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Ability of silica-immobilized *Medicago sativa* (alfalfa) to remove copper ions from solution

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Abstract

Preliminary screening laboratory batch experiments to determine the binding ability of seven different populations of *Medicago sativa* (alfalfa) showed good copper binding characteristics of the biomasses studied. All seven populations examined had similar trends for binding copper as a function of pH. The copper binding by the different alfalfa populations occurred within 5 min. All the alfalfa biomasses showed high copper binding, but the capacities varied according to the alfalfa sample studied. The pH dependence of the copper ion binding to the alfalfa biomasses suggested that it might be possible to recycle the system much like an ion-exchange resin. However, the alfalfa cells cannot be packed into a column because the cells clump together and restrict the flow. We immobilized the cells of Malone alfalfa shoots in a silica matrix. Column experiments for copper binding by the silica immobilized alfalfa demonstrated that the alfalfa tissues were capable of removing considerable amounts of copper ions under flow conditions. After every copper binding cycle most of the copper was desorbed with a few bed volumes of 0.1 *M* HCl. Our work indicates that the Malone-silica preparations are highly durable. We subjected the biomaterial to as many as 10 cycles of binding and elution without observing any significant decrease in copper binding capacity.

Keywords: Biofiltration; Phytoremediation; Medicago sativa; Alfalfa; Copper; Removal; Recovery

1. Introduction

Due to the accumulation in the environment of many toxic chemicals which threaten the public health, there has been an increase in research and development

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aimed at environmental remediation [1]. Traditionally, contaminated areas required soil removal and transport to hazardous landfills or wastewater treatment by activated charcoal and ion-exchange resin filters. Due to the high cost of these methods, there is a crucial need for the development of a method that is not only cost effective, but can be easily implemented. Biological systems are the target of recent research for environmental remediation. This is due in part to an increase in environmental awareness and governmental regulations and policies that favor natural technologies. Biological systems have great potential for remediation because they are easily obtained, low in cost, and prevent further pollution to the environment [2].

Bioremediation has emerged as a technology for accumulation of heavy metal contaminants using living organisms. Many researchers have conducted studies using live microbial and fungal systems to remove heavy metals from contaminated waters [3–8]. Bioremediation works well at low concentrations, but live systems are limited by the toxicological effects of high levels of metal contamination. The advantages of using inactivated systems are the freedom from toxicological effects from high levels of contamination and low maintenance as well as low cost. Dead or inactivated systems using peat moss have proven to be very effective for decontamination of water [9]. Recent research using dead algal biomass has shown a great potential for removal and recovery of heavy metal ions [10].

More recently, phytoremediation has emerged as one of the alternative technologies for removing pollutants from the environment. Interest in using plants for environmental remediation is increasing due to their natural capacity to accumulate heavy metals and degrade organic compounds [11–17]. Studies done with *Datura innoxia* (gypsum weed), the roots of garlic, and dried roots of the tomato plant have shown metal binding properties [18, 19]. Gardea-Torresdey and coworkers demonstrated that carboxyl groups found on the cell wall of algae are responsible for copper binding [20]. Therefore, higher plant cells which contain these functional groups might also be capable of metal binding.

Alfalfa may be a potential source of biomaterial for the removal of heavy metal ions from water. Alfalfa has been found growing in fields irrigated with water containing high levels of heavy metal contamination [21, 22]. Studies have shown that alfalfa has better tolerance levels for heavy metals than other plants [23–26]. This tolerance may be due to its high protein content or the evolution of chemical functional groups in the plant cells that inhibit the toxic effects of the heavy metals [27]. Because alfalfa shows a high affinity for metal ions and can be obtained inexpensively and easily, it has attributes with potential for the removal of metal ions from contaminated waters. We chose to perform experiments with dead alfalfa plant tissues in an effort to remove copper ions from solution through an environmentally safe technology.

The objective of our study was to investigate the binding of copper ions to silica immobilized alfalfa under flow conditions. Column experiments were performed with immobilized alfalfa biomass to examine copper removal and recovery, as well as the ability to recycle the column and determine its efficiency.

2. Methodology

2.1. Alfalfa collection

Seven alfalfa populations were selected as representatives of the many different varieties of alfalfa by their individual characteristics. The different characteristics of each population may be due to differences in plant composition and may provide different chemical functional groups that could affect copper binding to the biomasses.

Alfalfa plants were collected from field studies conducted by Dr. John Henning at New Mexico State University near Las Cruces, New Mexico. Four alfalfa basic germplasms (African, Peruvian, Flemish, Ladak) and two cultivars (Malone, Moapa 69) were obtained from plots that had received irrigation every 2 weeks during the growing season. One cultivar (Cal West 630) was taken from a dryland test, which received no irrigation. Plants were removed from the soil, washed, and the roots were separated from the shoot material (stems and leaves). All samples were oven dried at 90 °C while Malone and CW630 cultivars were also lyophilized. Dried samples were ground to pass through a 100-mesh screen using a Wiley mill.

2.2. pH profile studies for copper binding

Batch laboratory techniques were used for the pH studies. A 250 mg sample of biomass was washed twice with 0.01 *M* hydrochloric acid (HCl) to remove any debris or soluble biomolecules that might interact with metal ions. Washings were collected, dried, and weighed to account for any biomass weight loss. Each biomass sample was resuspended in 50 ml of 0.01 *M* HCl with tissue concentration of approximately 5 mg/ml solution. The pH was adjusted to 2.0, allowed to equilibrate and 2 ml aliquots of the suspension were transferred into three 5 ml plastic tubes. The pH was then adjusted and allowed to equilibrate at pH 3.0, 4.0, 5.0, and 6.0, and 2 ml aliquots of the suspension at each pH were transferred into 3 new tubes for each pH. The suspensions were centrifuged at 2500 rpm for 5 min and the supernatants were examined to determine if soluble materials in solution could be responsible for copper binding. A solution of 0.1 mM copper sulfate ($CuSO_4$) was prepared and the pH adjusted to 2.0, 3.0, 4.0, 5.0, and 6.0. At each pH, 2 ml of the copper solution were added to the respective pH biomass pellet, and to the separated supernatant solutions. In addition, at each respective pH, 2 ml of the 0.1 mM Cu^{2+} solution was transferred to 3 tubes for controls. All the tubes were equilibrated on a rocker for 1 h. The samples were then centrifuged at 3000 rpm for 5 min and the supernatants for the pellets were transferred to clean tubes. Final pHs for all tubes were recorded and analysis for copper was performed by flame atomic absorption.

2.3. Time-dependence studies for copper binding

A 500 mg sample of biomass was washed twice with 0.01 *M* HCl to remove any debris or soluble biomolecules that might interact with metal ions. The washings

were collected, dried, and weighed to account for any biomass weight loss. Each biomass sample was resuspended in 100 ml of deionized water with tissue concentration of approximately 5 mg/ml solution. The solution was then adjusted to pH 5.0 and allowed to equilibrate. Two milliliter aliqouts of the suspension were transferred to 24 tubes; 3 tubes for each time interval of 5, 10, 15, 20, 25, 30, 45, and 60 min. After centrifugation and decantation, 2 ml of 0.1 mM copper solution was added to each of the tubes and controls. All the tubes were equilibrated by rocking and were removed at the appropriate time intervals. The samples were then centrifuged at 3000 rpm for 5 min and the supernatants from the pellets were transferred to clean respective tubes. Final pHs for all tubes were recorded and copper analysis was performed by flame atomic absorption.

2.4. Copper binding capacity studies

Samples of 100 mg of biomass were washed twice with 0.01 *M* HCl and washings were collected and weighed to determine biomass loss. Washed biomass was resuspended in 20 ml of deionized water and the pH adjusted to 5.0. Two milliliter of the suspension were transferred to 3 tubes and then centrifuged. The supernatants were saved for testing. Two milliliter aliqouts of 0.3 mM Cu^{2+} solution was added to each of the tubes and controls. After equilibration for 10 min, the tubes and controls were centrifuged, and the decanted supernatants were stored for copper analysis and again 2 ml of 0.3 mM copper solution were added to each tube. This was repeated 12 times or until the saturation point was achieved and a final pH for all tubes was recorded. Samples were diluted as required to remain within the calibration linear range and analysis for copper was performed by flame atomic absorption.

2.5. Desorption of the adsorbed copper

Pellets from binding capacity studies with adsorbed copper were exposed to 2 ml of 0.1 *M* HCl, equilibrated by rocking for 5 min and then centrifuged. Supernatants were collected for analysis and diluted as required to stay within the calibration range. Pellets were then exposed to 2 ml of 1 *M* HCl to remove remaining metal and equilibrated by rocking for 5 min. After centrifugation, the supernatants were analyzed. All analysis for copper was performed by flame atomic absorption.

2.6. Immobilization of alfalfa biomass

The method for immobilization of biomass material within a polysilicate matrix was similar to that reported by Huei-Yang and Rayson [28]. A 5 g sample of biomass was washed twice by vortexing the sample with water and then centrifuged for 5 min at 3000 rpm to remove solubles and debris. Next, 75 ml of 5% sulfuric acid (H_2SO_4) was mixed with enough 6% sodium silicate (Na_2SiO_3) solution to raise the pH to 2.0. At pH 2.0, the 5 g of washed biomass was added to the silica solution and allowed to stir for 15 min. The pH was then raised slowly by addition of 6% Na_2SiO_3 to obtain a final pH of 7.0. The polymer gel was washed with water until

the addition of two drops of barium chloride ($BaCl_2$) did not produce a white precipitate. $BaCl_2$ was used to indicate whether the sulfates had been removed. The polymer gel with the immobilized biomass was dried overnight at 60 °C and then ground by mortar and pestle and sieved to pass the 20–40 mesh size.

2.7. Column experiments

A column was prepared using 6 ml of immobilized Malone alfalfa. One bed volume equals the volume of immobilized biomass inside the column. The column was washed with 10 bed volumes of 0.01 *M* sodium acetate buffer at pH 5.0 and the effluent pH was monitored to ensure that the column was at the optimal binding pH. A flow rate of 2 ml/min was used to pass 120 bed volumes of 5.0 ppm Cu solution in 0.01 *M* sodium acetate at pH 5.0. Each effluent bed volume was collected and analyzed by flame atomic absorption.

2.8. Recovery of copper from the column

To remove the bound copper, 0.1 *M* HCl was passed through the column at a flow rate of 2 ml/min. Each effluent bed volume was collected and analyzed by flame atomic absorption spectroscopy.

2.9. Analytical procedure

Analysis for copper was performed using a Perkin Elmer model 3110 Atomic Absorption Spectrometer with deuterium background subtraction. An impact bead was utilized to improve the sensitivity at a wavelength of 327.4 nm. Samples were read three times and a mean value and relative standard deviation were computed. The instrument was zeroed with distilled deionized water blanks. Calibrations were performed in the range of analysis and a correlation coefficient for the calibration curve of 0.98 or greater was obtained. The instrument response was periodically checked with known copper standards. The difference between the initial metal concentration and the remaining metal concentration in effluents was assumed to be taken up by the biomass.

3. Results and discussion

Previous screening experiments performed to determine the copper binding characteristics of seven populations of *Medicago sativa* alfalfa showed that the Malone variety had good binding capabilities [29]. Fig. 1 is the pH profile for copper binding by Malone roots and shoots. The shoots show only slightly higher binding than the roots. All seven samples of the populations examined had similar trends for pH versus copper binding. As can be seen in Fig. 1, the binding of copper ions by the Malone biomass is pH dependent, with a maximum binding observed between pH 5.0 and 6.0. At pHs higher than 6.0, the copper ions start to precipitate out of

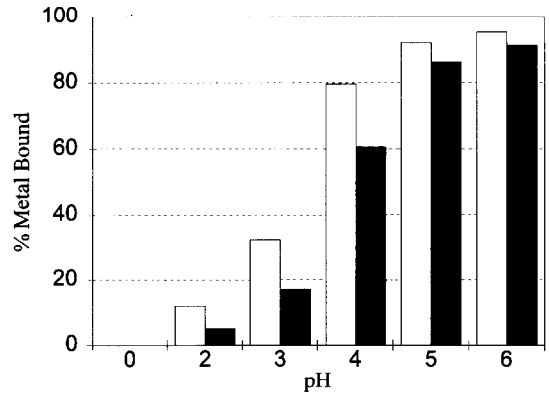

Fig. 1. Effects of pH on copper binding by malone shoots □ and roots ■. Biomass (5 mg/ml) was shaken for 1 h at the appropriate pH with 0.1 mM copper.

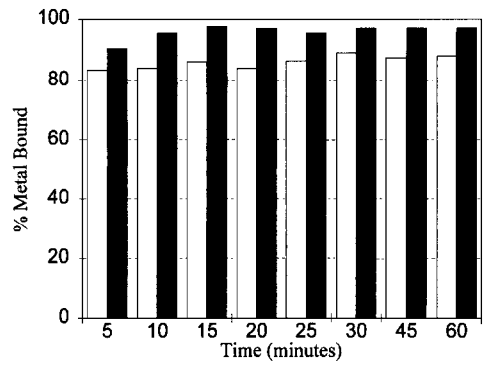

Fig. 2. Time-dependency studies for copper binding by malone shoots □ and roots ■. Biomass was shaken for appropriate time with 0.32 mM copper.

solution. This trend in pH dependent binding suggests that carboxyl groups may play a role in the copper binding by the biomass. The acid dissociation constants (pKa's) for various carboxyl groups have been reported to be around 3–4 [30, 31]. Free carboxyl groups are protonated at pHs lower than 3 and reduce any metal binding. At pHs greater than 4, the carboxyl groups are deprotonated and attract positively charged copper ions. Metal ions bind to the carboxyl groups through an ion-exchange type mechanism. Therefore, if carboxyl groups do play a role in the binding of metal ions, by lowering the pH, the metal ions would be released back into solution.

Studies were conducted to determine how long it would take the copper ions to bind to the alfalfa biomass. Fig. 2 demonstrates the percent of copper ions removed from solution by Malone germplasm roots and shoots when exposed to 0.32 mM copper solution at pH of 5 over a 60 min period [29]. It can be seen that the mechanism for copper ion binding occurs in less than 5 min. Even after 1 h of reaction,

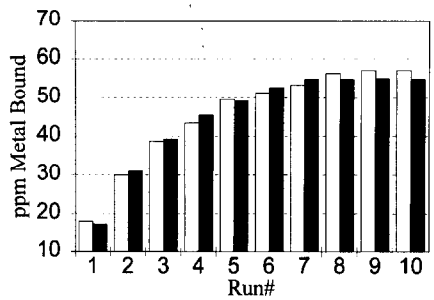

Fig. 3. Copper binding capacity by malone shoots □ and roots ■. Biomass was cycled with additions of 0.32 mM copper for ten runs. The amount of metal bound by 10 mg of biomass is represented in ppm (mg/l).

relatively the same amount of copper was bound. This shows that the binding of copper is relatively stable. Since all of the soluble components were eliminated in prior washing, the binding must be due to the alfalfa biomass. Because the alfalfa plant tissues were inactivated during drying, the rapid binding of the copper ions may be due to functional groups located on the cell wall and rather than cellular enzymatic processes.

Binding capacity experiments previously performed showed that all 7 populations of alfalfa were capable of binding copper ions from solution. Previous experiments indicated that the shoots bind more copper ions than the roots for most of the samples studied. This phenomena may be attributed to the different chemical composition of the plant's roots and shoots. Fig. 3 shows the amount of copper ions bound by the Malone sample [29]. Saturation of the Malone biomass was achieved by cycle 7. Malone shoots had proven to bind copper well and also showed good recovery rates by treatment with 0.1 *M* HCl, therefore Malone shoots were chosen as one of the biomasses that was to be further investigated. The reversibility of the copper binding could have very important implications for the reclamation of metal ions from contaminated waters.

Instead of removal of copper ions from solution by batch experiments, it would be most useful if the alfalfa plant tissues could be packed into a column so that contaminated waters could simply be passed through the column. Unfortunately, when alfalfa cells are packed into columns, the cells clump together and the effluent flow rates are reduced significantly. This problem can be solved by immobilizing the alfalfa cells in a polymer matrix. The immobilized biomass can then be packed into columns through which high flow rates can be achieved. In order to maintain optimal flow through the columns, a polysilicate matrix support material was used to immobilize the alfalfa biomass. This matrix material would have the physical properties of a polymer resin and the binding properties of the alfalfa. Column experiments were conducted to study the effects of copper binding by the alfalfa biomass under flow conditions. Fig. 4 shows the amount of copper remaining after a solution of 5 ppm copper at pH 5.0 was passed through the column of immobilized Malone shoots. Only after 60 bed volumes had been passed did trace amounts of

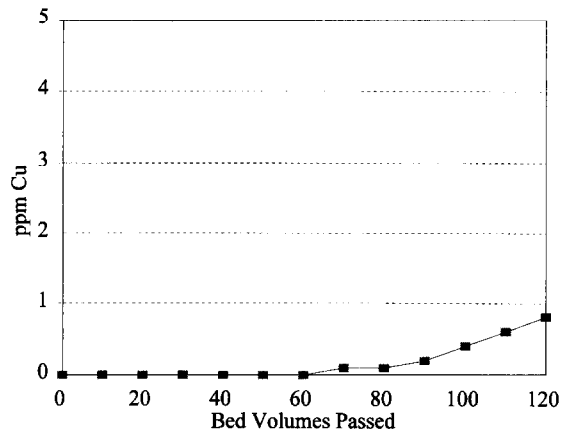

Fig. 4. Concentration of copper remaining in the effluent after being passed through a column of immobilized malone shoots. Five ppm copper solution was passed at a flow rate of 2 ml/min. Column effluents were analyzed for copper. One bed volume equals the volume of silica immobilized alfalfa in the column (6 ml).

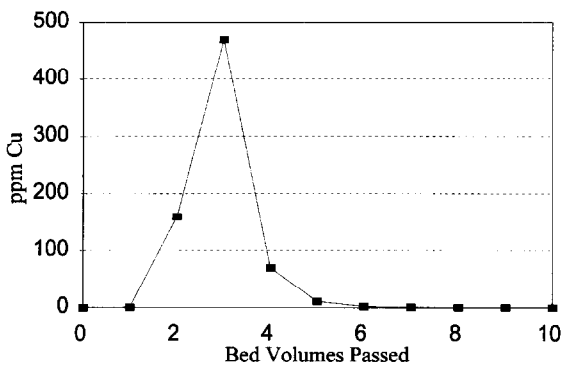

Fig. 5. Recovery of copper ions from column of immobilized malone shoots by the addition of 0.1 *M* HCl. Flow rate of 2 ml/min was used.

copper emerge in the effluent. Even after 120 bed volumes had passed, the concentration of copper in the effluent was still below 1 ppm and 97% of the copper passed was bound in the column. Some of the experiments were carried out to approximately 230 bed volumes and saturation of the column was still not achieved.

The pH profile experiments (Fig. 1) suggested that the copper ions could be removed by lowering the pH. Using low strength acid, the bound copper can be removed from the column without damaging the alfalfa biomass or the polysilicate matrix. Fig. 5 shows the effects of passing 0.1 *M* HCl through the column of immobilized shoots which contained bound copper ions. The initial lag was due to the movement of the solution through the tubing. The immediate effects of the acid are

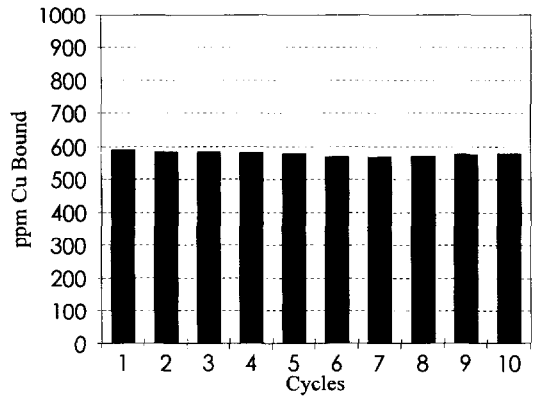

Fig. 6. Amount of copper bound per cycle by the immobilized malone shoots.

seen in bed volumes 1–5. Most of the bound copper was recovered in approximately 3 bed volumes of low concentration acid.

In order to verify that the alfalfa biomass was not affected during the recovery cycle, the column was cycled again with 120 bed volumes of 5 ppm copper solution at pH 5.0. As expected, the amount of copper bound in the second cycle was very similar to that bound in the first cycle. This demonstrates that the low concentration of acid used to remove the bound copper ions had little effect on the binding by the immobilized alfalfa biomass. Fig. 6 illustrates 10 binding/recovery cycles after 120 bed volumes per cycle of 5 ppm copper solution had been passed through the immobilized Malone shoots. It can be seen that the efficiency of the column remained relatively steady even after ten cycles of low concentration of acid were used for the recovery. This innovative technology has potential for the removal and recovery of copper ions from contaminated waters.

4. Conclusions

Alfalfa was shown to be successful in binding copper ions from aqueous solutions. These studies provide preliminary data that shows the potential for the silica immobilized alfalfa biomass to be used as a biosorption resin (biofilter) for the removal and recovery of metal ions from contaminated waters. The alfalfa silica polymer matrix functions like a "biological" mixed-bed ion-exchange resin. Like ion-exchange resins, the alfalfa silica biomaterial can be recycled. We sorbed and desorbed copper ions over as many as 10 cycles with no significant loss in binding efficiency. Alfalfa is inexpensive and easily obtained. This innovative technology provides a reusable material that is not only biodegradable, but also environmentally friendly.

Further experiments are being performed in our laboratory to determine the binding of several different metal ions by different populations of alfalfa. We will also be conducting interference studies to find what effects other cations will have on metal binding under flow conditions.

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