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Copper removal by filamentous and yeast-like morphologies of *Mucor indicus*: surface characterization and biosorption mechanism

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ABSTRACT

Two different morphologies of fungus *Mucor indicus* were used for the biosorption of copper ions from aqueous solutions. Potentiometric titration, chemical modification of functional groups, and Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) analyses were performed to characterize the biosorption process. The presence of carboxyl, phosphate, and amine functional groups on the biomass of both morphologies was indicated by potentiometric titration and FTIR analysis. Chemical modification of carboxyl and amine groups showed that these groups considerably affected the biosorption, while the extraction of lipids did not influence the process. Ions of Na^+, K^+, Ca^{2+}, and Mg^{2+} were released into the solution during copper adsorption, indicating that ion exchange was a key mechanism in the biosorption. Copper removal was accompanied by the adsorption of hydrogen ions on the filamentous form biomass; however, it was complemented with the release of hydrogen ions from the yeast-like biomass. Kinetics and isotherm data were appropriately fitted to Ho and Langmuir models, respectively. Maximum adsorption capacity predicted by Langmuir model for the filamentous form (24.5 mg g^{-1}) was higher than that of the yeast-like form (14.5 mg g^{-1}), which was attributed to the higher contents of phosphate, glucosamine, and active sites in the filamentous biomass. SEM micrographs revealed the difference between the surfaces of the two morphologies, resulting in different adsorption behaviors.

Keywords: Biosorption mechanism; Copper; Morphology; *Mucor indicus*; Potentiometric titration

1. Introduction

Copper is a heavy metal, released into the environment via agricultural fungicides, algicides, plumbing corrosion, fertilizers, and electroplating effluents [1–3]. A limited amount of copper is necessary as a nutrient, while its higher concentrations negatively influence the environment and human health. For instance, copper causes gastrointestinal disorder, liver and kidney malfunctioning, and anemia. According to WHO guidelines, the maximum permissible limit of copper ions in drinking water is 2 mg L^{-1}; therefore, there has been a great deal of effort to fulfill this requirement [4,5].

There are different methods for treatment of effluents containing heavy metals. Adsorption is a low-cost technique for treatment of dilute metal solutions. A number of adsorbents with biological origin and very low environmental impacts can be used. Fungi are among interesting biosorbents for heavy metals removal since fungi show excellent metal binding capacity and huge amounts of fungal biomass are available as industrial waste products [4].

Mucor indicus is a zygomycete fungus with a chitosan rich cell wall. Chitosan is a polysaccharide containing amino and hydroxyl groups, which makes it a suitable adsorbent

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for heavy metals removal. In the previous papers, the ability of *M. indicus* derivatives including cell wall, chitosan, and chitin for copper removal from aqueous solutions was verified [6,7]. This dimorphic fungus appears in filamentous or yeast-like morphologies. The main application of *M. indicus* (either in the form of yeast-like or filamentous cells) is in the fermentation of lignocellulosic hydrolysates to produce ethanol as the most important product of biotechnology [8]. Therefore, huge amounts of waste filamentous or yeast-like fungal biomass can be obtained from ethanol industries.

There are some investigations on the biosorption of heavy metals by the filamentous form of *M. indicus* biomass [7,9,10]; however, to the best of our knowledge, there is no study on biosorption of copper ions by different morphologies of *M. indicus*.

This study aimed to investigate and compare the mechanism of copper removal by filamentous and yeast-like morphologies of *M. indicus*. The role of lipids and different functional groups on biosorption capacity was investigated. Biomass surface characterization and Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) analyses were also performed to study the biosorption mechanism. Equilibrium data and adsorption rates were, respectively, described by appropriate isotherm and kinetics models.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade. Deionized water (Millipore Co., Germany) was used to prepare all solutions. Agar (Scharlau, Spain), glucose (Scharlau), and peptone (Fluka, USA) were purchased to prepare culture media. Bovine serum albumin (Sigma-Aldrich, USA) was used for protein determination. Other chemicals used in this study such as sodium hydroxide (≥ 97.0%), nitric acid (65.0%), sulfuric acid (98%), hydrochloric acid (37%), and $CuSO_4.5H_2O$ were obtained from Merck Chemicals, Germany.

2.2. Production of filamentous and yeast-like morphologies

Fungus *M. indicus* CCUG 22424 (Culture Collection, University of Göteborg, Sweden) was maintained on plates containing 40 g L^{-1} glucose, 10 g L^{-1} peptone, and 15 g L^{-1} agar and kept at 32°C for 5 d to grow and then stored at 4°C until use. The cultivation medium contained 90 g L^{-1} date syrup (contained 7.2% fructose, 7.0% glucose, and 1.1% sucrose), 0.5 g L^{-1} ammonium phosphate, and 3 g L^{-1} urea in distilled water, which was autoclaved for 20 min at 120°C.

Inoculum spore concentration and cultivation conditions (aerobic or anaerobic) are critical factors in the morphology induction for *M. indicus* [11]. In order to provide the yeast-like form, 250 mL flasks containing 100 mL medium (5 (±1) × 10^6 spores mL^{-1}) with loop traps were used. Pure nitrogen gas was introduced into the medium to provide anaerobic conditions and sterile water was kept in the loop trap to prevent back-diffusion of air and permit CO_2 to exit. The flasks were kept in a shaker-incubator at 32°C and 135 rpm for 43 ± 1 h. In order to provide the filamentous form, a fermenter containing 12 L medium (3 (±1) × 10^4 spores mL^{-1}) with an inlet flow of sterile air (2 volume air per liquid volume per min) was used. During the cultivation period (43 ± 1 h), the temperature was kept at 32°C. In both cases, the medium pH was adjusted at 5.0–5.5 using H_2SO_4 and NaOH solutions at the beginning of cultivations. After complete consumption of the sugars (43 ± 1 h), the fungal biomass was separated and washed three times with distilled water to remove the traces of the cultivation medium and then freeze-dried.

2.3. Basic pretreatment of the biomass

Dried filamentous and yeast-like forms were pretreated with 0.5 M NaOH solution (30 mL solution per g dry biomass) at 121°C for 20 min. Afterward, the residual materials were centrifuged, washed with distilled water to neutral pH, and freeze-dried. The alkali insoluble material (AIM) was considered as a representative for the fungal cell wall.

2.4. Modification of different functional groups

Biomass amine groups were methylated by adding 0.5 g biomass to 10 mL formaldehyde and 20 mL formic acid and agitating the suspension for 6 h at room temperature according to the following equation [12]:

$$RCH_2NH_2 \xrightarrow{HCHO+HCOOH} RCH_2N(CH_3)_2 + CO_2 + H_2O$$

Carboxyl groups were esterified by suspending 0.5 g biomass in 32.5 mL methanol and 0.3 mL concentrated hydrochloric acid and agitating the suspension for 6 h at room temperature [12]:

$$RCOOH + CH_3OH \xrightarrow{H^+} RCOOCH_3 + H_2O$$

Lipids were extracted by heating the suspension of 0.5 g biomass in 75 mL acetone under reflux condition for 6 h [12].

After chemical modifications or lipid extraction, the biomass was separated by centrifugation (6,000 rpm, 5 min), washed five times with distilled water, and freeze-dried.

2.5. Determination of pH of zero charge

In order to determine pH of zero charge (PZC) for the filamentous and the yeast-like form of *M. indicus*, 100 mL deionized water was boiled for 20 min, quickly cooled in an ice bath, and capped to get CO_2-free water. 0.2 g biomass was added to 15 mL of the CO_2-free water, sealed, and placed in a rotary shaker at room temperature. After 48 h, the pH of the suspension was measured as PZC [13].

2.6. Potentiometric titration of the biomass

In order to determine the amounts of acidic sites on the biomass, 0.25 g of biomass was added to a 50 mL solution of 0.1 M HCl and agitated on a magnetic stirrer at room temperature for 2 h. Afterward, the suspension was titrated by stepwise addition (0.25 mL) of 0.1 M NaOH solution and the pH was measured after reaching equilibrium. To determine the amounts of basic sites on the biomass, similar titration was performed on the suspension of 0.25 g biomass in 50 mL 0.1 M NaOH solution using 0.1 M HCl as the titrant. For both types of titration, a blank solution without the

biomass was also titrated as the control. The titration curve was obtained by plotting the measured pH values against the volume of the titrant used [14]. Total basic and acidic sites as well as the type of acidic groups and their pK_a values were obtained using the titration curves.

2.7. Determination of proteins, glucosamine, N-acetyl glucosamine, and phosphates

To measure the protein content of the filamentous and the yeast-like forms of the biomass, the Biuret method [15] was applied. The biomass (0.01 g) was mixed with NaOH 1 M (3 mL), boiled for 10 min, immediately cooled in an ice bath, and added to 1 mL of 2.5% $CuSO_4.5H_2O$. After 5 min, it was centrifuged at 3,400 × *g* for 4 min. The absorbance of the supernatant was measured at 555 nm after 15 min.

In order to determine the phosphate content of the biomass, the hydrolysate of the biomass obtained by using sulfuric acid was mixed with ascorbic acid and an acid molybdate reagent to form a blue complex. The absorbance of the complex was measured at 880 nm according to European standard ISO 6878 [16].

Glucosamine (GlcN) and *N*-acetyl glucosamine (GlcNAc) contents of AIM were measured based on the method presented by Mohammadi et al. [17]. A combination of a two-step sulfuric acid hydrolysis and nitrous acid degradation that produces 2,5-anhydromannose and acetic acid were applied. The concentrations of anhydromannose and acetic acid were measured by HPLC (Jasco International Co., Japan) with an ion exchange Aminex column (HPX-87H, Bio-Rad Laboratories, Inc., USA) at 60°C with 0.6 mL min^{-1} eluent of 5 mmol L^{-1} sulfuric acid. The anhydromannose was measured by chromatogram prepared by RI detector, while acetic acid was monitored by UV detector. The Borwin chromatography software was used for analysis of chromatograms and concentration calculations. The moles of *N*-acetyl glucosamine were considered to be equal to the moles of acetic acid, while the moles of glucosamine were equal to the difference between the moles of anhydromannose and acetic acid.

2.8. Biosorption experiments

A stock solution containing 1,000 mg L^{-1} Cu^{2+} was prepared by dissolution of $CuSO_4.5H_2O$ in deionized water and used to prepare solutions with different concentrations. The initial pH was adjusted using NaOH and H_2SO_4 solutions (0.1 M). All the adsorbents used were grounded and sieved to obtain particles with sizes less than 0.5 mm. The experiments were carried out in 250 mL Erlenmeyer flasks containing 50 mL metal solution and different amounts of the adsorbent in a shaker-incubator at 135 rpm and 32°C. Control samples, without the adsorbent but containing the same metal concentrations, were also prepared. After reaching the equilibrium, the adsorbent was separated by centrifugation (6,000 rpm, 5 min) and the concentration of copper ions in the supernatant was measured. The following biosorption experiments were performed:

- The effect of initial pH on biosorption capacity was studied by addition of 0.03 g biomass to a copper solution (5 mg L^{-1}) at pH values of 3.0, 4.0, and 5.5.
- The effect of chemical modification of biomass was investigated by adding 0.03 g of unmodified and modified biomass to a copper solution (5 mg L^{-1}) at pH 4.
- The effect of alkali pretreatment on biosorption was studied by adding 0.05 g of the untreated and treated biomasses to a 20 mg L^{-1} copper solution at pH 4.
- In order to investigate the biosorption mechanism, 0.03 g of the biomass was added to a copper solution (50 mL, 5 mg L^{-1}) at pH 4. A control sample was also provided by adding the same amount of the biomass to 50 mL deionized water at pH 4. After reaching equilibrium, the final pH value as well as the concentration of Ca^{2+}, K^+, Mg^{2+}, and Na^+ ions in the control sample and the copper solution was measured for both morphologies.
- Kinetics of biosorption was studied by adding 0.03 g of biomass to a copper solution (5 mg L^{-1}) at pH 4. Each datum was achieved from individual flasks at different times. To describe the kinetics of copper removal, pseudo-first-order [18], pseudo-second-order [19], and Elovich models [20] were employed.

Pseudo-first-order model is represented by the following equation:

$$\log\left(q_e - q_t\right) = \log q_e - \frac{K_1}{2{,}303}t \tag{1}$$

where K_1 (min^{-1}) is the first-order adsorption rate constant and q_e and q_t (mg g^{-1}) are the adsorption capacity at equilibrium and time t (min), respectively. Adsorption capacity is the amount of adsorbed copper ions by unit mass of the adsorbent.

Pseudo-second-order model is expressed as:

$$\frac{t}{q_t} = \frac{1}{K_2 q_e^2} + \frac{t}{q_e} \tag{2}$$

where K_2 (g mg^{-1} min^{-1}) is the pseudo-second-order adsorption rate constant.

Elovich model is as follows:

$$q = \frac{1}{b}\ln\left(ab\right) + \frac{1}{b}\ln\left(t + \frac{1}{ab}\right) \tag{3}$$

where a (mg g^{-1} min^{-1}) and b (g mg^{-1}) are the model's parameters.

- Adsorption isotherms were evaluated by addition of 0.05 g biomass to solutions with initial concentrations of 5, 10, 15, 20, and 25 mg L^{-1} at pH 4. Langmuir, Freundlich, Redlich–Peterson, Temkin, and Scatchard models were used to describe adsorption isotherm.

Langmuir model assumes the formation of a monomolecular layer with no interaction between the adsorbed molecules [21]. The linear form of this equation is as follows:

$$\frac{1}{q_e} = \frac{1}{q_{\max}} + \frac{1}{q_{\max} K C_e} \tag{4}$$

where K (L mg^{-1}) is a constant related to the energy or net enthalpy of adsorption, q_{max} (mg g^{-1}) is the amount of adsorbed ions to saturate unit mass of the adsorbent, and C_e (mg L^{-1}) is the equilibrium concentration of copper ions.

The linear form of Freundlich model, which is a multisite adsorption isotherm for heterogeneous surfaces [22], is as follows:

$$\ln q_e = \ln K_F + \frac{1}{n}\ln C_e \quad (5)$$

where K_F ((mg g^{-1}) (mg L^{-1})$^{-1/n}$) and n show the capacity and intensity of adsorption, respectively.

Temkin model [23] is as follows:

$$q_e = \frac{RT}{b_T}\left(\ln C_e + \ln A_T\right) \quad (6)$$

where A_T (L g^{-1}) is the equilibrium binding constant corresponding to the maximum binding energy and b_T (kJ mol^{-1}) is a constant related to the heat of sorption, R (0.0083 kJ mol^{-1} K^{-1}) is the universal gas constant, and T (K) is the temperature.

Scatchard model [10] is as follows:

$$\frac{q_e}{C_e} = q_m \cdot k_b - k_b q_e \quad (7)$$

where q_m (mg g^{-1}) and K_b (L mg^{-1}) are Scatchard model's parameters.

Redlich–Peterson model [22] is as follows:

$$\frac{C_e}{q_e} = \frac{1}{K_R} + \left(\frac{a_R}{K_R}\right) C_e^{\beta} \quad (8)$$

where K_R (L g^{-1}), a_R (L mg^{-1})$^{\beta}$, and β ($0 < \beta < 1$) are the model's parameters.

2.9. Measurement of copper ions concentration

The concentration of copper ions was measured by an atomic absorption spectrophotometer (210VGP, Buck Scientific Instrument Manufacturing Co., England). The amount of solute adsorbed by unit weight of the adsorbent at equilibrium (adsorption capacity) was calculated as

$$q_e = \frac{(C_o - C_e)v}{m} \quad (9)$$

where C_0 (mg L^{-1}) and C_e (mg L^{-1}) are the initial and the final concentrations of copper ions, respectively, m (g) is the mass of the adsorbent, and V is the volume of the solution which was 0.05 L in all experiments.

All experiments were performed in duplicate and the average value was reported.

The graphical representation of this study was provided as the supplementary Fig. 1.

3. Results and discussion

3.1. The effect of pH on copper biosorption and pH of zero charge

The effect of initial pH on Cu^{2+} adsorption capacity was studied at pH values of 3.0, 4.0, and 5.5. At pH values higher than 5.5, copper precipitates as copper hydroxide [13]. The adsorption capacities (mg g^{-1}) at these pH values were, respectively, 7.43, 8.02, and 8.15 for the filamentous form and 3.46, 7.90, 8.09 for the yeast-like form of *M. indicus*. It was observed that for both morphologies, the adsorption capacity was enhanced by increasing pH. Furthermore, the yeast-like form was more affected by the pH than the filamentous form. Generally, pH affects the surface charge of the adsorbent and is usually considered as an effective parameter in adsorption processes [10]. At low pH values, there is competition between H_3O^+ and copper ions to be adsorbed on the surface, which reduces the adsorption capacity, while at high pH values, the adsorbent surface becomes more negative, which facilitates binding of copper ions on the adsorbent surface [24]. Similar results were also observed in copper removal by three species of fungi *Cladosporium cladosporioides*, *Gliomastix murorum*, and *Bjerkandera* [25].

The PZC values of the filamentous and the yeast-like forms were 6.35 and 5.98, respectively. At pH < PZC, the surface charge of the biomass is positive resulting in low Cu^{2+} adsorption, while at pH > PZC, the surface charge is negative and the positive copper ions are attracted to the surface more easily. Therefore, by increasing the pH, the amount of adsorbed copper ions increases.

3.2. Kinetics of Cu^{2+} removal

It is necessary to find the optimum conditions for biosorption processes; therefore, the kinetics of metal removal is important to study [26]. Variation of copper ions concentration with time is plotted in Fig. 1. For both morphologies, the adsorption rate was very high at the beginning of the adsorption and about 67% of the total adsorbed copper ions were removed within the first 3 h. After the rapid initial phase, adsorption rate was gradually decreased to reach equilibrium state at the end. The rapid initial rate of adsorption results from the presence of a high number of free sites on the adsorbent surface and a high copper ions concentration gradient between the bulk and the biomass surface. Upon gradual occupation of the sites, the cations had to penetrate to inner layers. Therefore, the rate of adsorption declined at longer times [27].

Both adsorbents reached equilibrium in about 48 h. Different equilibrium times have been reported for copper

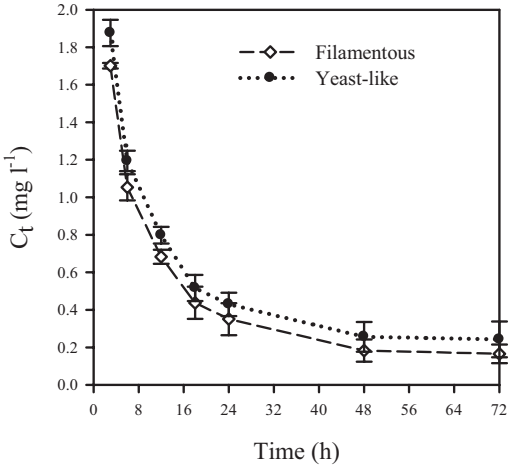

Fig. 1. Biosorption capacity of copper ions by filamentous (◊) and yeast-like (•) forms of *M. indicus* at different contact times.

removal from aqueous solutions by different adsorbents, e.g., the equilibrium time was about 6 h for *Phanerochaete chrysosporium* biomass [28] and 12 d for chitosan [29].

In order to describe the kinetics data, pseudo-first-order, pseudo-second-order, and Elovich models were used. Results are presented in Table 1. Pseudo-first-order and Elovich models cannot describe the kinetics data appropriately, as the corresponding correlation factors are low. Generally, pseudo-first-order model is just appropriate for the initial phase of the adsorption process and not over the whole range of contact time [30]. Pseudo-second-order model can appropriately predict the kinetics data, as the correlation factors are high and the calculated equilibrium adsorption capacities are close to the experimental values. This model assumes that the rate-limiting step in adsorption is chemisorptions, which involves valence forces through sharing or exchange of electrons between the adsorbent and metal ions. Unlike pseudo-first-order model, Ho's pseudo-second-order model is appropriate over the whole range of contact time [31]. Ho's model was also the best model to describe the kinetics data obtained for copper ions removal by different fungal species [25,32].

3.3. Adsorption isotherm

Adsorption isotherms were evaluated by addition of 0.05 g biomass to copper solutions with initial concentrations of 5, 10, 15, 20, and 25 mg L^{-1} at pH 4. Different models were used to describe the isotherm data and the results are presented in Table 2. The correlation factors obtained by fitting the isotherm data to Langmuir and Redlich–Peterson models are high for both morphologies ($R^2 > 0.99$). Therefore, these models are appropriate to describe the isotherm data. Furthermore, the Temkin model can also describe adsorption process of the filamentous form of *M. indicus* accurately. It has been reported that the Langmuir model is appropriate for describing isotherm data of copper ions adsorption by *Rhizopus oryzae* [32] and *P. chrysosporium* [28].

According to Table 2, the maximum adsorption capacities predicted by the Langmuir model are about 24 and 14 mg g^{-1} for the filamentous and the yeast-like form of *M. indicus*, respectively. Therefore, the ability of the filamentous form to remove copper ions is higher than the yeast-like form.

Variations in the equilibrium adsorption capacity of copper ions versus equilibrium concentration for the two morphologies are shown in Fig. 2. Each point corresponds to a different initial copper ions concentration. As the initial concentration increased, adsorption capacity was also increased, probably because of a higher copper ions concentration gradient as a driving force for mass transfer.

Table 2
Different models' isotherm constants for biosorption of copper ions into filamentous and yeast-like forms of *M. indicus*

Model	Parameters	Morphology	
		Filamentous	Yeast-like
Langmuir	R^2	0.9981	0.9911
	q_{max} (mg g^{-1})	24.52	14.53
	K (L mg^{-1})	2.15	2.47
Freundlich	R^2	0.9506	0.8894
	n	2.25	3.65
	K_F (mg g^{-1} (mg $L^{-1})^{-1/n}$)	14.79	8.62
Temkin	R^2	0.9993	0.9290
	$\frac{RT}{b_T}$	5.14	2.45
	A_T	22.59	47.38
Scatchard	R^2	0.9856	0.9614
	q_m (mg g^{-1})	24.42	14.95
	K_b (L mg^{-1})	2.16	2.27
Redlich–Peterson	R^2	0.9996	0.9993
	β	0.915	1.00
	K_R (L g^{-1})	59.87	36.94
	a_R	2.68	2.51

3.4. Chemical modification of the biomass

Different chemical modifications were performed to investigate the role of the functional groups of the biomass on copper ions removal. Results are presented in Table 3.

Treatment with methanol and hydrochloric acid esterified the carboxyl groups and reduced copper ions adsorption by 54% ± 2% for both morphologies, which indicates the involvement of carboxyl groups in copper ions removal. Carboxyl groups also played an important role on metals ions removal by *Rhizopus arrhizus* biomass [33].

Formaldehyde and formic acid were used for methylation of primary and secondary amines. A considerable reduction in copper ions removal was observed as the result of methylation of amines present in the biomass of filamentous and yeast-like forms. This indicates the effective role of amine groups on copper ion removal for both morphologies. The electron pairs of nitrogen atoms in the amine groups of chitin and chitosan bind with copper ions and form stable metal complexes. Similar results indicating the significant role of carboxyl and amine groups on Pb, Cd, Zn, and Ni

Table 1
Different models' correlation factor (R^2) and Ho's model parameters for the biosorption of copper ions by filamentous and yeast-like forms of *M. indicus*

Morphology	Pseudo-first order	Pseudo-second order			Elovich	Experimental
	R^2	R^2	K_2 (g mg^{-1} min^{-1})	Calculated q_e (mg g^{-1})	R^2	q_e(mg g^{-1})
Filamentous	0.9098	0.9999	0.0784	8.24	0.9082	8.06 ± 0.03
Yeast-like	0.9229	0.9999	0.0725	8.13	0.9066	7.92 ± 0.02

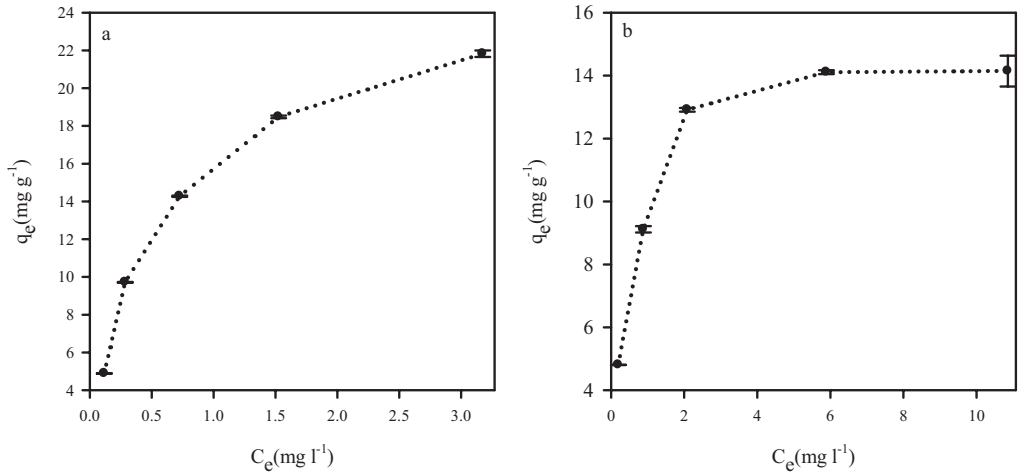

Fig. 2. Equilibrium copper biosorption capacity of filamentous (a) and yeast-like (b) forms of *M. indicus*.

Table 3
Effect of different chemical modifications of filamentous and yeast-like forms of *M. indicus* on adsorption capacity (mg per g modified biomass)

	Chemical modification			
Morphology	No modification	Amine methylation	Carboxyl esterification	Lipid extraction
Filamentous	8.04 ± 0.02	3.60 ± 0.02	3.81 ± 0.02	8.01 ± 0.02
Yeast-like	7.92 ± 0.03	2.86 ± 0.02	3.52 ± 0.03	7.89 ± 0.02

ions biosorption by the filamentous form of *M. indicus* were reported by Yan and Viraraghavan [34].

Removal of lipids from the biomass of the two morphologies did not affect copper ions removal. This indicates that lipids do not contribute to copper ions biosorption. Tobin et al. [33] also reported that acetone treatment of *R. arrhizus* biomass did not affect metal ions uptake capacity.

3.5. Alkali treatment of the biomass

Both filamentous and yeast-like forms of *M. indicus* were subjected to basic treatment of the biomass. Biosorption capacities of untreated filamentous and yeast-like biomass were 8.04 and 7.89 mg g^{-1}, respectively, while the corresponding values were 8.16 and 8.29 mg g^{-1} for alkali-treated biomass. It was observed that the treatment of biomass with NaOH slightly increased the biosorption capacities of both morphologies. Alkali treatment may disrupt the microbial cell wall, which exposes more functional groups. It may remove surface impurities, rupture cell membrane, destroy autolytic enzymes that cause putrefaction of biomass, and remove proteins that make reactive sites unavailable. Alkali pretreatment also improved copper ions removal compared with live biomass of *R. oryzae* [32].

3.6. Potentiometric titration of the biomass

The quantity of the acid functions involved in copper ions biosorption can be estimated through potentiometric titration. The total organic acidity (A_{TO}) of a heterogeneous surface, that is, the fungal biomass, consists of three types of acidity based on the apparent ionization constant. Strong acidities (A_S) are based on the presence of high-affinity acidic groups such as sulfonate and carboxylic, linked to aromatic, at pH < 4. Weak acidities (A_W) are results of the ionization of carboxylic groups at 4 < pH < 7. Very weak acidities (A_{VW}) are derived from the phenolic and amine groups, including the ionization of amino groups of proteins at pH > 7 [35].

The acidities and their pK_a values were determined using the equations derived by Naja et al. [35], based on the NaOH into HCl titration curves. In these equations, Gran's equations were applied to a heterogeneous surface containing acid sites and the reactions that occurred during an acid–base titration were considered. The plot of the second derivative of pH with respect to the added volume of NaOH was also provided. The dissociation coefficients of the acidities (α) and the pK_a value were related to the pH according to the Henderson–Hasselbalch equation [35].

Using the titration curve (Fig. 3(a)), the amount of acidity for the two morphologies were calculated and presented in Table 4. The filamentous and the yeast-like forms contained 1.97 and 1.72 me total acidity groups per g dry biomass, respectively. The total acidity of *M. indicus* biomass is high in comparison with other bioadsorbents such as *Aspergillus niger* biomass (0.81 me g^{-1}) [35] or biomass of brown alga *Fucus vesiculosus* (0.53 me g^{-1}) [36]. The higher acidity may result from the high amount of amine groups in the chitin and chitosan rich cell wall of *M. indicus* biomass.

The values of the pK_a obtained for the weak acidity were 6.33 and 5.97 for the filamentous and the yeast-like forms,

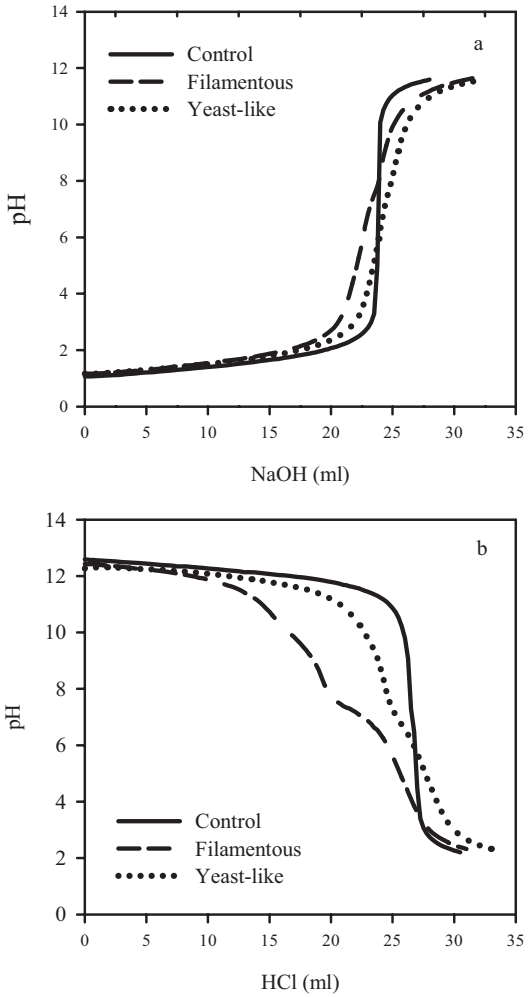

Fig. 3. Potentiometric titration curves of filamentous (– –) and yeast–like (···) forms of *M. indicus* for determination of acidic (a) and basic (b) sites.

respectively, which are attributed to carboxyl groups' links to aliphatic rather than aromatic groups (pK_a ~5.7) and phosphate groups (pK_a 6.1–6.8) [37]. The corresponding pK_a values for very weak acidity were 10.23 and 9.75, which corresponds to amine groups present in amino acids of proteins and chitosan (pK_a 8–11) [37], abundant in *M. indicus* cell wall.

The A_{TO} value indicates the available sites on the surface of biomass for adsorption of metal cations and is considered as the upper limit for biosorption. In fact, a fraction of total organic acidity may be involved in copper ions sorption by the fungus. It was previously shown that the carboxylic and amine groups play a key role as metal-binding sites in the biomass. Differences in the metal ions biosorption capacity of the two morphologies may be attributed to differences in the amount of these groups in the biomass. Therefore, the higher amounts and higher deprotonation capacity of the acidic groups in filamentous form resulted in the higher copper ions uptake capacity in comparison with the yeast-like form.

The concentrations of basic sites on the biomass were also calculated based on acid into base titration (Fig. 3(b)). Obtained values are presented in Table 4. Accordingly, the concentrations of basic sites were higher than acidic sites for the two morphologies, which make the surfaces of the two adsorbents basic.

3.7. Characteristics of the adsorbents

Proteins and phosphate content of the biomass and glucosamine and *N*-acetyl glucosamine content of the biomass cell wall were measured and presented in Table 5. The protein content of the filamentous form (58%) was higher than that of the yeast-like form (45%). Furthermore, the glucosamine content of the filamentous biomass (8.5%) was higher than that of the yeast-like biomass (5.7%). These findings indicated that the amount of amines in filamentous cells is higher than in yeast-like cells, which was also demonstrated by potentiometric titration. Amines contain electron pairs, which facilitate copper ions binding to biomass.

In order to provide better insight on the performance of the filamentous and the yeast-like morphologies for copper ions removal, FTIR analysis was performed (Fig. 4). The band at 3,271 cm^{-1} may have resulted from the overlapping of amine N–H and hydroxyl O–H stretching vibrations. The bands at 2,925–2,800 cm^{-1} are for –CH stretching vibration of C–CH_3. The band at wavelength of 1,639 cm^{-1} is for the C=O stretching mode of the amide I band. Amide II derives mainly from the N–H bending and C–N stretching, which is found at 1,537 cm^{-1}. The COO^- of carboxylate group appears at 1,396 cm^{-1}. The band around 1,250 cm^{-1} was assigned to the –C–O–C– or O–H bonds. The bands in the range of

Table 4
Total basic and acidic (A_{TO}) sites, weak acidity (A_W), very weak acidity (A_{VW}), and pK_a values derived for filamentous and yeast-like forms of *M. indicus*

	A_W		A_{VW}		A_{TO}	Total basic sites
Morphology	me g^{-1}	pK_a	me g^{-1}	pK_a	(me g^{-1})	(me g^{-1})
Filamentous	1.128	6.33	0.844	10.23	1.972	2.1046
Yeast-like	1.020	5.97	0.700	9.75	1.720	2.1847

Table 5
Characterization of the filamentous and the yeast-like forms of *M. indicus*

Morphology	Protein (g per g biomass)	Phosphate (g per g biomass)	AIM yield (g per g biomass)	GlcN (g per g AIM)	GlcNAc (g per g AIM)
Filamentous	0.58 ± 0.02	0.061 ± 0.002	0.15 ± 0.001	0.57 ± 0.03	0.18 ± 0.003
Yeast-like	0.45 ± 0.01	0.054 ± 0.002	0.25 ± 0.002	0.23 ± 0.02	0.10 ± 0.001

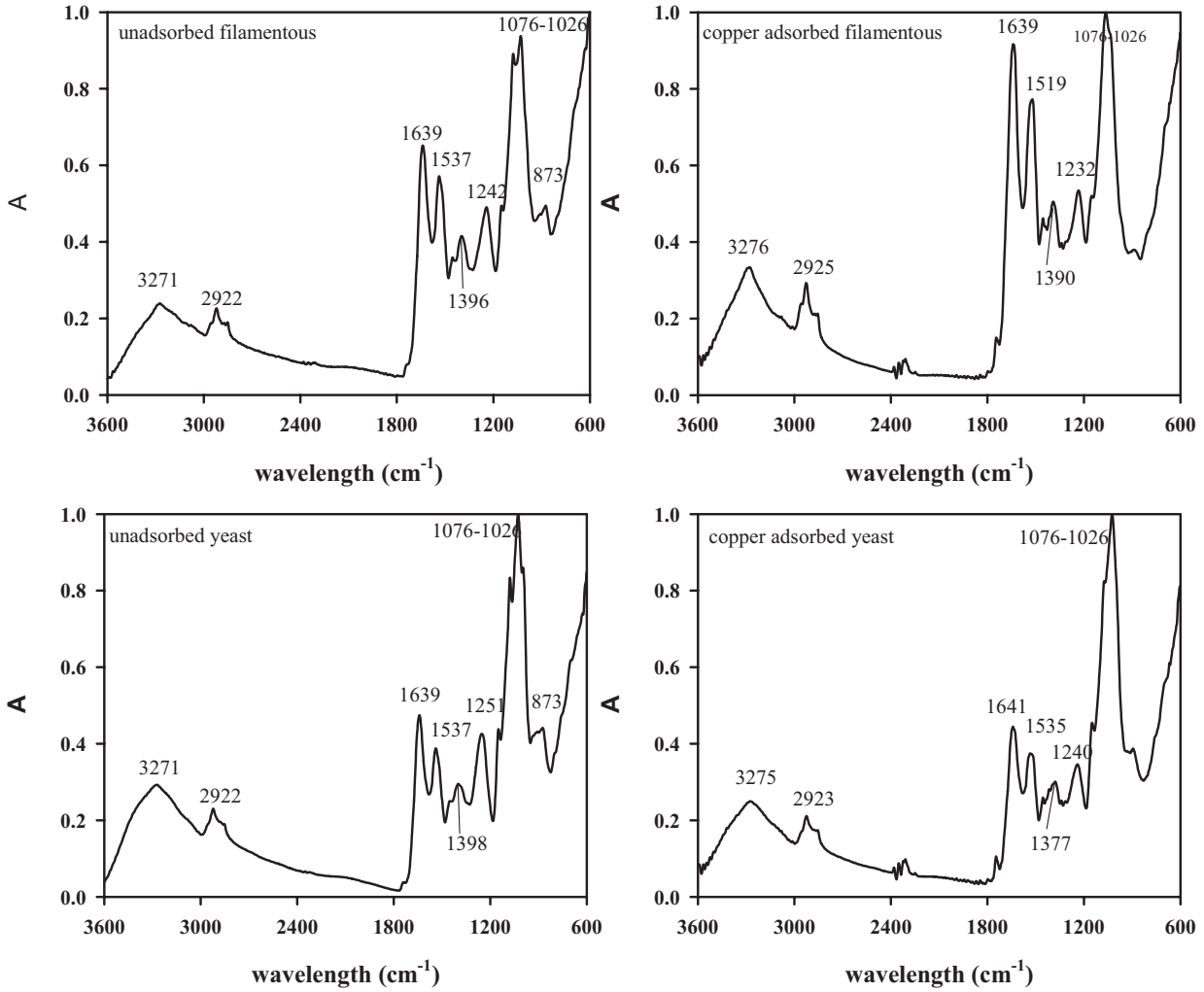

Fig. 4. FTIR spectra of different morphologies of *M. indicus* before and after copper adsorption.

1,076–1,026 cm^{-1} are for C–O stretching vibrations [10] or P–O–C bond of phosphate groups [35]. The band at 873 cm^{-1} indicated the presence of phosphate groups [10] or saccharide structure in the macromolecule [38].

The spectra of the pristine biomass of both morphologies were similar and absorbance peaks are present in the same wavelength numbers. This signals the presence of the same functional groups in the biomass of filamentous and yeast-like morphologies. For both morphologies, the spectra of copper-loaded biomass are considerably different with those of pristine biomass. For the filamentous form, the peaks at 3,271, 1,537, and 1,396 cm^{-1} are shifted to 3,276, 1,519, and 1,390 cm^{-1} due to adsorption, which shows the interaction of copper ions with amine and carboxyl groups. Furthermore, the peak at 873 cm^{-1} has disappeared, indicating the role of phosphate groups in the biosorption process. For the yeast-like form, the peaks at 3,271 and 1,398 cm^{-1} have shifted to 3,275 and 1,377 cm^{-1} and the peak at 873 cm^{-1} has disappeared, showing the involvement of amine, carboxyl, and phosphate groups in the process.

In order to study the surface morphology of the two forms of *M. indicus*, SEM was used (Fig. 5). There is an obvious difference in the surface morphology of the two pristine adsorbents. Filamentous morphology shows a fibrillar structure of the fungal mycelia, while the yeast-like form has a spherical structure. For both morphologies, there is a considerable difference between pristine biomass and copper-loaded biomass. For the filamentous form, the empty blade-like surface of biomass was more compact due to the adsorption of copper ions, and the empty spaces were filled with copper ions. For the yeast-like form, there was also an obvious change in the morphology of biomass, and the presence of metal ions on the spherical surface was observed. It seems that the mycelia of filamentous biomass provide a larger surface area for adsorption of copper ions compared with the yeast-like biomass, which results in a better performance for adsorption of copper ions.

3.8. Mechanism of copper ions removal by different morphologies

In order to investigate the biosorption mechanism, the biomass was added into a copper ions solution (5 mg L^{-1}) and deionized water as the control at pH 4. After reaching equilibrium, the final pH value and the concentration of

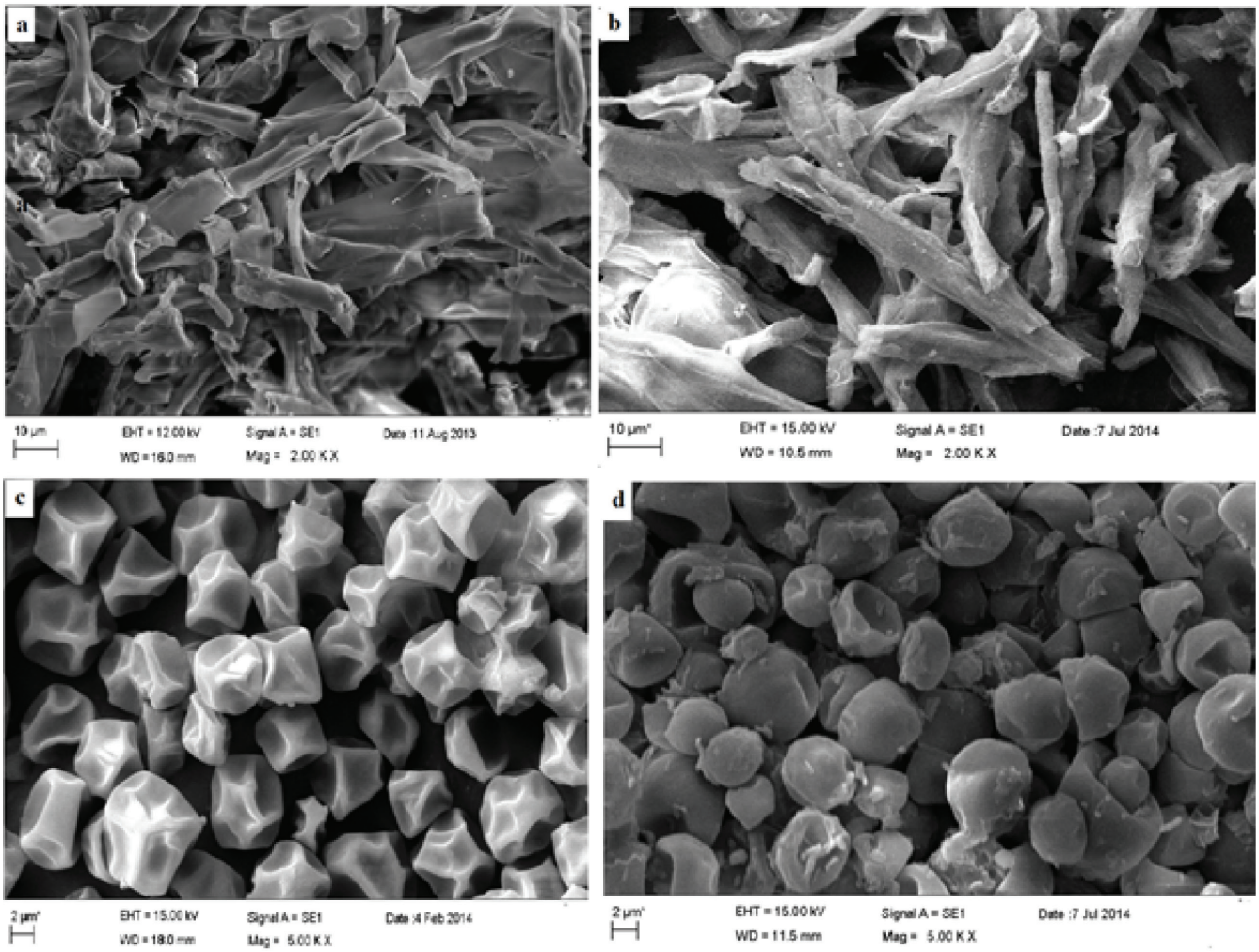

Fig. 5. SEM images of (a) pristine filamentous biomass, (b) Cu-loaded filamentous biomass, (c) pristine yeast-like biomass, and (d) Cu-loaded yeast-like biomass.

Ca^{2+}, K^{+}, Mg^{2+}, and Na^{+} in the control sample and the copper solution were measured for both morphologies. The amount of released ions during the copper adsorption process was considered to be equal to the difference between the concentrations of the ions in copper and control solutions.

Concentrations (mg L^{-1}) of the released Ca^{2+}, K^{+}, Mg^{2+}, and Na^{+} were, respectively, 2.13, 1.59, 0.35, and 0.26 for the filamentous and 1.98, 1.15, 0.26, and 0.25 for the yeast-like form. This indicates that biosorption of copper ions accompanied the release of the mentioned ions, similar to metal removal by ion-exchange resins. This indicates that ion exchange was involved in the copper ions removal process. The ratio of the total released Ca^{2+}, K^{+}, Mg^{2+}, and Na^{+} to the adsorbed copper ions is 0.9, and 0.76 for the filamentous and the yeast-like form, respectively. The observed nonstoichiometric release of the ions is explained by the following reasons: other ions may also be released during the biosorption. The exchange of ions may not have taken place as in balanced chemical equations, and the biomass surface may not be homogenous in nature. Potassium or sodium ions carry a single positive charge, while copper ions carry a double charge; therefore, two moles of potassium or sodium ions may be exchanged for one mole of copper ions. Yan and Viraraghavan [34] and Kapoor and Viraraghavan [39] also observed nonstoichiometric release of ions in heavy metal removal using fungi.

The final pH value of the control sample and the copper solution was also measured. The respective values were 6.71 and 7.32 for the filamentous form, while they were 7.97 and 6.93 for the yeast-like form. The lower pH value of the control sample in comparison with the copper solution for the filamentous form indicates that hydrogen ions as well as copper ions were adsorbed on the filamentous biomass. In contrast, hydrogen ions were released during the copper ions removal by the yeast-like form. Accordingly, pH plays a more important role on copper ions removal by the yeast form rather than the filamentous morphology. The more intensive dependence of copper ions removal on the pH for the yeast-like form in comparison with the filamentous form was previously shown in pH experiments. Additionally, the final pH of the control sample is higher than the initial value

for the two morphologies, which confirms the basic characteristic of biomass surface.

As mentioned earlier, at low pH values the binding of hydrogen ions to the biomass surface reduces the net negative charge of the biomass surface, resulting in a lower adsorption capacity. Therefore, electrostatic attraction is also involved in copper ions removal. It seems that the association of metal ions with the functional groups within the biomass is also involved in biosorption of metal ions. The fungal cell surface contains different functional groups where complexes with metals can be formed. Complexation with the nitrogen of the chitin and chitosan present in the biomass play a key role on biosorption, since Mucorales have a considerable amount of chitin and chitosan with amine functional groups. Carboxyl, amide, and phosphate are among effective functional groups, as shown through FTIR analysis and potentiometric titration. The biosorption capacity reduction by modification of the functional groups also indicated their involvement for the copper ions binding due to complex formation. Therefore, the adsorption of copper ions on the biomass is complex, involving more than a single mechanism, such as ion exchange, surface complexation, and electrostatic attraction.

4. Conclusion

Filamentous and yeast-like forms of the fungus *M. indicus* can be used for copper ions removal from aqueous solution. FTIR analysis and potentiometric titration showed the presence of carboxylic, phosphate, and amine groups on the biomass cell surface. The involvement of different functional groups in copper ions removal was confirmed by chemical modification of the groups. Lipids did not show a considerable effect on biosorption. For both morphologies, the quantities of basic sites were higher than acidic sites, suggesting that the biomass surface is basic. The adsorption occurred through exchanging ions such as Ca^{2+}, K^{+}, Mg^{2+}, and Na^{+}, complexation, and physical adsorption. In the process of copper ions removal, hydrogen ions were adsorbed on the filamentous biomass, while they were released from the yeast-like form. Ho's pseudo-second-order model appropriately fitted the kinetics data. Langmuir model described isotherm data and predicted a higher adsorption capacity for the filamentous form rather than the yeast-like form. Alkali treatment especially enhanced Cu^{2+} removal by the yeast-like form.

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