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TECHNICAL ARTICLE

Biosorption and Bioaccumulation of Cu^{2+} from Aqueous Solution Using Living *M. amorphae* Isolated from Mine Tailings

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Abstract Mining generates large amounts of wastewater, which may contain elevated levels of potentially toxic metals. We evaluated the use of live cells of *Mesorhizobium amorphae* CCNWGS0123 as a new, relatively inexpensive and environment-friendly adsorbent material for removal of toxic metals from aqueous solutions, such as mine tailings leachate. Maximum copper uptake and removal efficiency was attained at a dosage of 0.5 g L^{-1}, a pH of 5.0, an agitation speed of 150 rpm, and at a temperature of 28 °C for an initial Cu^{2+} concentration of 100 mg L^{-1}; equilibrium was achieved within 30 min. The adsorption isothermal data matched Langmuir and Freundlich isotherms well, with correlation coefficients of 0.931 and 0.932, respectively. FT-IR analysis indicated that many functional groups with a negative charge on the cell surface were involved in the interaction between live CCNWGS0123 and copper ions. Scanning electron microscope results confirmed that there was some copper sediment on the cell surface, causing deformation, aggregation, and cell surface damage. Cu^{2+} accumulation was related to cell growth, and copper uptake was not only due to cell-surface binding, but also to intracellular accumulation. Live cells of CCNWGS0123 are a new biosorbent that have the potential to be economical when small batches of biomass are used to remove Cu^{2+} from an aqueous solution.

Keywords Biosorption · Bioaccumulation · Copper · Live cells · *Mesorhizobium* · Mine water

Introduction

Toxic metals in the aquatic environment are a major source of environmental concern. Tailings from mining operation can release high concentrations of Cu, Zn, Fe, Mn, Ni, Pb, and Cd (Monica et al. 2008). Copper (Cu), though an essential element for human life, can cause series problems, such as liver and kidney damage, stomach and intestinal distress, and anemia (Dönmez and Aksu 2002; Kandah et al. 2002; Özer et al. 2004). The World Health Organization (WHO) recommends a maximum acceptable concentration of 1.5 mg L^{-1} of Cu in drinking water (Rao 1992). The United State Environmental Protection Agency (USEPA) specifies a threshold aqueous concentration limit, defined as the maximum contaminant level, of 1.3 mg L^{-1} (USEPA 2002).

The use of biomass as sorbents has been studied as a potentially inexpensive, simple, and effective technology for removal of toxic metals from aqueous solution (Abu Al-Rub et al. 2004; Demir and Arisoy 2007; Hawari and Mulligan 2006; Volesky 2001). Studies have reported the removal of toxic metals ions onto different organism such as bacteria, yeast, and molds (Galli et al. 2003; Ozdemir et al. 2003). The cell walls of these materials have many functional groups with a negative charge (e.g., carboxyl,

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hydroxyl, phosphate, imidazole, amine), which can form coordination complexes with metals (Abu Al-Rub et al. 2004; Wong et al. 2000). CCNWGS0123 is a gram-negative soil bacterium isolated from a mining region in the Gansu province of China that has profound scientific and agronomic significance due to its ability to establish nitrogen-fixing symbiosis with leguminous plants, which is very important to the maintenance of soil fertility (Somasegaran and Hoben 1994). We chose live cells of *Mesorhizobium amorphae* strain CCNWGS0123 for the biosorption of Cu (II) as an alternative biomaterial for the treatment of wastewater because it is easy to manipulate and cost effective.

The major goal of this study was to investigate the metal uptake, removal efficiency, and bioaccumulation of copper (II) removal from aqueous solution with regard to the effects of pH, initial copper concentration, contact time, temperature, and biomass dosage. FT-IR analysis was used to study the biosorption mechanism before and after biosorption. In addition, scanning electron microscopy (SEM) was used to study the surface structure and cell morphology. To our knowledge, this is the first time that live cells of *M. amorphae* strain CCNWGS0123 have been used as an absorbent for copper ions.

Materials and Methods

Isolation and Screening of Copper-Resistant Rhizobia

Robinia pseudoacacia root nodules were collected from heavy metal mine tailings. Tryptone yeast (TY) liquid medium (5 g tryptone, 3 g yeast extract, and 0.7 g $CaCl_2{\cdot}2H_2O$ per liter, pH 7.2) and standard methods described by Vincent (1970) were used for the isolation of root nodule bacteria CCNWGS0123. Single colonies were picked and repeatedly streaked on TY medium. A total of 76 isolates were screened for copper resistance by growth on TY medium supplied with $CuSO_4{\cdot}5H_2O$ at final concentrations of 50, 150, 250, 350, and 700 mg L^{-1}. The bacteria were incubated at 28 °C for both the isolation and screening.

Biosorbent Preparation

Strain CCNWGS0123 was pre-cultured in TY medium at 28 °C for 3 days with shaking (150 rpm). At the end of the exponential phase the cells were collected by centrifugation at 12,000 rpm for 15 min. The collected cells were rinsed three times with sterilized distilled deionized (dd) H_2O, and then re-suspended in designated toxic-metal solutions for biosorption experiments.

Metal Solution Preparation

Solutions of Cu^{2+} ions were prepared by dissolving predefined amounts of $CuSO_4{\cdot}5H_2O$ in distilled water, so as to achieve concentrations of 1,000 mg L^{-1} in each flask. Different initial concentrations of metal ions were prepared by diluting the stock solution with dd H_2O. The pH of the solutions was adjusted using 0.3 N HCl and 0.1 N NaOH to achieve the desired values. All treatments in this study were replicated three times and mean values and standard error were calculated.

Metal Ions Uptake and Initial Metal Ions Concentration Studies

Effects of different dosage and initial metal concentration on copper uptake and biosorption percentage by live cells of CCNWGS0123 were studies in order to determine the optimum biosorption conditions. All samples were agitated at 150 rpm and 28 °C for 24 h in an initial Cu^{2+} concentration ranging from 50 to 350 mg L^{-1} of Cu^{2+} with biosorption doses ranging from 0.5 to 2.5 g L^{-1} dry weight. The residual Cu ions in the supernatant were measured using an atomic absorption spectrophotometer (AAS) (Hitachi Z-5000, Japan) after centrifugation at 10,000 rpm for 10 min. The amount of copper adsorbed by the live biomass was calculated using the following equation (Kandah et al. 2002)

$$q_e = (C_0 - C_e)V/X$$

where q_e is metal uptake (mg g^{-1} of dry cells); C_0 and C_e are the concentrations of the metal ions before and after biosorption was the initial metal ion concentration mg L^{-1}; V the initial volume of copper solution ml L^{-1}; and X is the mass of the live cells (g L^{-1} of dry cells).

The values of biosorption percentage ratio were evaluated as follows (Chen et al. 2008).

$$\text{Removal ratio } (\%) = \frac{C_0 - C_e}{C_0} \times 100$$

Optimal Agitation Speed, pH and Temperature

The effects of varying agitation speed, pH, and temperature on copper ions uptake were studied with a biosorption dose of 0.5 g L^{-1} and an initial metal concentration of 100 mg L^{-1} Cu^{2+}. The agitation speed was adjusted from 60 to 210 rpm and the pH was adjusted using a digital pH-meter in our lab from 1 to 6. Temperature was adjusted from 20 to 40 °C. After 24 h, the supernatant was collected by centrifuging (12,000 rpm for 10 min) and the metal ion concentrations were analyzed immediately using atomic absorption spectrometry (AAS).

Sorption Isotherms

The adsorption isotherms were characterized by the Langmuir (1918) and the Freundlich (1907) isotherm models at a dosage 0.5 g L^{-1}, with the biosorbent was suspended in a copper ion solution with initial concentrations ranging from 50 to 500 mg L^{-1}. The residual metal concentration in the suspension was measured after 24 h of incubation at 28 °C and agitation at 150 rpm. The Langmuir model is usually used to characterize the equilibrium between the biomass and aqueous solution as a reversible chemical equilibrium between species. A linear form for the Langmuir model is given as:

$$q_e = Q_{max} LC_e / 1 + LC_e$$

where q_e is metal uptake (mg g^{-1} dry cell); C_e is residual metal concentration in solution at equilibrium with the biosorbent (mg L^{-1}); Q_{max} indicates the maximum monolayer biosorption capacity of the biosorbent (mg g^{-1}) and L is the Langmuir constant (L/mg) and is related to the affinity of adsorption.

The Freundlich model was originally proposed as an empirical equation to describe the data for heterogeneous adsorbents. A linear form of the Freundlich model is given as:

$$\log q_e = \log K + 1/n \log C_e$$

where K (mg g^{-1}) and $1/n$ (g L^{-1}) are the Freundlich constants, which represent sorption capacity and sorption intensity, respectively.

Effects of Contact Time

The live cell biomass was suspended in 300 mL of ddH_2O containing 100 mg L^{-1} of copper ion solution in a 500 mL Erlenmeyer flask with a bacterial concentration of 0.5 g L^{-1}. The suspension was maintained at an agitation speed of 150 rpm, a temperature of 28 °C and a pH of 5.0. Samples were taken every 5 min from the solution for the first hour and then every 60 min up to 420 min. Samples were centrifuged at 10,000 rpm for 5 min. The residual Cu ions in the supernatant were measured by AAS.

Determination of the Functional Groups on CCNWGS0123 Cells

Infrared analysis was performed with a 330 FT-IR (Fourier transform-infrared spectrometer, NICOLET AVATRA, USA Nicolet Co) at a range of 4,000–400 cm^{-1} of live *M. amorphae* CCNWGS0123 biomass before and after the biosorption process at a dosage of 0.5 g L^{-1} and 100 mg L^{-1} of Cu^{2+}. Two milligram of powdered (dry weight) cells was mixed and ground with 200 mg of KBr in an agate mortar in order to determine the functional groups and possible binding site involved in copper sorption.

Scanning Electron Microscopy Analysis (SEM)

The strain surface structure and cell morphology of CCNWGS0123 were examined after being loaded with 100 mg L^{-1} of copper ions at different magnifications using a scanning electron microscope (SEM, JEOL- model:JSM-6360 LV, USA). The cells were fixed with 3 % glutaraldehyle for 24 h followed by dehydration with a graded series of ethanol concentrations (30, 50, 70, 80, 90, and 100 %) for 30 min each. Cells were then dried in a critical-point dryer using CO_2 (EMITECH, K850-Englaned). The metal loaded samples were mounted on a stainless steel stab with double stick tape with a thin layer of gold in a high vacuum and examined with the SEM.

Bioaccumulation of Copper by *Mesorhizobium amorphae* CCNWGS0123

0.2 g of cells (dry weight) was inoculated into 200 mL TY medium containing 100 mg L^{-1} of Cu^{2+} and incubated at 28 °C at 150 rpm. Cells were harvested at 48, 72, 96, and 122 h, respectively after inoculation by centrifugation at 6,000 rpm for 25 min. After washing three times with sterilized dd H_2O, the cell pellets were agitated with 10 mM sterilized EDTA at 28 °C with agitation at 150 rpm for 10 min in order to remove copper ions adsorbed to the cell surface. After further centrifugation at 14,000 rpm for 25 min, the cells were suspended in 5 mL of 0.1 M HNO_3, and the solution was centrifuged at 14,000 rpm for 30 min. The supernatant was used for determination of cell wall bound Cu^{2+} (Carrasco et al. 2005) with AAS. The pellets, after centrifuging, were dried at 105 °C for 48 h, and the cells were digested in Teflon bombs with H_2SO_4–$HClO_4$ (V:V = 3:1) at 110 °C for 3 h. Then, the cells were diluted with ddH_2O (Sousa et al. 2008) for determination of intracellular accumulated Cu^{2+} (Carrasco et al. 2005).

Results and Discussion

Screening of Copper-Resistant Rhizobia

Heavy metal contaminated soils and some plants grown in metal mine tailings and leachate can serve as a source of metal resistant bacteria (Monica et al. 2008). In this study, a total of 72 isolates were obtained but only *M. amorphae* CCNWGS0123 could grow in TY medium supplemented with 2.2 mM of Cu^{2+}. Therefore, strain CCNWGS0123 was selected as a copper resistant bacterium and used in this study.

Effect of Bacterium Cells Dose and Initial Copper Concentration on Biosorption

The effect of varying the live CCNWGS0123 cells dosage with various copper ions concentration ranging from 50 to 350 mg L^{-1} on copper uptake is shown in Fig. 1. The findings showed that as the dosage concentration was increased from 0.5 to 2.5 g L^{-1}, the biosorption capacity, in mg g^{-1}, decreased with increasing initial copper concentration. The highest biosorption capacity was at 0.5 g L^{-1} (Fig. 1); these results are in agreement with Tangaromsuk et al. (2002). This decrease in copper uptake has many possible explanations, such as solute availability, binding sites interference, and electrostatic interaction (Dönmez and Aksu 2002). Therefore, live CCNWGS0123 strain binding sites remain unsaturated due to a lower adsorptive capacity utilization of biomass. According to these results, a more economical design for the removal of copper ions from aqueous solution would involve using small batches of biomass (Abu Al-Rub et al. 2004).

The data also shows that the copper removal ratio decreases as the initial copper concentration was increased from 50 to 350 mg L^{-1}. The lowest copper removal (12.7, 18.6, 18.3, 23.2, and 37.4 %) was achieved at the highest initial copper (II) concentration (350 mg L^{-1}). This may be due to insufficient surface area to accommodate all of the dissolved metal available. At a lower concentration (100 mg L^{-1}), all of the copper (II) in solution could interact with the available binding sites in the live CCNWGS0123 cell walls at each dosage and so the copper (II) adsorption percentage was higher (34.6, 44.3, 50.9, 59.8 and 60.9 %) (Aksu and Balibek 2007). According to our results, the optimum biosorption dosage and initial copper concentration was 0.5 g L^{-1} and 100 mg L^{-1}, respectively. The following experiments were conducted under these conditions.

Effect of pH on Desorption Efficiency

The pH has a major effect on metal uptake (Abu Al-Rub et al. 2004; Iyer et al. 2004; Ozdemir et al. 2004) due to the interaction between the biomass and the dissolved metals, such as the change in metal speciation and the available negative functional groups on the cell surface at different pHs (Maurya et al. 2006). So, the copper (II) removal by live *M. amorphae* CCNWGS0123 was studied as pH ranged from 1 to 6 (Fig. 2).

At low pH (1.0), there were less amiable binding sites, because carboxyl groups are acidic; this decreased copper uptake and the removal ratio. As the pH was increased, the electrostatic attraction between live CCNWGS0123 and copper ions increased gradually and the competing effect of hydrogen ions decreased; thus, the highest copper (II) uptake and removal efficiency was achieved at pH 5.0 (79.1 mg g^{-1} and 39.7 %, respectively) (Abu Al-Rub et al. 2004; Ekmekyapar et al. 2006; Meena et al. 2008; Pagnanelli et al. 2003). Copper could precipitate as hydroxides at pH 6 (Harris and Ramelow 1990; Villaescusa et al. 2000). Therefore, the optimal pH for live CCNWGS0123 desorption is at pH 5.0 for Cu^{2+}.

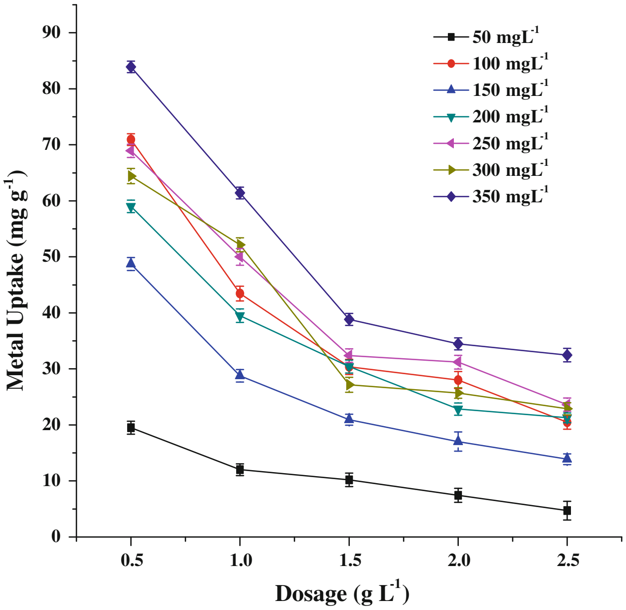

Fig. 1 Copper uptake by live cells of CCNWGS0123 with different biosorbtion dosage range from 0.5 to 2.5 g L^{-1} over an initial copper concentration range from 50 to 350 mg L^{-1}

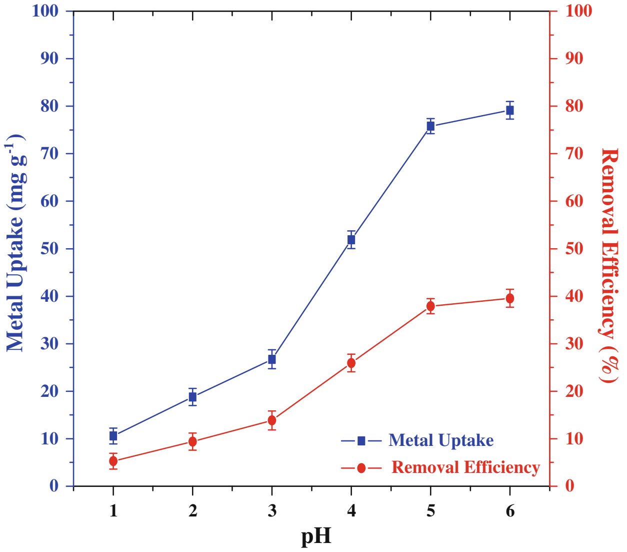

Fig. 2 Effect of pH on desorption efficiency of copper (II) by cells of CCNWGS0123 (initial Cu^{2+} concentration: 100 mg L^{-1}; temperature 28 °C; agitation speed 150 rpm; dosage: 0.5 g L^{-1})

The effect of five different temperatures on metal uptake by live CCNWGS0123 was studied in batch experiments. There was no obvious difference in metal uptake and removal efficiency with increasing temperature from 22 to 40 °C; this agrees with Yuan et al. (2009).

Effects of Shaking Time

Figure 3 shows the effect of varying the agitation speed from 60 to 210 rpm on copper (II) uptake and the removal ratio by live CCNWGS0123. The monitored biosorption capacity and removal ratio increased gradually until reaching a maximum at 150 rpm (78.7 mg g^{-1} and 39.4 %, respectively). It has been reported that agitation promoted the transference of copper ions to biosorbent by increasing contact. According to our results, the copper uptake by live *M. amorphae* CCNWGS0123 was more effective with a moderate agitation speed of 150 rpm (Bai and Abraham 2001; Li et al. 2010).

Effects of Contact Time

Contact time was also evaluated as an important factor affecting copper uptake. Figure 4 shows the biosorption capacity of copper ions by live CCNWGS0123 as a function of contact time ranging from 5 min to 24 h. The biosorption of copper by live CCNWGS0123 was significantly enhanced by increased contact time up to 30 min. After this, equilibrium was reached so that increasing the removal time to 24 h did not have notable effects. This is quite normal as biosorption is considered to be a spontaneous process and often occurs very rapidly (Hashim and Chu 2004; Lin et al. 2012; Sari and Tuzen 2008; Volesky 2001). This rapid sorption indicated that passive surface uptake happens on the biomass surfaces and suggests that intercellular bioaccumulation might contribute to the copper (II) uptake.

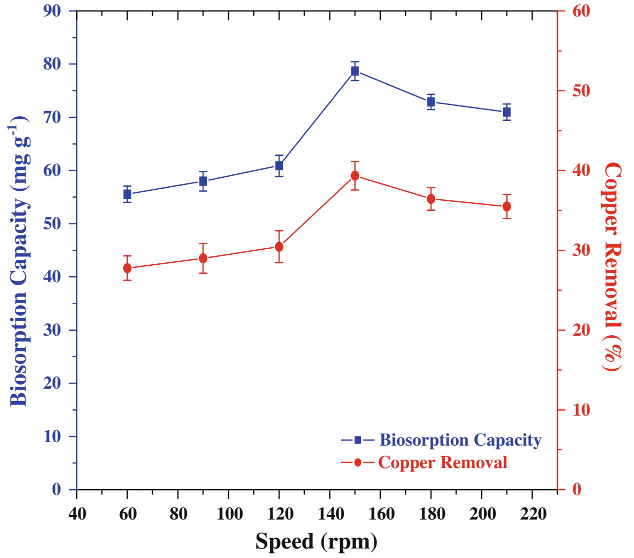

Fig. 3 Effect of shaking time on metal uptake and copper (II) removal (II) by live cells of CCNWGS0123 (initial Cu^{2+} concentration: 100 mg L^{-1}; temperature 28 °C; pH 5.0; biosorbent dose: 0.5 g L^{-1})

Biosorption Equilibrium Isotherms

Langmuir and Freundlich adsorption isotherms are simple mathematical relationships and are widely applied in the equilibrium analysis to understand the sorption mechanisms. They can be used to explain the relationship between copper concentration in solution and the amount of copper adsorbed on an adsorbent at equilibrium. The experimental isotherm data were fitted to Langmuir and Freundlich isotherms for nonlinear regression analysis. The plots are presented in Fig. 5.

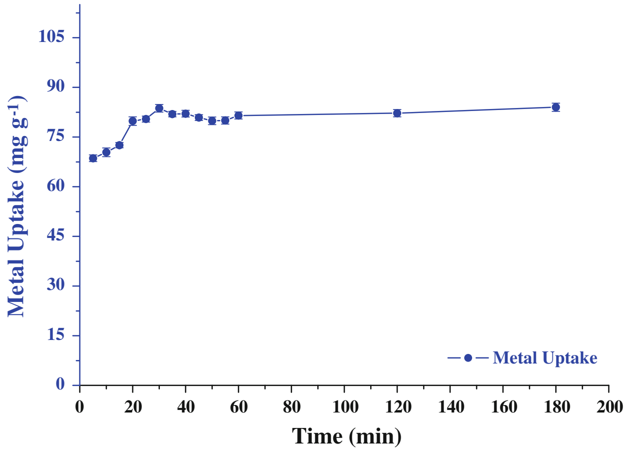

Fig. 4 The adsorption kinetic curves of Cu (II) by the biomass of live *M. amorphae* CCNWGS0123 (initial Cu^{2+} concentration: 100 mg L^{-1}; temperature 28 °C; agitation speed 150 rpm; dosage: 0.5 g L^{-1})

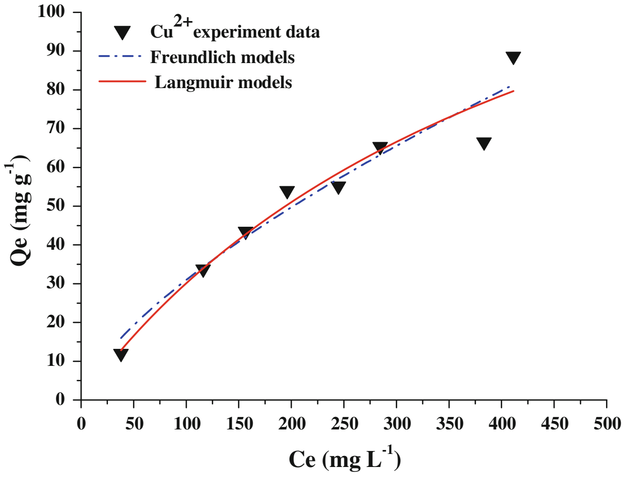

Fig. 5 Biosorption equilibrium isotherms fitting plots of copper (II) uptake onto live CCNWGS0123 strain

Table 1 Adsorption constants estimated from simulations with Langmuir and Freundlish models equation simulations for biosorption of Cu^{2+} by live strain CCNWSGS0123

Strain	Langmuir constants			Freundlich constants		
	Q_{max} (mg g^{-1})	b (mg L^{-1})	R^2	K_f (mg g^{-1})	$1/n$ (mg $L^{-1})^n$	R^2
Live	169.71	0.00215	0.93439	1.3322	0.6831	0.93001

As shown in Table 1, the correlation coefficient of Langmuir and Friendilish isotherms was 0.931 and 0.932, respectively. These results indicated that the live CCNWGS0123 was saturated on a monolayer (Olak et al. 2009; Tunali et al. 2006) and demonstrates heterogeneous energetic distribution (Sawalha et al. 2006).

FT-IR Spectroscopic Study

IR spectra were used in this study to determine the functional groups involved in metal binding before and after the sorption process. The IR spectra obtained in the range of 4,000–500 cm^{-1} showed several peaks Fig. 6, reflecting the complex nature of cell wall lipopolyscharides and other cell surfaces, and membrane polymerase, which contains anionic ligands, such as OH^-, CO_3^{2-}, HCO_3^-, PO_4^-, and S^{2-} (Volesky and Prasetyo 1994).

The broad, intense adsorption peaks around 3,423–3,415 cm^{-1} are indicative of amide A bonding, which is most likely due to N–H stretching of a protein. The peaks observed between 2,928–2,929 cm^{-1} can be assigned to the C–H group, while peaks that have shifted from 1,737 to 1,738 cm^{-1} are related to the C=O bond. It is noteworthy that there is a strong amide I, II, III group shift from 1,638, 1,543, and 1,177 to 1,655, 1,522, and 1,183 cm^{-1}, respectively. In addition, the peaks that have shifted from 1,382 to 1,383 cm^{-1} mainly indicate stretching of the C–N bond. All peaks below 1,059 cCm^{-1} are related to unsaturated alkenes and aromatic (R=C=H) bonds.

The FT-IR analysis indicates that there are many functional groups involved in the live CCNWGS0123 biosorption process, such as OH, C–H, C=O, N–H, C–N, amide I, II, III and unsaturated alkenes groups on the cell surface and cell wall. Similar FT-IR results have been observed by others (e.g., Bankar et al. 2009; Li et al. 2010; Lin et al. 2012).

Cu (II) Bioaccumulation

Copper accumulation by *M. amorphae* CCNWGS0123 showed differences between the intracellular and extracellular components that were related to incubation times Fig. 7. The highest copper ion uptake was shown in day 5 on the cell surface, cell wall, and cytoplasm: 112, 97, and 88 mg g^{-1}, respectively. These results demonstrated that copper (II) accumulation was associated with cell growth

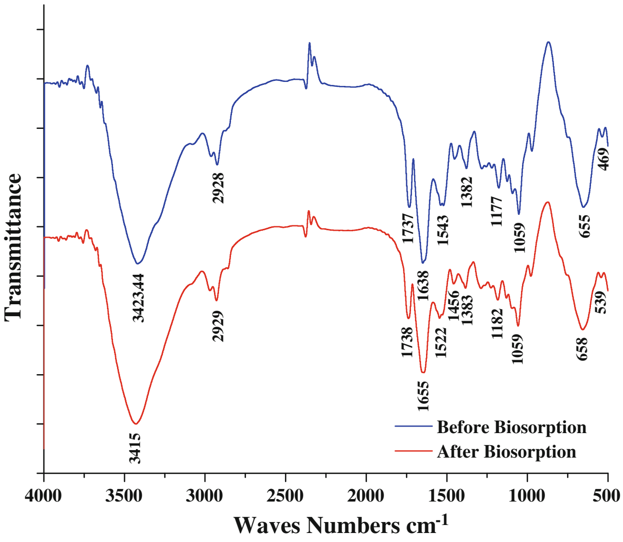

Fig. 6 Fourier transform infrared absorption spectra of live CCNWGS0123 biosorbent before and after loaded 100 mg L^{-1} copper (II)

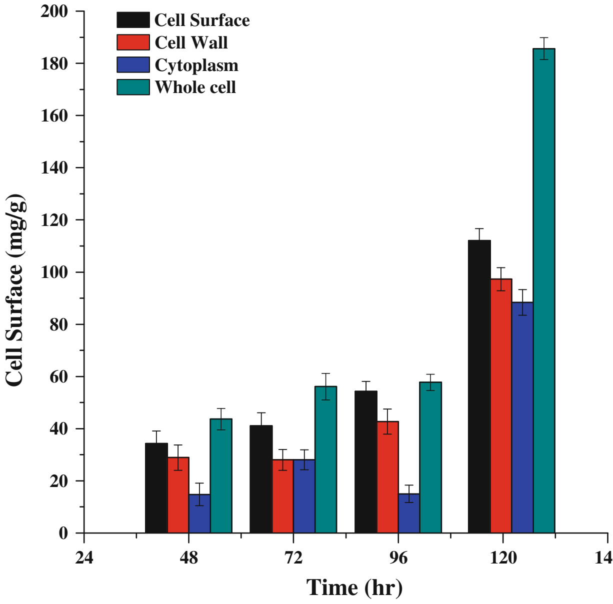

Fig. 7 Time-depence of copper (II) bioaccumulation by *M. amorphae* CCNWGS0123 at cell surface, cell wall, intracellular section and whole cell

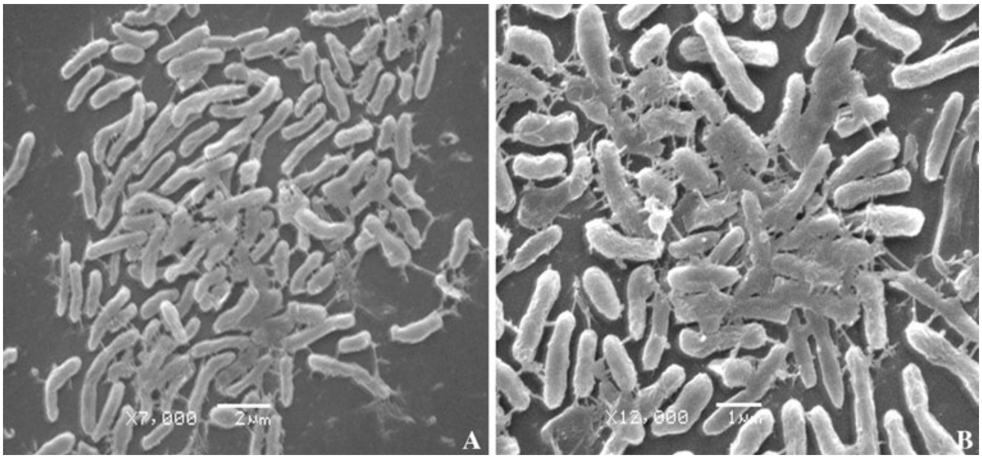

Fig. 8 SEM micrographs after copper sorption by *M. amorphae* CCNWGS0123 at different magnification (**a** ×7,000; **b** ×12,000)

or increased biomass (Fan et al. 2010). The cell surface had the highest metal uptake at 48, 72, 96, and 122 h of incubation time, followed by the cell wall, and then the cytoplasm. This means that copper (II) uptake was not only due to cell-surface binding, but also to intracellular accumulation, as observed by Volesky and Holan (1995). After 72 h, the accumulation of Cu^{2+} in whole cells totaled 56 mg g^{-1} of dry biomass, which was 1.3 times as much as at 48 h. Then, the amount of Cu^{2+} at 124 h was 4.3 times greater than that at 72 h. Again, these results demonstrate that Cu^{2+} accumulation was associated with cell growth or increased biomass. The CCNWGS0123 strain could have produced the intracellular polysaccharide and immobilized the copper ions in their cells, or secreted compounds onto the extracellular space and cell wall.

SEM Analysis

Scanning electron microscopy is an extremely useful tool for visual confirmation of surface morphology and the physical state of a surface Fig. 8. We observed irregular and changed cellular morphology of the bacterial cells after copper sorption by live CCNWGS0123. The deformation and cell surface damage may have been due to the secretion of extracellular polymeric substances during metal biosorption (Chen et al. 2000; Lin et al. 2012).

Conclusions

Experimental evidence indicates that live *M. amorphae* CCNWGS0123 biomass could be used as an efficient biosorbent for the removal of Cu (II) ions. The copper uptake and removal efficiency was highest at pH 5.0, at an agitation of 150 rpm, within 30 min at 28 °C. Scatchard plot analysis for the adsorption data fit the Langmuir and the Freundlich isotherms well. FT-IR spectra implicated the role of different functional groups in metal biosorption and SEM observations confirmed the presence of Cu (II) on the bioadsorbed cells. Thus, the present work showed that live cells of CCNWGS0123 can be used as a new biosorbent. It appears that the most economical way to use it would be to use small batches of biomass to remove toxic concentrations of Cu^{2+} ions from aqueous solution.

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