HEAVY METAL TOLERANCE AND BIOSORPTION POTENTIAL OF *ASPERGILLUS NIGER* ISOLATED FROM SOLID MINING WASTE

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Abstract

The fungi are the most common and efficient group of the heavy metal resistant microbial family which have potential for metal biosorption study. In this research work, ten fungi were isolated from solid mining waste and soil sample. The isolated fungi were screened for their heavy metals tolerance to different concentrations (0-20 mM) of Cr^{6+}, Cu^{2+}, Cd^{2+}, and Ni^{2+} solutions. Minimum inhibitory concentrations (MICs) for (Cr^{6+}, Cu^{2+}, Cd^{2+}, and Ni^{2+}) were also determined by the agar diffusion method. Most of the isolates were tolerant of the metals. Among all fungal strains, isolated from solid mining waste, *Aspergillus niger* was the highest resistant to Cu^{2+} ion up to 20 mM. Thus *A. niger* exhibiting great tolerance to metal ion was used for biosorption study. The optimum parameters for biosorption (pH, contact time, initial metal concentration and adsorbent dose) were studied. The maximum removal efficiency of copper was observed around 60.27 % at pH 4.5 with 0.1g adsorbent dose for 5h. Metal sorbed adsorbents were characterized by FT IR and SEM analysis. In FT IR spectra, changes in spectral data of biomass were observed after absorption of Cu (II) by *A.niger*. Scanning electron microscopy indicated that the morphology of the biomass considerably changed after metal sorption. It could be concluded that *A.niger* possessed significant heavy metal tolerance and biosorption potential against Cu^{2+}ions.

Keywords: *Aspergillus niger*, metal tolerance, heavy metals, MIC, biosorption

Introduction

Heavy metal pollution is one of the most important environmental problems today because of their toxicity, bio-accumulation tendency, the threat to human life and the environment. Heavy metals are presented in nature and industrial wastewater, so the presence of heavy metals in surface and groundwater pose a contamination problem. A large number of industries can produce and discharge wastes containing different heavy metals into the environment. The main sources of heavy metal pollution are metal plating, mining, battery manufacturing, tanneries, petroleum refining, pigment manufacture, pesticides, etc. (Igwe and Abia, 2003).The release of large quantities of hazardous materials into the natural environment has resulted in several environmental problems and due to their non-biodegradability and persistence, can accumulate in the environmental elements such as food chain, and thus may pose a significant danger to human health (Hlihor *et al*., 2013).

In recent years, microbial biomass has emerged as an option for developing an economic and eco-friendly wastewater treatment process, therefore, applying biotechnology in controlling and removing metal pollution has been paid much attention, and gradually becomes a hot topic in the field of metal pollution control because of its potential application. An alternative process is a biosorption, which utilizes various certain natural materials of biological origin, including bacteria, fungi, yeast, algae, etc. Fungal organisms like *Aspergillus niger*, *Sreptomyces noursei*, *Pseudomonas aeruginosa*, and *Rhizopus arrhizus* have been reported for removal of heavy metals, such as Pb, Cd, and, in particular, Ni (II) (Sar *et al*., 2000). Heterotrophic fungi such as *Mucor* sp., *Aspergillus* sp., *Penicillium* sp., and *Yarrowta* sp. can remove both soluble and insoluble metal species from solutions and can leach metal cations from solid waste (Heinfling *et al*., 1997). Metal

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resistance is defined as the ability of an organism to survive metal toxicity using a mechanism produced in direct response to metal species concerned. This ability of the microorganisms to grow in the presence of heavy metals has potential use in bioremediation of polluted waters (Desai *et al*., 2015).

The application of fungal organisms in the field of biosorption technology has become a part of active research by environmental scientists. Heavy metal tolerance fungi might be present in metal contaminated places. The objective of this study is to isolate and screen heavy metal tolerant (Pb, Cd, Cr, Cu) fungi from contaminated waste and soil and to evaluate their biosorptive potential against heavy metals under laboratory conditions.

Materials and Methods

Sample Collection and Heavy Metals Analysis

The solid mining wastes were collected from near Lepantaung copper mine, Salingyi Township, Sagaing Region. The solid waste samples were air-dried and sieved to achieve the homogeneity and stored in the plastic container for subsequent experiments. Samples were stored in the refrigerator (4ºC) for the isolation of fungi. Soil samples were utilized for the isolation of microorganisms especially fungi. These soil samples were collected from Kanyin-pin-hla village, Laymyethna Township, Ayeyarwady Region. Soil texture and pH were analyzed at the Department of Agriculture (Land Use), Giorgione, Insein Township, Yangon. Soil samples and mining waste were analyzed by Atomic Absorption Spectrometry (AAS) for the concentration of copper, cadmium, nickel, and chromium at the Innovation Center, Department of Higher Education.

Isolation of Fungi from Mining Waste and Soil

The fungal strains were isolated from soil and solid mining waste by serial dilution method (Hayakawa and Kobayashi, 2005).The dilution technique was made by placing one gram of the sample in the test tube containing 10 mL of sterile distilled water and tenfold serial dilution was made by transferring 1 mL of the suspension to another test tube containing 9 mL of distilled water. This step was repeated ten times to obtain a dilution of 10^{-10}. Each volume 0.1 mL from the test tubes (10^{-3}), (10^{-6}), (10^{-9}) was taken and placed on the plate containing Lignocellulose Agar (LCA) culture medium. Chloramphenicol was added to the medium after autoclaving for 15 min at 121 °C to arrest bacterial growth. After 5 to 7 days incubation, larger identical colonies from each plate were isolated. Isolated fungi from master plates were transferred to the medium plate Potato Dextrose Agar (PGA) to obtain pure cultures. The pure cultures were stored in (PGA) slant culture before further analysis.

Screening for Metal Resistance Fungi

The metal resistance properties of ten isolated fungi were determined by the spot plate's method (Zafar *et al*., 2007) using K_2CrO_4, $CuSO_4$, $CdSO_4$ and $NiSO_4$ metal solutions Potato dextrose agar medium was used for heavy metal resistance experiments. The metal solution was added to the sterile medium and made up the concentrations of (5, 10, 15 and 20 mM).Then the test fungi were spotted on metal-containing plate and control plates. The duplicated cultures were carried out in this work. The plates were incubated for 10 days to observe the growth of the spotted area. Metal tolerance was observed as the minimum inhibitory concentration (MIC) of the heavy metal that inhibited the visible growth of test fungi.

Morphological Characterization of Highest Metal Resistant Fungal Isolates

The highest Cu resistance fungus (MR-02) was characterized. The fungus was cultured on Potato Dextrose Agar (PDA), Czapek-Dox Agar (CZA), and Meat Extract Agar (MEA) medium.

After seven days, fungal isolates were studied for its morphological features under the light microscope (at 40x). The isolates were mounted on slide with the help of lactophenol cotton blue. The fungal isolates were identified by comparing these morphological characteristics by using fungal identification manuals.

Preparation of Fungal Biomass

A liquid medium of potato dextrose broth (PDB) with a pH value adjusted to 5 was prepared. 5 days old culture of fungi from LCA plate culture was inoculated into the 500 mL flask containing 100 mL sterile medium incubated at 25°C for 7 days. The fungal cell mass was determined by filtering the culture medium through weighed Whatman filter paper No. (1). Mycelium was thoroughly washed with generous amounts of deionized water, re-suspended and washed again. The biomass thus obtained was suspended in 0.5N sodium hydroxide solutions for 15 min. The pretreated biomass was washed with deionized water until the pH of the solution was in a near neutral range (pH 6.8-7.2). The pretreated fungal biomass was autoclaved for 15min at 121°C. Then the fungal biomass was dried in an oven at 60ºC for constant weight. When the biomass was dry, it was powdered and stored in the desiccator.

Studies on Biosorption

Testing for biosorption efficiency different concentrations of *Aspergillus niger* biomass was combined with 100 mL of $CuSO_4$ solution in 250 mL Erlenmeyer flasks. The flasks were placed on a shaker with a constant speed of 150 rpm and left to equilibrate. Samples were collected at predefined time intervals. The adsorbate was decanted and separated from the adsorbent using Whatman No.1 filter paper. The supernatant was analyzed by AAS. The effect of pH on the copper biosorption using *A.niger* was investigated on a wide pH ranged from 3 to 6. The effect of initial metal ion concentration was also altered. The dosage of biosorbent was also investigated with varying concentration of dead fungal biomass (0.02- 0.14 g). The contact time of the biosorbent with the metal ion solution (1- 6 h) was also optimized further. The removal percentage of copper was calculated by using the following formula:

$$E\ (\%) = \left(\frac{C_i - C_f}{C_i}\right) \times 100$$

where, C_i = the initial copper concentration

C_f = the final copper concentration

E = metal removal efficiency (%)

Characterization of Fungal Biomass

FT IR analysis

The functional groups present on the dried fungal biomass *Aspergillus niger* before and after biosorption were analyzed by FT IR Spectrophotometer, Perkin Elmer. The spectra were recorded over the range 4,000 – 400 cm^{-1} using the recommended standard procedures as described in FT IR Spectrophotometer.

SEM analysis

The surface morphology and fundamental physical properties of *Aspergillus niger* biomass before and after biosorption were characterized by using scanning electron microscope (SEM) for a visual inspection of external porosity and micro texture.

Results and Discussion

Characteristics of Soil Samples and Heavy Metals Concentrations

The properties of soil that was used for isolation of fungi were analyzed. The pH range of soil conditions was 6.24. The soil type was silt loam. The soil texture was determined depending upon the percentage of sand, silt, and clay (Table 1). It was observed that Cu^{2+} had the highest concentration in the solid mining waste 106.26 mg kg^{-1} and the Ni^{2+} , Cr^{6+}, and Cd^{2+}, were 76.73, 40.33 and 76.82 mg kg^{-1}. The Cr^{6+}, Cu^{2+}, Cd^{2+}, and Ni^{2+} contents in soil sample were very lower and some were not detected (Table 2).

Table 1 Analytical Data of Soil Sample

Samples	Texture (%)					
	Sand	Silt	Clay	Total	pH	Soil type
Soil	4.30	76.00	18.20	98.50	6.24 (slightly acid)	silt loam

Table 2 Concentrations of Metal in Soil and Solid Mining Waste Samples

Samples	Metal concentration(mg/kg)				pH
	Cu^{2+}	Ni^{2+}	Cr^{6+}	Cd^{2+}	
Soil	0.004	0.014	0.007	ND	6.24
Solid Waste	106.26	76.73	40.33	76.82	8.2

* ND - Not Detected

Isolation of Fungi from Soil and Solid Mining Waste

Ten fungal strains were isolated from soil and mining waste by the serial dilution method. They were designated as MR-01, MR-02, MR-03, MR-04, and MR-05 which were isolated from metal contaminated waste and MR-06, MR-07, MR-08, MR-09, and MR-10, which were isolated from soil. It can be seen that fungi are able to grow in the heavy metals contaminated places.

Screening for Metal Resistance Fungi

The isolated fungi tolerance to metal ions such as Cr^{6+}, Cu^{2+}, Cd^{2+}, and Ni^{2+} were determined by varying concentration The MICs of fungal isolates against on the four metals ion are shown in Table 3. Most of isolated fungi resisted the individual metal in different concentration except MR-03 and MR-07. MR-01 showed resistance up to 5 mM concentration of Cu^{2+}, Cd^{2+}, and Cr^{6+}. The highest metal resistance fungus (MR-02) is shown in Figure 2. Some fungal isolate can resist at higher concentration of heavy metals due to the various biological factors. Malik (2004) have been reported that the concentration of heavy metal was affected on the growth of fungi. Heavy metal resistant microorganisms play an important role in the bioremediation of heavy metal contaminated places. (Ray and Ray, 2009). Among all fungal isolates, MR-02 showed the highest resistance up to 20 mM concentration (Figure 2). Thus MR-02 was selected for biosorption study of Cu^{2+} ions.

Table 3 Minimum Inhibitory Concentration of Heavy Metals Tolerated by Resistant Fungi

Strain No.	MIC of Heavy Metal Ions (mM)			
	Cd^{2+}	Cr^{6+}	Ni^{2+}	Cu^{2+}
MR-01	5	5	-	5
MR-02	-	-	5	20
MR-03	-	-	-	-
MR-04	-	-	-	15
MR-05	-	-	-	5
MR-06	-	10	-	10
MR-07	-	-	-	-
MR-08	-	5	5	-
MR-09	-	5	5	5
MR-10	-	5	-	5

(-) = No growth

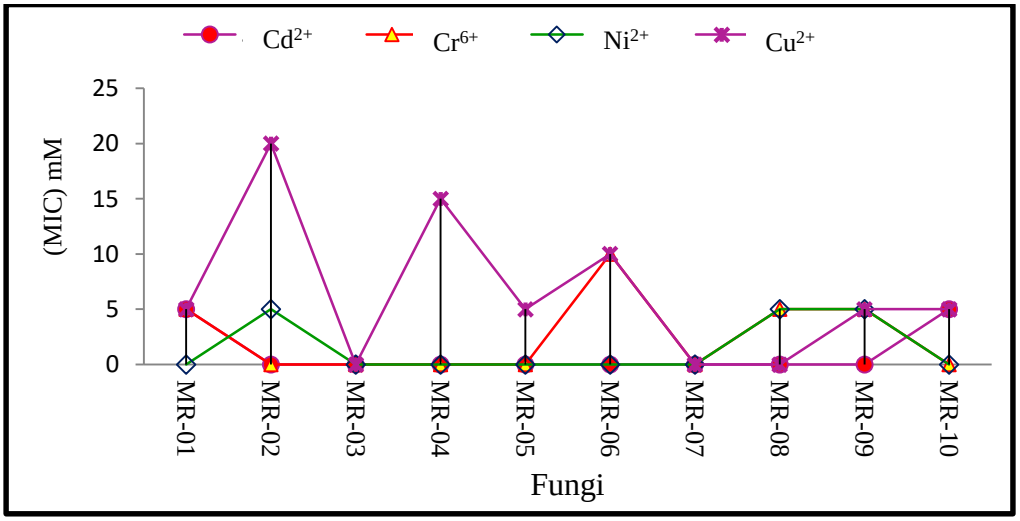


Figure 1 MIC of all fungal isolates against metals

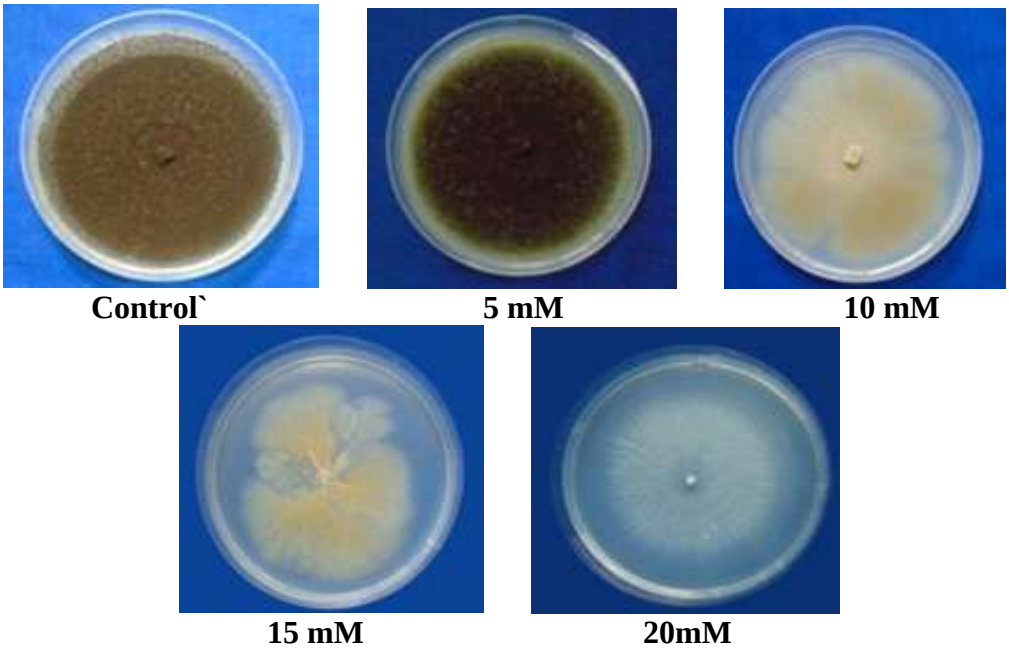


Figure 2 The growth of *Aspergillus niger* with different concentration of (Cu^{2+}) ionsfor 10 days

Morphological Characteristics

Microscopical Characters of isolates MR-02 was observed under compound microscope. Isolates MR-02, colony showed typically black powdery. Conidiophores arising from long, broad, thick-walled, mostly brownish. Conidia in large, radiating heads, mostly globose, irregularly roughened 4.0 - 5.0 µm diameters. Based on the works of literature, references (Domsch, 1993; Ando, 2016) and morphological and microscopically characters, the characters of fungus MR-02 was identified as *Aspergillus niger*.

Evaluation of Biosorption Efficiency

Aspergillus niger biomass showed biosorption efficiency on Cu (II) ions. Maximum biosorption was 60.27% and minimum absorption was 11.11%. The effect of the Cu (II) ions removed by *A. niger* biomass are shown in Figures 3 and 4. Results are in agreement with (Gadd, 1993) who reported that fungi are able to grow in the presence of heavy metals due to physiological adaptation and such adaptation may be associated with increased metal sorption capacity.

Effect of biosorbent dosage and initial metal concentration on Cu(II) ions on biosorption

In the effect of biomass dosage on biosorption, biomass dosages were varied between 0.02 -0.14 g while the initial copper ion concentration and pH were 0.2-1.4mg/mL and 3-6. It was found that optimum adsorption was 60.12 % at 0.1g of *A. niger* biomass for Cu^{2+} (Figure 3-a). Further increase in the biosorbent dosage, the biosorption efficiency had not increased.

The initial heavy metal ions concentration is an important parameter in adsorption since a certain amount of adsorbent can adsorb a certain amount of heavy metal ions. The biosorbent optimum dose was found to be 0.1g. The results represented in Figure 3-b, showed that maximum percent removal of *A. niger* for Cu^{2+} ion was 60.23 % with initial concentration at 1.0 mg/mL. After that the adsorption capacity was decreased with the increasing metal ion concentration. It may be due to the surface of the biomass has less adsorption sites. The results are in agreement with the literature studies reporting high metal ion biosorption at high metal ion concentrations (Gulnaz *et al.*, 2005). Percent copper ion removal decreased from 40.31 to 21.11% when the initial Cu^{2+} concentration was raised from 1.2 to 1.4 mg/mL.

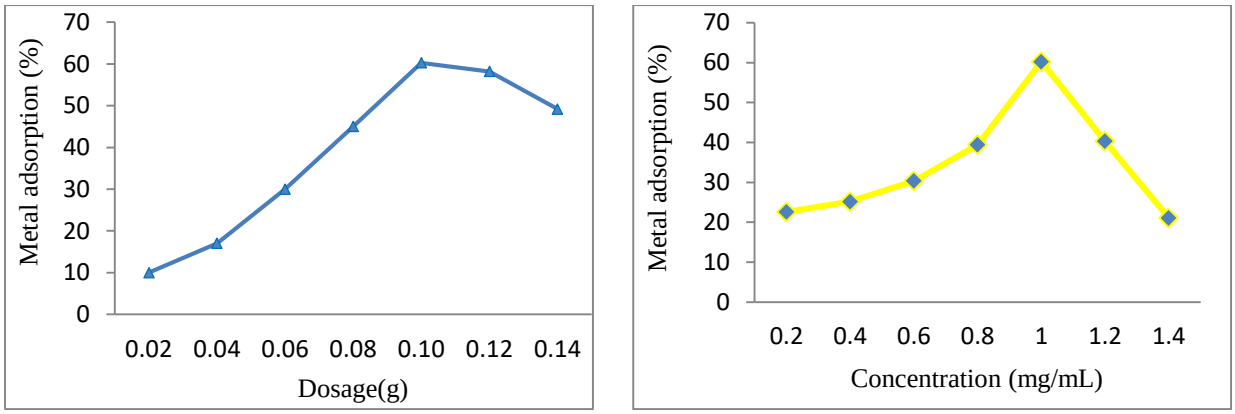
Effect of pH

The pH was an important parameter for adsorption of metal ions from aqueous solution because it affected the solubility of the metal ions, concentration of the counter ions on the functional groups of the adsorbent and the degree of ionization of the adsorbate during reaction. Biomass had active sites capable of binding metal ions. Such bond formation could be accompanied by displacement of protons and was dependent in part on the extent of protonation which was determined by the pH (Volesky, and Holan, 1995). To examine the effect of pH on the Cu^{2+} removal efficiency, the pH was varied from 3 to 6. Pretreated fungal biomasses of *Aspergillus niger* was contacted Cu^{2+} ions in separate solutions at concentration of 1.0 mg/mL for 5 h respectively. Figure 4(b) showed that the uptake of metal ions depended on pH. It was observed that *Aspergillus niger* exhibited maximum sorption capacity for the Cu^{2+} in the pH of 4.5 with maximum efficiency 60.27 %. Above this pH substantial declined in metals uptake was evidenced which represented the pH factor being highly sensitizing element. In similar findings by earlier investigators it has been attributed to protonation or poor ionization of acidic functional group of cell wall at low pH, inducing a weak complex affinity between the cell wall and the metal ions (Chergui *et al.*, 2007). The reduction in metal ions uptake by fungus at high pH can be explained on the basis that at higher pH values the metal ions may accumulate in the cells or the intra-fibular capillarity of the cell walls by a combined sorption of micro precipitation mechanism (Beveridge,

1986). With an increase in pH, the negative density on cell surface increased due to deprotonation of the binding sites thus improved biosorption of heavy metal ions.

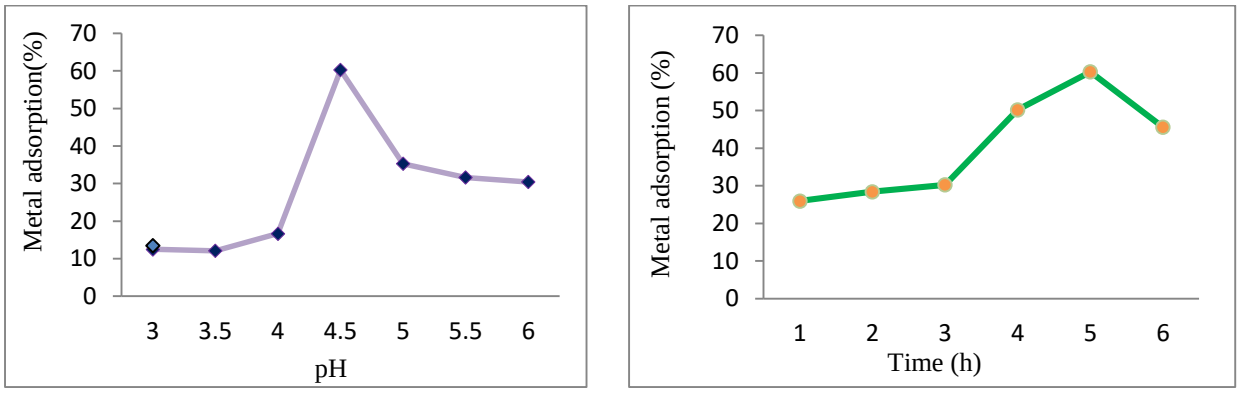
Effect of contact time

In the effect of contact time, metal solution concentration of 1.0 mg/mL was adjusted to the maintain optimum pH 4.5 and biosorbent 0.1 g. The filtrate was collected by using Whatmann filter at different time intervals of 1, 2, 3, 4, 5 and 6 h. As can be seen in Figure 4(b) the percentage removal of ions increased with increasing the shaking time. A sharp increase was observed at optimum time of 5 h for biosorption of Cu^{2+} by *A.niger*. At the equilibrium maximum adsorption for Cu^{2+} was observed as 60.26 %. This result was important, as equilibrium time was one of the important parameters for an economical wastewater treatment system.



(a) (b)

Figure 3 (a) Effect of biosorbent dosage on Cu(II) ions biosorption by *Aspergillus niger*
(b) Effect of initial metal concentration on Cu(II) ions biosorption by *Aspergillus niger*



(a) (b)

Figure 4 (a) Effect of pH on Cu (II) ions biosorption by *Aspergillus niger* (b) Effect of contact time on Cu (II) ions biosorption by *Aspergillus niger*

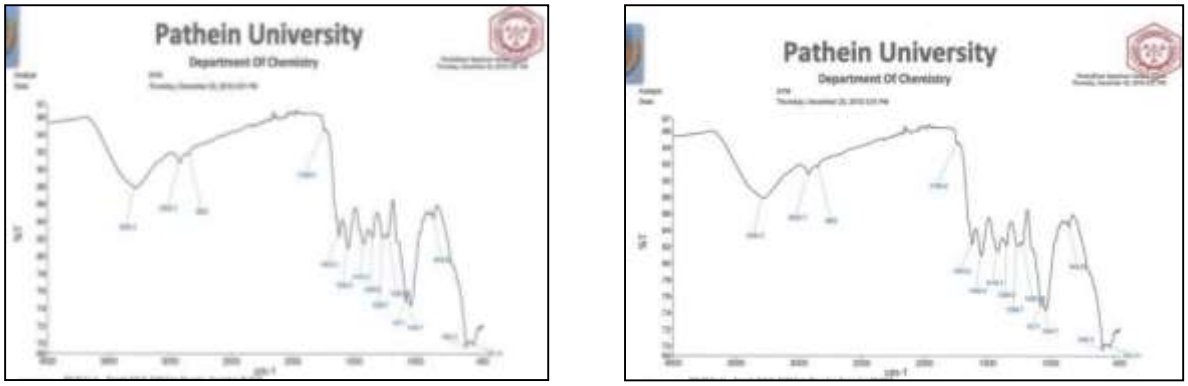
FT IR Analysis of Fungal Biomass Before and After Cu II Biosorption

The negatively charged functional groups present in the biomass were identified by FT IR spectrum. Table 4 shows the FT IR spectral data of *Aspergillus niger* biomass. In Figure 5-a , *A. niger* fungal biomass shows stretching vibrations at 3285, 1740 and 1040 cm^{-1}, which indicates the presence of the (-OH,); (-C=O); (-C-O) respectively. It was found that the FT IR spectra of before and after biosorption of heavy metal Cu II were not significantly different but after biosorption some of the peaks were shifted and gave the more sharp (Figure 5-b). Changes in wave number at

3285, 2852, 1740, and 1040 cm^{-1} in metal-bounded biomass could be assigned to participation of -OH, C-H, C=O and -C-O groups in biosorption process of copper II ions .

Table 4 FT IR Spectral Data for Pretreated *Aspergillus niger* Biomass Before and After Cu^{2+}Biosorption

Fungal biomass	Functional groups	Wave number (cm^{-1})	
		Before Cu^{2+} biosorption	After Cu^{2+} biosorption
Aspergillus niger	υ_{O-H} of alcohol	3285	3276
	υ_{C-H} of CH_2 and CH_3	2924, 2852	2924, 2855
	$\upsilon_{C=O}$	1740	1746
	$\upsilon_{C=C}$ of aromatic ring	1622, 1549	1622, 1543
	δ_{C-H} of CH_3	1416, 1346	1410, 1370
	υ_{C-O} of alcohol	1040	1028

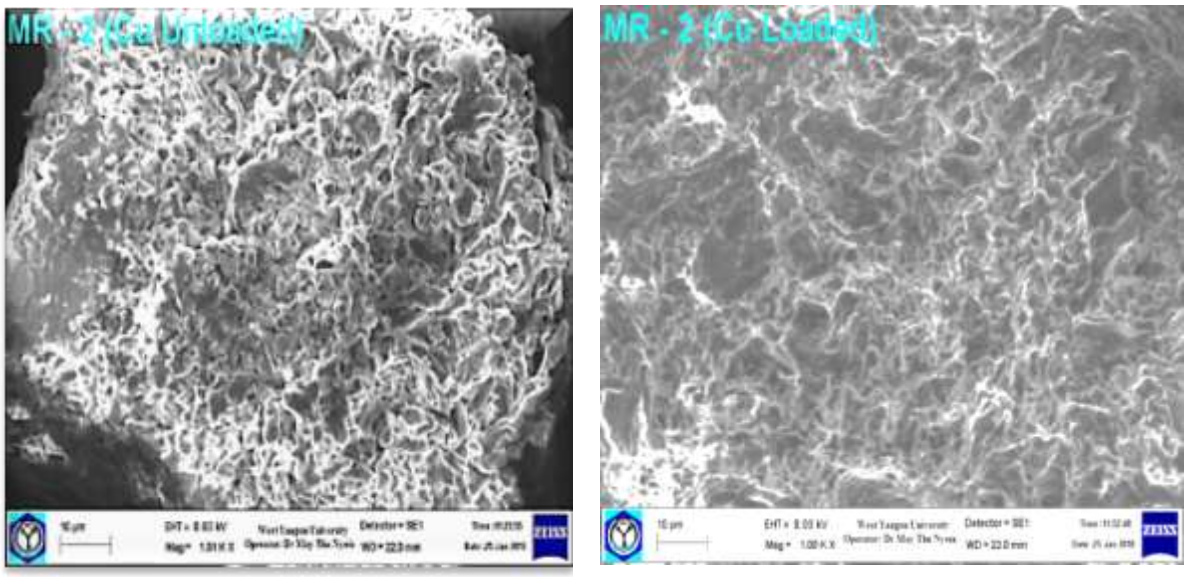


(a) **(b)**

Figure 5 FT IR analysis of alkali pretreated *Aspergillus niger* (a) before and (b) after biosorption

SEM Analysis of Fungal Biomass Before and After Cu II Biosorption

Scanning electron microscopy (SEM) had been a primary tool for characterizing the surface morphology and fundamental physical properties of the adsorbent surface. It was useful for determining the particle shape, porosity and appropriate size distribution of the adsorbent (Arami *et al.*, 2008). SEM micrographs of *Aspergillus niger* biomass before (Figure 6-a) and after (Figure 6-b) adsorption of Cu^{2+} were compared. The SEM micrographs of *Aspergillus niger* without metal stress (control) possessed a rough, heterogeneous and a large surface area. As seen in Figure, the adsorbents had some heterogeneity which is supposed to be the active site for metal Cu^{2+} binding. After Cu^{2+} adsorption, the surface of fungal biomass was flattened (Figure 6-b). The SEM micrographs of fungal biomass *Aspergillus niger* showed the changes in the structure after sorption indicating the effective adsorption of copper II ions in the interstices and cavities on the external surface of this biosorbent. Results are in agreement with the Mondal, *et al*. (2017) who indicated that the surface changes occurred after binding of Cu^{2+} ions onto the surface of *A. niger* biomass.



(a) **(b)**

Figure 6 SEM micrographs of *Aspergillus niger* fungal biomass (a) Cu^{2+} ions unloaded (b) Cu^{2+}ions loaded

Conclusion

In this study, ten fungal strains were isolated from solid mining waste and soil sample. The isolated fungal strains were screened for their tolerance to four metal ions ($Cu^{2+,}$ $Cd^{2+,}$ Ni^{2+} and $Cr6^{+}$). Among them, fungus *Aspergillus niger* isolated from metals contaminated waste showed the highest $\mathbf{Cu^{2+}}$ tolerence up to (20 mM). This study reveals that heavy metal contaminated places might be considered as a precious natural source of the resistant fungal strains. Thus the fungal biomass of *A. niger* was evaluated for biosorption study of Cu (II) ions from aqueous solution. The maximum sorption efficiency of biomass *A.niger* for the Cu^{2+} ions was 60.27 % at pH 4.5. Biomass of *A. niger* exhibited sorption potential to bind with Cu^{2+} ions and uptake Cu (II) ions from the aqueous solution. Though the mechanism of sorption potential with Cu^{2+} ions should be fruited studied. The results of the present study suggest that *Aspergillus niger* isolated from solid mining waste in metal contaminated places can be useful for the biosorption of heavy metals from wastewater.

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