence.

RESULTS AND DISCUSSION

Identification of copper tolerant fungal strain

Morphological characterization of the isolated copper 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 fluffy 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

Biosorption medium initial pH is considered as one of the most important environmental factors affecting the metal biosorption by biosorbents as it could change biosorbent surface charge, degree of ionization, and availability of the functional groups such as hydroxyl (R-OH), carboxyl (R-COOH), amino (-NH2), and sulphydryl (-SH) groups (Chen *et al*., 2008; Al-Fakih, 2011). Copper removal increases as pH increases in the metal solution up to pH 9 and decreases thereafter (Figure 3). At pH value higher than 9, as observed in this study, 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). Inhibition of copper (II) biosorption at low pH (less than 3) could be because in an 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).

Effect of temperature

The removal of copper 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 copper 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; Al-Fakih, 2011).

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 copper (II) biosorption was increased with the increasing copper (II) concentration of up to 30 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).

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 Cu(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 Cu(II) adsorbed 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 *et al*., 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 *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 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 copper(II) at an agitation speed of 150 rpm indicates least mass transfer resistance experienced by the system.

Effect of contact time

Cu(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 (Engle and 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.

Effect of biomass age

The effect of biomass ages (ranging from 3-7 days) on the biosorption of copper (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 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 KR706303* loaded with copper ions and showed that the fungus can absorb copper from aqueous solutions and forms insoluble copper precipitates on the cell wall within the matrix of fungal mycelia (Figure 10). The figure showed clearly the structural modification of the mycelia possibly due to metal ions absorption by the biomass of the tested fungi. In comparison with the control, that is, the biosorbent without metal, the mycelial mat is without any structural modification. 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 become 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 (Rahmiana *et al*., 2015).

FTIR spectral analysis

The FTIR spectra of free and metal loaded fungal biomass in the range 4000-400 cm^{-1} were taken and compared with each other to obtain information on the nature of the possible biomass-metal ions interactions. The results obtained are presented in Fig. 11. The broad adsorption bands at 3700-3000 cm^{-1}, representing –OH groups of the glucose and the –NH stretching of the protein and chitosan. In the range 3000-2800 cm^{-1} the bands are representative of symmetric and asymmetric vibrations of stretching of CH3 and CH2 groups. The peaks at 1643.80 and 1557.76 cm^{-1} of *F. equiseti,* can be assigned to a carbonyl group (C=O) stretching in carboxyl or amide groups. The following observations were noted, after metal biosorption:

Little shifting of peaks (corresponding to –OH and –NH groups) from 3264.40 to 3262.27 and 3264.23 cm^{-1} after lead(II) and copper(II) biosorption by *F. equiseti*, respectively. This shifting indicates that these groups were involved in lead(II) and copper(II) biosorption. Changed absorption bands (corresponding to symmetric and asymmetric vibrations of stretching of CH3 and CH2 groups) at 2920.76 to 2919.43 and 2919.29 cm^{-1} after lead(II) and copper(II) biosorption by *F. equiseti,* respectively.

Mechanism of biosorption

It has been reported previously by the same author, that the electrostatic attraction and complexation seem to be the most important mechanism of biosorption of lead (II) and copper (II) ions (Wahab *et al* 2016). This is in agreement with the report of Al-Fakih, 2011, that electrostatic attraction and complexation are key to biosorption of lead(II) and cobalt(II) ions.

CONCLUSION

In this study, an indigenous copper-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 appreciable effects on copper biosorption by *Fusarium equiseti* KR706303 with maximum efficiency at pH 9.0, temperature of 30°C, initial concentration of 40 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* KR706303 from mangrove soil environments has the potential application for the copper removal from aqueous solution.

Conflict of interests

The author(s) did not declare any conflict of interest.

Acknowledgements

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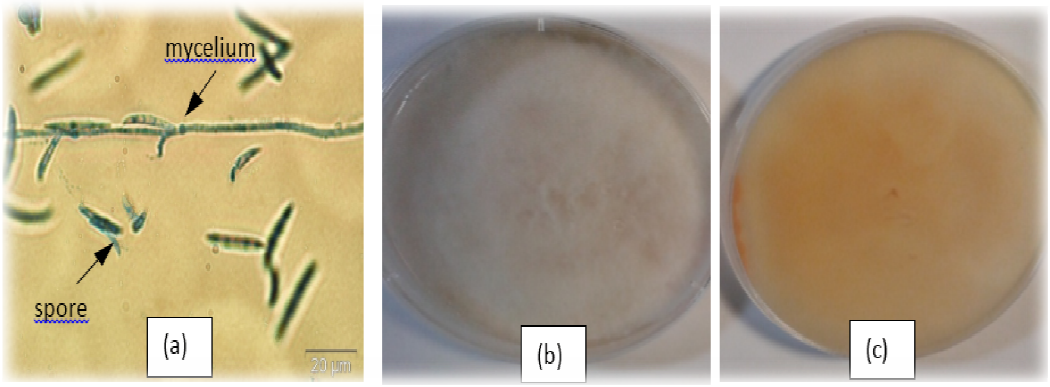

Figure 1: UMAS A0 grown on PDA after day 7, expected to be genus *Fusarium*. (a) A rapidly growing fungi producing characteristics such as sickle-shaped multiseptate microconidia (mag. 1000x). (b) Colony surface; colonies are rapidly growing woolly to cottony, flat, spread, white mycelium. Conidiation causes the white fluffy mycelium to clump, forming thick fluffy tufts. (c) Reverse colony surface; white with pale brownish mycelium, no pigment production and the pale brownish mycelium is the colour of the growth medium.

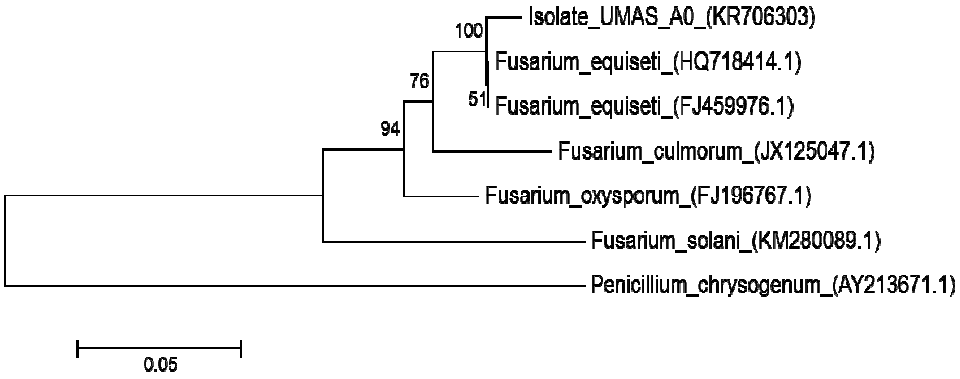

Figure. 2: Neighbour-joining tree from ITS sequences showing the relationship between the isolated indigenous fungus UMAS A0 and other closely related *Fusarium* species retrieved from the GenBank(accession number). Bootstrap values >70% (1000 replicates) are shown on the branches. Bar = 5 nucleotide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

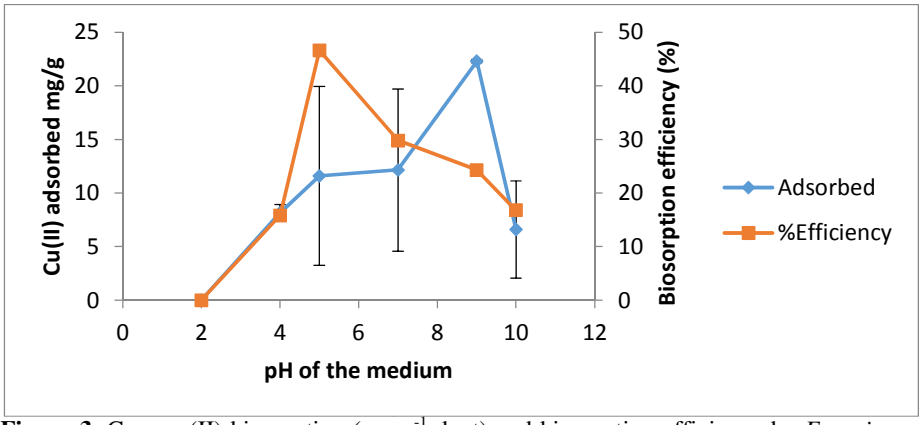

Figure 3: Copper (II) biosorption (mg g^{-1} d.wt) and biosorption efficiency by *Fusarium equiseti* KR706303 at different pH values. Amount of dried biomass: 0.04g; initial metal concentration (Ci): 50 mg L^{-1}; suspension

volume: 20 mL; temperature: 30^{0}C; agitation speed: 150 rpm. The data are the mean values of 3 replicates, and the bars indicate the standard deviation of the mean.

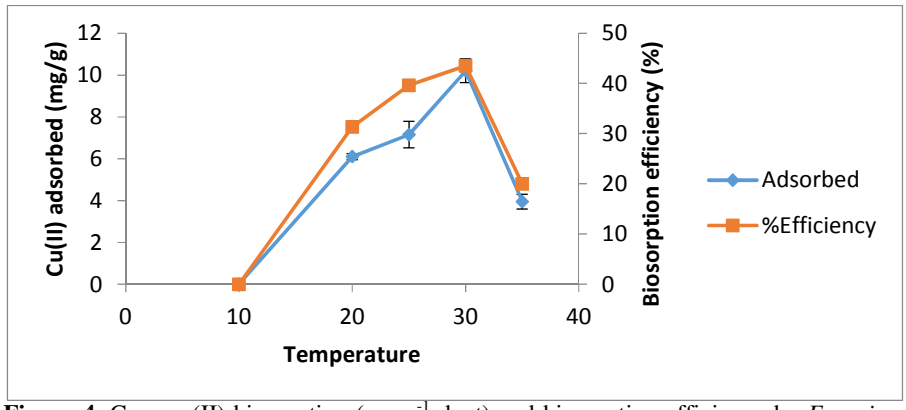

Figure 4: Copper (II) biosorption (mg g^{-1} d.wt) and biosorption efficiency by *Fusarium equiseti* KR706303 at different temperatures. Amount of dried biomass: 0.04 g; initial metal concentration (Ci): 50 mg L^{-1}; suspension volume: 20 mL; pH: 9; agitation speed:150 rpm. The data are the mean values of 3 replicates, and the bars indicate the standard deviation of the mean.

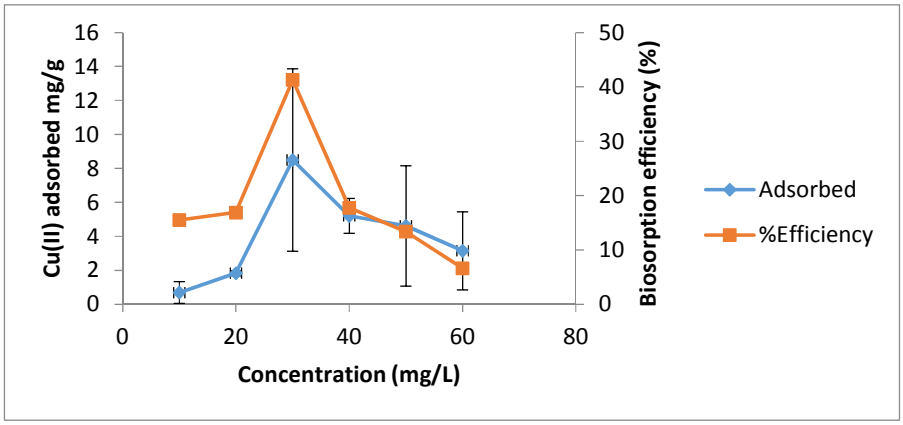

Figure 5: Copper (II) biosorption (mg g^{-1} d.wt) and biosorption efficiency by *Fusarium equiseti* at different concentrations. Amount of dried biomass: 0.04 g; suspension volume: 20 ml; temperature: 30^{0}C; pH: 9; agitation speed: 150 rpm. The data are the mean values of 3 replicates, and the bars indicate the standard deviation of the mean.

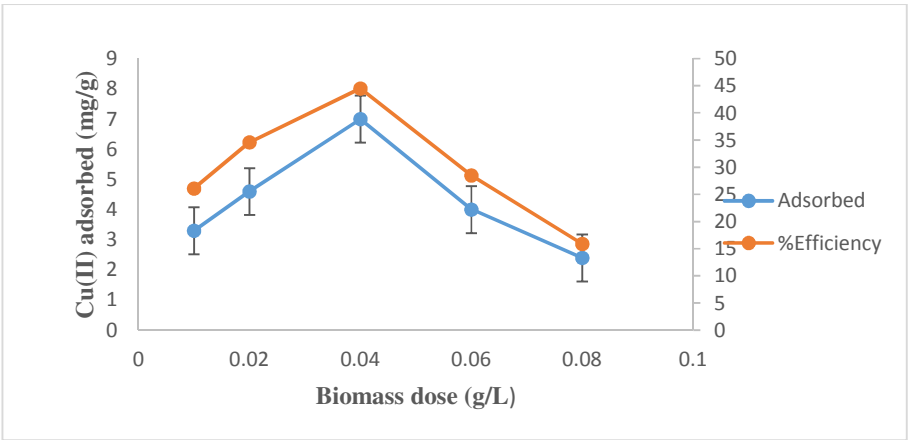

Figure 6: Copper (II) biosorption (mg g^{-1} d.wt) and biosorption efficiency by *Fusarium equiseti* KR706303 at different biomass dose. Initial metal concentration (Ci): 50 mgL^{-1}; suspension volume: 20 ml; temperature:

30^0C; pH: 9; agitation speed: 150 rpm. The data are the mean values of 3 replicates, and the bars indicate the standard deviation of the mean.

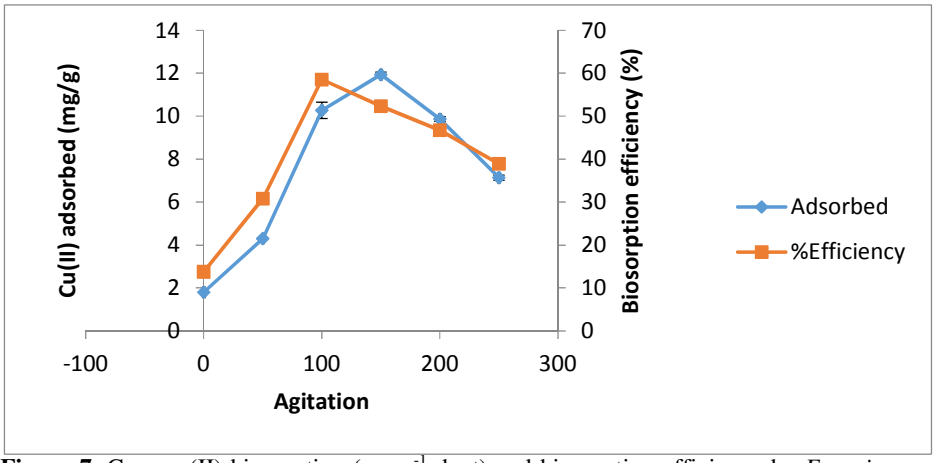

Figure 7: Copper (II) biosorption (mg g^{-1} d.wt) and biosorption efficiency by *Fusarium equiseti* KR706303 at different agitation speed. Amount of dried biomass: 0.04 g; initial metal concentration (Ci): 50 mgL^{-1}; suspension volume: 20 ml; temperature: 30^0C; pH: 9. The data are the mean values of 3 replicates, and the bars indicate the standard deviation of the mean

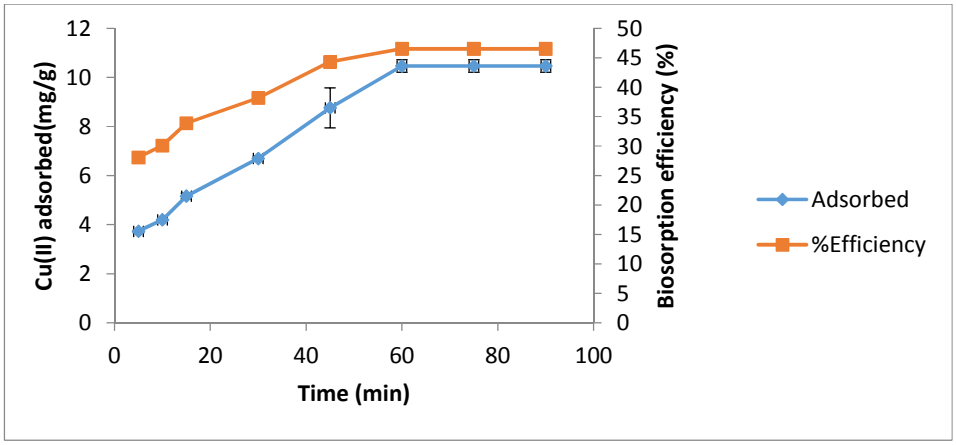

Figure 8: Copper (II) biosorption (mg g^{-1} d.wt) and biosorption efficiency by *Fusarium equiseti* KR706303 at different time intervals. Amount of dried biomass: 0.04 g; initial metal concentration (Ci): 50 mg L^{-1}; suspension volume: 20 mL; temperature: 30^0C; pH: 9; agitation speed: 150 rpm. The data are the mean values of 3 replicates, and the bars indicate the standard deviation of the mean.

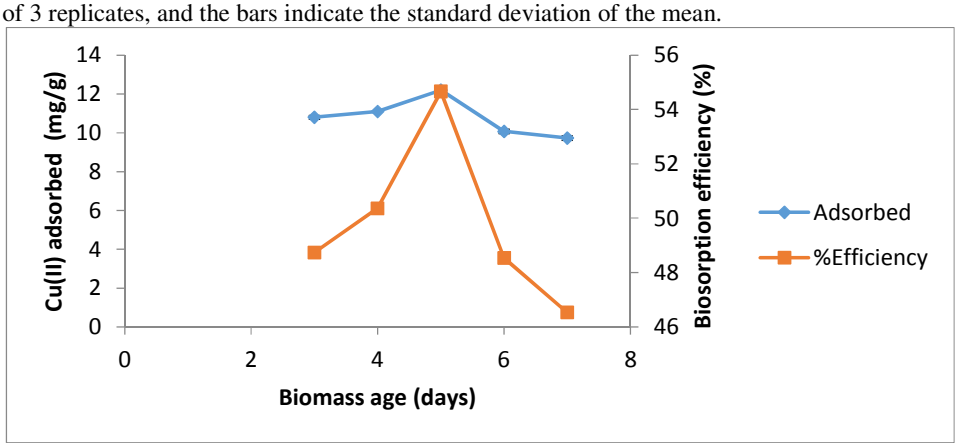

Figure 9: Copper (II) biosorption (mg g^{-1} d.wt) and biosorption efficiency by *Fusarium equiseti* KR706303 biomasses cultivated at different growth periods. Amount of dried biomass: 0.04g; initial metal concentration

(Ci): 50 mgL^{-1}; suspension volume: 20 ml; temperature: 30^0C; pH: 9; agitation speed: 150 rpm. The data are the mean values of 3 replicates, and the bars indicate the standard deviation of the mean.

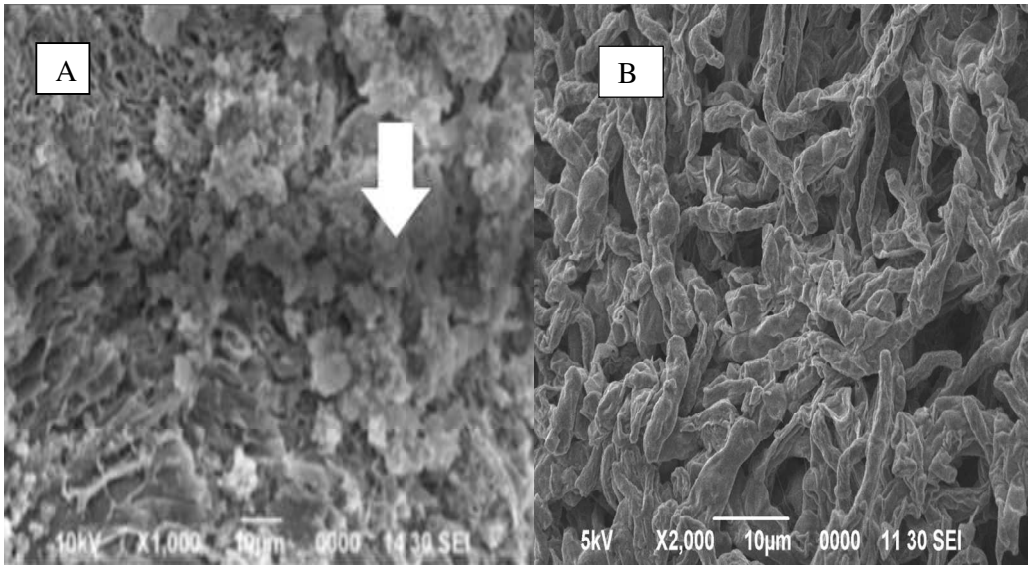

Figure 10: SEM micrographs of *Fusarium equiseti* KR706303 loaded with copper (A), and unloaded with metal (B). Arrow indicate the metal ions.

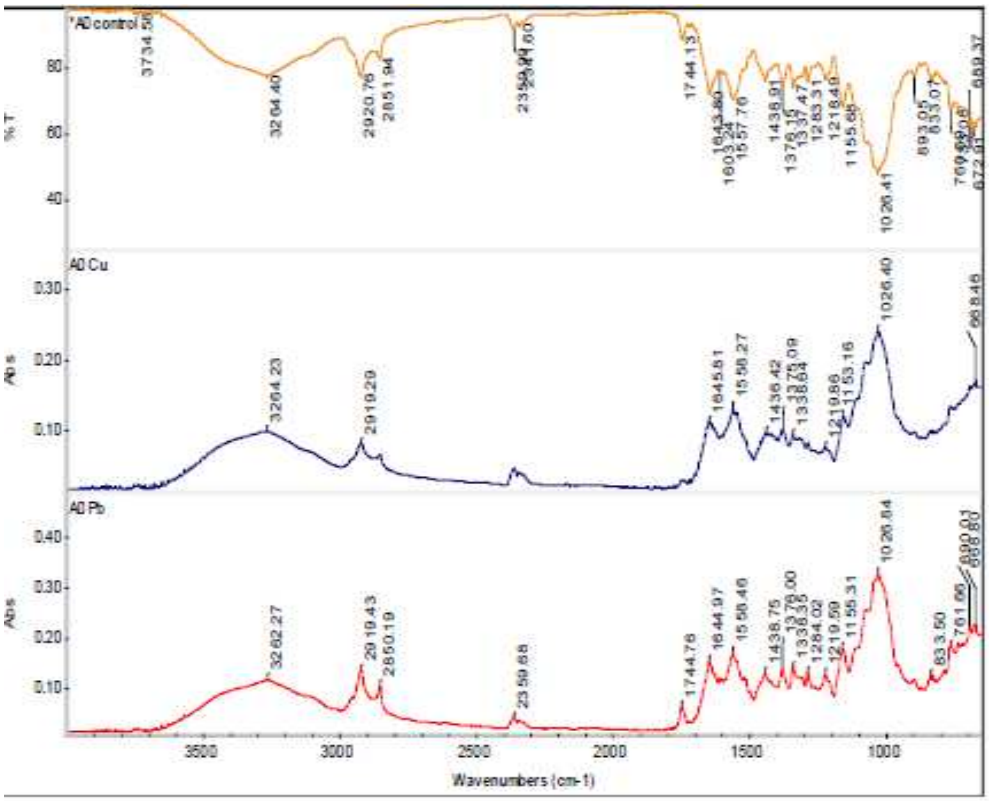

Fig. 11: FTIR spectra of dried unloaded (A0 control), lead(II)-loaded (A0 Pb), and copper(II)-loaded biomass of *F. equiseti* (A0 Cu).

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