

COPPER BINDING BY INACTIVATED CELLS OF MUCOR ROUXII

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ABSTRACT

Inactivated copper-sensitive and copper-tolerant strains of *Mucor rouxii* cultured at high copper concentration were tested for their potential to adsorb Cu(II) ions from solution. We performed batch experiments to determine the pH profile for Cu(II) binding, binding capacity and the possibility of removing the Cu(II) that had been adsorbed. The biomasses studied showed a high affinity for Cu(II) ions as the pH increased from 2 to 5. The copper-tolerant strain exhibited a higher copper adsorption than the copper-sensitive strain. Most of the Cu(II) adsorbed by the inactivated cells was desorbed by treatment with 0.1 M HCl. We investigated the mechanisms involved in Cu(II) binding. We chemically modified the possible carboxyl groups present on the inactivated cells of *Mucor rouxii* by esterification with methanol. Cu(II) binding decreased approximately 25% after the carboxyl groups were esterified. The Cu(II) binding ability of the esterified carboxyl groups was regained after base hydrolysis. These preliminary findings suggest that the carboxyl groups could be at least in part responsible for Cu(II) binding. This phenomenon can have useful applications for metal-removal from contaminated water.

KEY WORDS

Mucor rouxii, fungi, copper, metal binding, metal-contaminated water

INTRODUCTION

The utilization of biomaterials for the accumulation and concentration of heavy metals from aqueous solutions has been proposed by many researchers as an alternative and relatively inexpensive method for water treatment and other metal recovering processes [1-7]. These properties are well documented in fungi, which are ubiquitous and sometimes the predominant life form in metal-polluted habitats [8]. Decontamination of industrial wastes using viable and nonviable fungal biomasses has been reported [9-11].

Fungal strains belonging to the taxonomic group of Zygomycetes are of interest due to the presence of chitin, chitosan and glucan in their cell walls [12]. These polysaccharides have shown to be efficient metal

biosorbents [13]. Fungi have been reported to be useful in the removal of valuable metals, particularly uranium [14-15]. Fungal biomasses of *Aspergillus niger* and *Mucor rouxii*, two representative soil fungi, were also evaluated for adsorption of several metals [16]. The protective action of mycorrhizal fungi may be based on the adsorptive properties of fungal surfaces [17]. Metal binding to the cell walls of *Saccharomyces cerevisiae* has been attributed to coordination with amino, carboxyl and hydroxyl groups [18].

Preliminary data have indicated that growing cells of the copper-resistant strain *M. rouxii* bound higher amounts of the metal in the cell wall fraction and in a mixture of cytosol and membranes, as compared to the copper-sensitive parental strain [19]. However, no studies on the binding of copper

ions by the inactivated biomasses obtained from copper-sensitive and copper-tolerant strains of *M. rouxii* have been reported. In addition, no research has been conducted to determine the mechanisms of metal ion binding to inactivated cells of *M. rouxii*. For metal removal and recovery from aqueous solutions, dead fungal biomass seems to offer several advantages; the biomass may be obtained inexpensively as a by-product of some industrial processes; it is not subject to metal toxicity since there is no metabolic activity; the biomass needs no nutrient supply; and recovery of surface-bound metals is not a detrimental process for the biomass [20].

The purpose of this study was to investigate Cu(II) adsorption by inactivated cells of *Mucor rouxii*. In addition, we investigated one of the mechanisms of copper binding to the dead fungal cells. We hypothesized that the carboxyl groups present in the surface cells play an important role in this process. Therefore, chemical modification of the carboxyl groups with methanol was performed. A decrease in Cu binding after esterification would strongly suggest the involvement of these groups in Cu binding. Results of these experiments are reported herein.

METHODOLOGY

Strains and culture conditions

Copper-sensitive (IM-80) and copper-tolerant (P1) strains of *Mucor rouxii* were utilized in this study. The copper-tolerant strain was derived from the sensitive parental strain by exposing cells to gradually increasing copper (as sulfate salt) concentrations up to 4.8 mM in the culture medium. The strains were maintained and propagated in yeast extract-peptone-glucose (YPG) medium [21]. Spores were harvested in sterile distilled water from three- to five-day-old cultures, stored at 4°C and used within two weeks of harvest. The harvested spores were inoculated in two flasks

to a final density of 5×10^5 spores/ml. The two flasks contained 500 ml liquid YPG medium with pH adjusted to 4.5 and supplemented with copper sulfate solution to a final concentration of 3.2 mM. Cultures were grown aerobically for 13-18 hr at 28°C on a reciprocating shaker bath at 180 rpm. After 48 hr, cells were collected by filtration onto Whatman #1 filter paper in a Millipore funnel. The resulting mycelium was washed, freeze-dried (in a Labconco freeze-dryer), ground and sieved to pass a 100 mesh screen.

pH profile studies of copper binding

A batch procedure was utilized for the pH profile studies. Samples of each biomass were washed by centrifugation (3000 rpm, five minutes) twice with 0.01 M hydrochloric acid (HCl). After the washing, the pellet fractions were resuspended in 0.01 M HCl (5 mg/ml). Aliquots of each sample, adjusted to corresponding pH (2, 3, 4, 5 and 6), were distributed into three tubes, centrifuged, and decanted. The pellets and supernatant fractions were resuspended in 3 ml of 0.1 mM copper sulfate solution adjusted to the corresponding pH. The suspensions were equilibrated for 1.0 hr by continuous agitation and then centrifuged. Each supernatant fraction was subsequently analyzed for copper by flame atomic absorption spectrophotometry in order to record the concentration of the remaining targeted metal. The final pH of each supernatant solution was measured and recorded. The difference between the initial metal concentration (indicated by the control blank) and the remaining concentration was assumed to be adsorbed to fungal biomass.

Copper binding capacity studies

Samples of each biomass (5 mg/ml, pH 5) were suspended in a solution containing 0.32 mM Cu(II) as copper sulfate in 0.01 M sodium acetate at pH 5. The suspensions

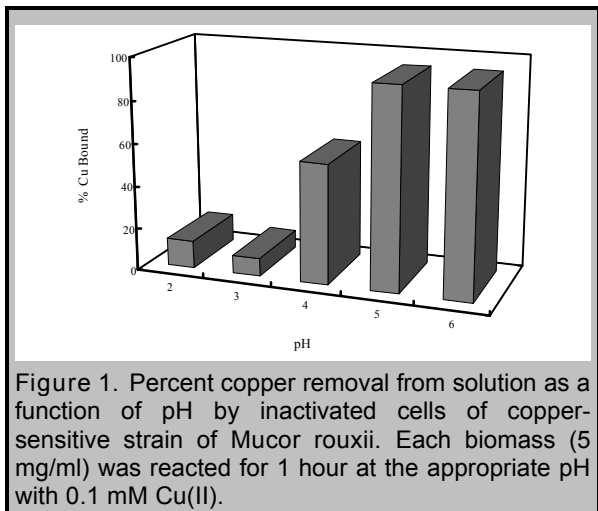


Figure 1. Percent copper removal from solution as a function of pH by inactivated cells of copper-sensitive strain of *Mucor rouxii*. Each biomass (5 mg/ml) was reacted for 1 hour at the appropriate pH with 0.1 mM Cu(II).

were shaken for 30 minutes, centrifuged and decanted. The supernatants were analyzed for the target metal, as indicated before. The same biomaterials were resuspended several more times in a fresh metal solution, repeating the procedure until the saturation capacity of the materials was attained (i.e., the metal concentration in the supernatant was the same as the initial solution). The samples were diluted as required and analyzed for copper content. The amount of metal ion bound to the fungi was calculated from the total metal accumulated from these separate metal-containing solutions.

Recovery of copper adsorbed

To remove the bound metal, the pellets with adsorbed copper from capacities studies were treated twice with 2 ml of 0.1 M HCl, equilibrated by shaking for 5 minutes and centrifuged. After centrifugation the supernatants were removed, diluted as required, and analyzed for copper content by flame atomic absorption spectroscopy.

Methanol esterification of fungal biomass

In an attempt to determine the mechanism of Cu(II) binding to the fungal biomass, carboxyl groups on fungal cells were esterified with methanol. For methanol esterifica-

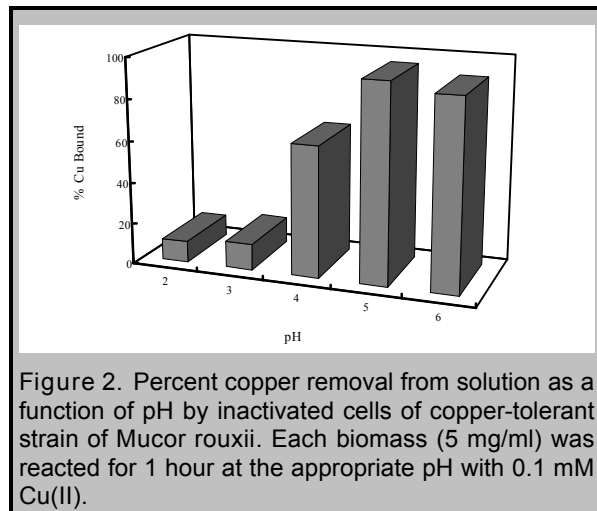


Figure 2. Percent copper removal from solution as a function of pH by inactivated cells of copper-tolerant strain of *Mucor rouxii*. Each biomass (5 mg/ml) was reacted for 1 hour at the appropriate pH with 0.1 mM Cu(II).

tion, inactivated biomasses of *M. rouxii* were treated with methanol in 0.1 M HCl at a biomass concentration of 5 mg/ml and refluxed at 60°C while stirring. After 5, 30, 60 and 360 minutes, samples were centrifuged, washed with cold distilled deionized water, resuspended in deionized water (5 mg/ml), and adjusted to either pH 2 or pH 5, respectively, in order to determine their copper binding ability as indicated previously.

Hydrolysis of esterified carboxyl groups

Upon determination of the copper binding ability of the methanol-esterified fungal biomasses, the inactivated cells were reacted with 0.1 M HCl to remove all adsorbed Cu(II). The cells were subsequently washed with distilled deionized water. After centrifugation the cells were treated with 0.1 M sodium hydroxide (NaOH) in order to hydrolyze the esterified carboxyl groups. Following five-minute agitation, the biomasses were centrifuged, washed, resuspended in deionized water (5 mg/ml), and adjusted to pH 5 in order to determine their copper binding ability as indicated previously.

Copper analysis by atomic absorption spectroscopy

All of the analyses for copper were performed by flame atomic absorption using a Perkin Elmer model 3110 Atomic Absorption Spectrometer with deuterium background subtraction. Impact bead was utilized to improve the sensitivity at a wavelength of 327.4 nm. Samples were read three times, and the mean value was computed. Calibration was performed in the range of analysis, and the correlation coefficients for the calibration curves were 0.98 or greater. Controls of the metal solution were introduced to detect possible metal precipitation.

RESULTS AND DISCUSSION

Experiments were performed to determine the pH dependence of copper binding to inactivated biomasses of both copper-sensitive and copper-tolerant strains of *M. rouxii* grown at high copper concentrations. Figures 1 and 2 show that all biomasses exhibited maximum binding for copper as the pH increased from 2 to 5. The pH dependent binding patterns were similar between the two strains. This behavior suggests similar metal-binding sites on the fungal cells. We believe that the main copper binding sites could be the carboxyl groups present on components of the cell walls, such as poly-uronic acid and proteins [22]. The ionization constants (pK 's) for these carboxyl groups have been reported to be 4-5 [23].

Experiments were performed to determine the copper binding capacities of copper-sensitive and copper-tolerant strains of *M. rouxii* grown at high copper concentration. Table 1 shows that the inactivated cells of the copper-tolerant strain of *M. rouxii* exhibited slightly higher copper binding capacities than the inactivated cells of the copper-sensitive strain. We had expected that the biomass of the copper-tolerant strain would have a higher binding capacity than the biomass of the copper-sensitive strain grown at the same condition. Experiments with *M. rouxii* grown at trace copper concentration (not shown) exhibited lower copper binding capacities than the copper binding capacities shown herein. These results may indicate that upon exposure to high levels of copper in the culture medium, *M. rouxii* develops (as first defense mechanism) polymers with chemical functional groups that may bind copper ions on the surfaces of the cells.

We investigated the possibility of desorbing the copper adsorbed to the fungal cells. Since we obtained low copper binding at pH 2, we expected that using HCl would protonate the carboxyl groups and, thus, the bound copper could be displaced and released into solution. Table 2 shows that 85-100% of copper bound by the biomasses of *M. rouxii* was removed by treatment with 0.1 M HCl.

It is important to understand the chemical nature of the fungal copper binding process. Therefore, it is necessary to determine

Mucor rouxii strains ^a	Cu Bound (mg/g biomass) ^b
Copper-sensitive (IM-80)	20.64 ± 0.03
Copper-tolerant (P1)	23.14 ± 0.10

^acultured at 3.2 mM Cu(II) Sulfate

^beach result represents the mean of 5 replicates and ± is the confidence interval of 95%

Table 1. Copper binding capacities of inactivated cells of *Mucor rouxii*.

Mucor rouxii strains ^a	% Cu Desorbed ^b
Copper-sensitive (IM-80)	85 ± 2.16
Copper-tolerant (P1)	100 ± 1.78

^acultured at 3.2 mM Cu(II) Sulfate

^beach result represents the mean of 5 replicates and ± is the confidence interval of 95%

Table 2. Percent of copper removal from inactivated cells of *Mucor rouxii* by treatment with 0.1 M HCl.

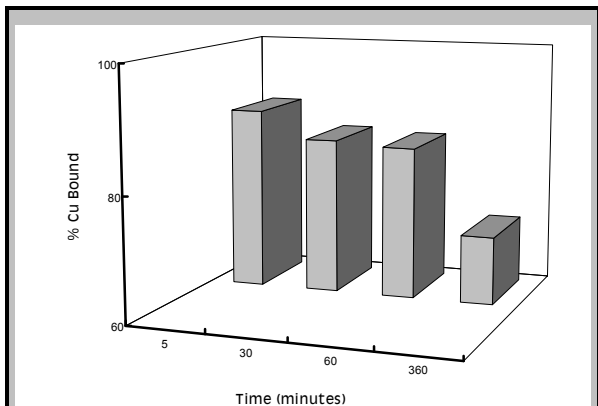


Figure 3. Percent copper removal from solution at different reaction times after methanol esterification by inactivated cells of copper-sensitive strain of *Mucor rouxii*. Each modified biomass (5 mg/ml) was reacted for appropriate times with 0.32 mM Cu(II) in 0.01 M of sodium acetate at pH 5.

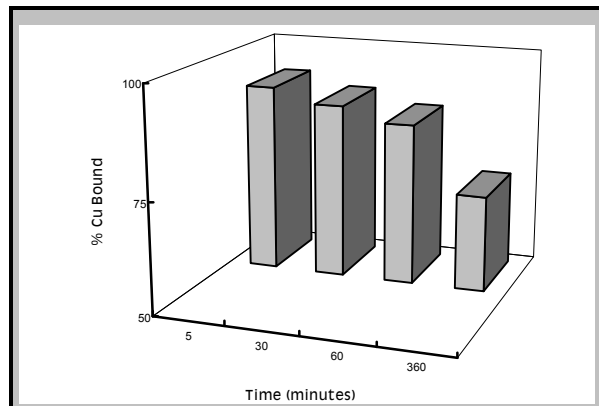
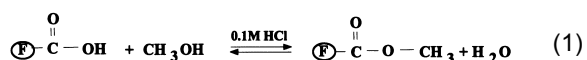


Figure 4. Percent copper removal from solution at different reaction times after methanol esterification by inactivated cells of copper-tolerant strain of *Mucor rouxii*. Each modified biomass (5 mg/ml) was reacted for appropriate times with 0.32 mM Cu(II) in 0.01 M of sodium acetate at pH 5.

which chemical groups on the fungal cell walls are responsible for binding copper ions. One approach to obtaining this information is chemical modification of the copper binding functional groups on the fungal surface. For example, if binding of Cu(II) occurs through interaction with carboxyl groups, a chemical modification rendering the carboxyl groups unavailable for metal binding should cause a marked reduction of Cu(II) binding. Carboxyl groups were esterified with acidic methanol. The basic chemical reaction is shown below (1).



Experiments were conducted on the methanol-modified biomasses to determine whether modification had altered Cu(II) binding characteristics. These studies were performed using a Cu(II) concentration of 0.32 mM in 0.01 M sodium acetate at pH 5. Figures 3 and 4 display the results of Cu(II) binding with the biomasses of the copper-sensitive and copper-tolerant strains of *M. rouxii*, respectively. At pH 5 the adsorption of Cu(II) by the fungal biomasses was decreased dramatically by incubation with methanol, and the loss of binding capacity is proportional to the length of incubation

period in both *M. rouxii* biomasses. However, even after chemical modification of carboxyl groups, there is still 70-75% Cu(II) bound by the copper-tolerant and the copper-sensitive strains of *M. rouxii*. This implies that carboxyl groups may not be fully responsible for Cu(II) binding, and groups such as amino, hydroxyl phosphoryl, oxalate, sulfate and sulfonate may also be involved in the copper binding [24]. Other investigators have found similar results with algal biomasses [25]. However, these results could also imply that the esterification may not be fully completed due to steric hindrance. Research is underway to investigate this further.

As expected, no significant changes in Cu(II) binding by the esterified and unesterified biomasses occurred at pH 2. This should be due to the fact that the carboxyl groups are protonated at pH 2 and the affinity of Cu(II) ions will be inhibited in the fungal biomasses. Esterification of the carboxyl groups also renders the groups unavailable for Cu(II) binding.

Copper binding studies after base hydrolysis of esterified groups were performed in order to demonstrate that the modification of carboxyl groups had indeed occurred

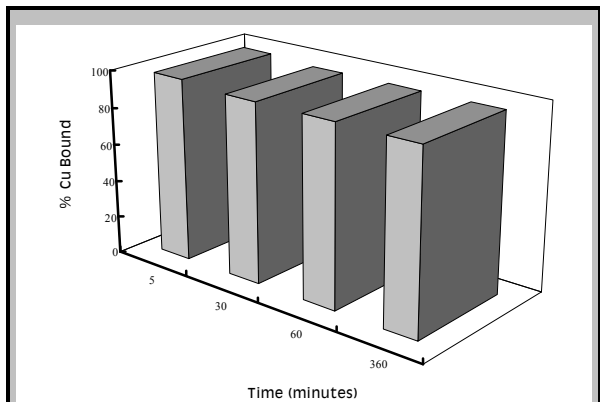


Figure 5. Percent copper removal from solution at different reaction times after base hydrolysis of modified carboxyl groups by inactivated cells of copper-sensitive strain of *Mucor rouxii*. Each modified and hydrolyzed biomass (5 mg/ml) was reacted for appropriate times with 0.32 mM Cu(II) in 0.01 M of sodium acetate at pH 5.

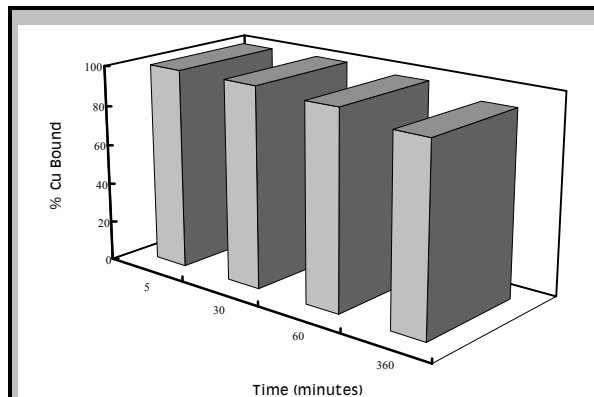


Figure 6. Percent copper removal from solution at different reaction times after base hydrolysis of modified carboxyl groups by inactivated cells of copper-tolerant strain of *Mucor rouxii*. Each modified and hydrolyzed biomass (5 mg/ml) was reacted for appropriate times with 0.32 mM Cu(II) in 0.01 M of sodium acetate at pH 5.

and that the decreased copper binding by fungal biomasses was not a result of cell destruction. Our hypothesis would be supported if the Cu(II) binding ability of the modified biomasses is regained after base hydrolysis (which would free the carboxyl groups to bind). Figures 5 and 6 show that the total binding at pH 5 was recovered in both copper-sensitive and copper-tolerant strains of *M. rouxii*. Therefore, the Cu(II) binding ability of the esterified carboxyl groups was regained after base hydrolysis.

SUMMARY AND CONCLUSIONS

These studies provide preliminary information for the potential use of inactivated cells of *Mucor rouxii* for the removal and recovery of copper ions from solution and suggest furthermore the possibility to reuse these inactivated biomasses. The inactivated cells of the copper-tolerant strain showed a higher Cu(II) binding capacity than the inactivated cells of the copper-sensitive strain of *Mucor rouxii*. The carboxyl groups present on the surface of cells play an important role in copper binding. However, other functional groups such as amino, sulfhydryl, hydroxyl, phosphoryl,

oxalate, sulfate and sulfonate may be also involved. Further studies will be performed in order to establish solid bases for the usefulness of these biomaterials in metal recovery processes and to investigate the complete mechanisms involved in metal binding by inactivated cells of *Mucor rouxii*.

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