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APPLICATION OF *ASPERGILLUS ORYZAE* AND *RHIZOPUS ORYZAE* FOR Cu(II) REMOVAL

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Abstract—Biosorption of copper(II) by the untreated and acid-treated fungal biomass has been studied in both batch and column modes. Two species, *Aspergillus oryzae* and *Rhizopus oryzae*, were used in this study. *A. oryzae* mycelia (undergoing acid-washing) exhibit a clear advantage in Cu(II) removal, compared with other adsorbents. The acid-washing process can strongly enhance the adsorption capacity for *A. oryzae* mycelia. However, acid-washing does not alter the metal adsorption capacity of *R. oryzae* mycelia. The result indicates that acid-washing is not only a pretreatment step but also a regeneration step in the heavy metal removal process. These dual purposes, therefore, give the acid-washing biomass an indication of successful prospect.

Cultivating *A. oryzae* in pellet form is an effective means of mycelium immobilization. The method established in this study provides the high-yield, uniformly-sized particles (2–3 mm in diameter), which are effective in solid–liquid separation. This pellet column can completely remove metals before breakpoint. After the breakpoint, a significant amount of Cu(II) removal over a long period has been observed. This is thought to be the result of intracellular uptake. Copyright © 1996 Elsevier Science Ltd

Key words—fungi, copper, adsorption, *Aspergillus oryzae*, *Rhizopus oryzae*, heavy metal, immobilization

INTRODUCTION

Heavy metals at low concentrations are difficult to remove. Chemical precipitation, reverse osmosis, and any other methods become inefficient when heavy metal contaminants are present at trace concentrations, in a large volume of solution. Adsorption is one of the few alternatives available for such a situation. Work has been carried out on the use of adsorbents such as activated carbon, low-cost industrial products, and microbial materials. Compared with other adsorbents such as oxides, clays, and activated carbon, biosorbents for heavy metal removal have received relatively little attention. Biosorbents such as fungal, algal, and bacterial have been found to accumulate heavy metals. Fungi in particular have demonstrated unique metal adsorption characteristics and are easy to cultivate (Gadd, 1987).

The application of immobilized biomass has many advantages over the use of suspended biomass; one of these is that the immobilized biomass can be easily regenerated. Another merit is that the immobilization process requires only minimal solid–liquid separation operation. In addition, reactors may be operated at higher flow rates and with a lesser chance of clogging under the immobilized condition.

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Methods for mycelia immobilization include films on solid supports (Anderson, 1983), growth within reticulated support particles (Lewis and Kiff, 1988), entrapment within gels (Mackaskie and Dean, 1984), and simple biomass flocculation or self-granulation (Atkinson, 1986). It is well known that many filamentous fungi form pellets when grown in a submerging liquid culture. The physiological properties of the mycelium and the physico-chemical factors that contribute to pellet formation have been studied by several researchers (Whitaker and Long, 1973).

Difficulties in using the dead ground microbial biomass as the adsorbent are small particle size and low mechanical strength (Tsezos *et al.*, 1989). It has been realized that immobilization of the fungal biomass—either into the supporter or by self-pelletization of a desirable size, mechanical strength, and settling characteristics—is the effective way for applying the biosorption process in practice.

A total of 11 fungal species has been studied in our laboratory (Huang *et al.*, 1988). *Aspergillus oryzae* were chosen for this study because of their high metal adsorption capacity. In addition, several filamentous fungi have been reported to be effective as biosorbents in other studies. Tsezos and Volesky (1982) and Tobin *et al.* (1984) have examined uranium and heavy metal biosorption by *Rhizopus oryzae* to some extent, and the results have shown that *R. oryzae* possess a great potential for binding

cations. Therefore, this species has been chosen in this study for comparison with the *A. oryzae* species. This study aims to explore the potential of fungal biosorbent, with acid treatment, for the removal of Cu(II) ions from dilute aqueous solutions. The experiment consists of two parts. The first part is the batch test of mycelium performance during the adsorption–desorption cycle. The second part is working on the development of a pellet-packed reactor to remove and recover heavy metals with a continuous laboratory-scale operation.

MATERIALS AND METHODS

Pure cultures of *Aspergillus oryzae* and *Rhizopus oryzae* were obtained from Carolina Biological Supply Company, Burlington, NC (15-5950) and the American Type Culture Collection (ATCC 58106), Rockville, MD, respectively. *A. oryzae* stock cultures received from the source were immediately transferred to sterile fungal slants of Sabourand's Agar (SDA). Fungal slants were cultivated in the dark, at 25°C, for a minimum of three days to assure vigorous growth, and then examined to determine purity of the culture. Freeze-dried *R. oryzae* cultures received from ATCC were activated in the liquid medium and transferred to the slant of PDA.

Fungal spores and conidia, grown for 7 days in a SDA or PDA, were suspended in 5 ml of sterile distilled water. Filamentous fungi grow as nearly spherical pellets in a liquid shake culture for a minimum of two days. Mycelia were then aseptically transferred to a stirred chemostat reactor of 1 l working volume, to produce large volumes of mycelia. Reactors were operated in a batch mode for approximately five days at room temperature to produce a consistently uniform mycelial pellet. Approximately 20 g of mycelia on the basis of fresh weight was generated in each batch. Mycelia were then filtered through a steel mesh kitchen strainer, vacuum filtered with distilled water, and rinsed several times to remove any remaining growth media. Mycelia were placed in a Buchner funnel with a Whatman #1 filter, and excess moisture was drawn off under *ca.* 250 mm Hg vacuum.

Harvested mycelia were re-suspended in the solution, with an electrolyte of same ionic strength as in the following experiments, and were stored in the refrigerator at 5°C for no longer than two days. Pretreated mycelia could be preserved in the refrigerator for a month without changing their metal adsorption capacity. Most of the mycelia were washed twice with an acid solution (5×10^{-3} M $HClO_4$) for 15 min, to remove the surface impurities and to stabilize the surface activity. The experiment found that *R. oryzae* undergoing acid-washing was still viable, while acid-washed *A. oryzae* was non-viable when placed on SDA. The fungal mass utilized in various experiments were measured on the basis of dry weight for consistency of comparison.

The fungal biomass were treated by acid solution to evaluate the potential for altering the biomass surface for adsorption enhancement. Biomass were washed with $HClO_4$ solution with controlled concentration for a given time period to remove surface impurities and to condition the surface activities. This washing reagent also served as the eluent during the regeneration experiment. After pretreatment, the filamentous fungi could be easily collected by vacuum filtration through a 0.45 μm Millipore membrane filter.

Acid-washed *A. oryzae* pellets were packed into plexiglass columns (1.6 cm in diameter and 40 cm in height). Each column was packed to a height of 36 cm. Metal solutions were continuously mixed with a magnetic stirrer during the column operation. The ionic strength of the metal solution was maintained with 5×10^{-2} M $NaClO_4$. The solution pH was adjusted to 5.0 with $HClO_4$ or NaOH. The column was operated in an upflow fashion at the specified flow rate. Upon column exhaustion, the total column effluent was collected and analyzed for residual metal concentration using an atomic absorption spectrophotometer.

Scanning electron microscopy (SEM) was conducted with a Philips 501 SEM. X-ray energy dispersion analysis (EDAX) was then performed on an EDAX 9100 mode, energy range 0–20 keV, with an ECON windowless detector.

RESULTS AND DISCUSSION

Comparison with other adsorbents

Fungal adsorbents remove heavy metals from solutions as a function of pH and metal concentration in a manner analogous to a variety of adsorbents. It is striking to know that the metal removal capacity of fungal biomass is comparable with other biosorbent and conventional solids. Figure 1(a) and (b) illustrate the Cu(II) adsorption isotherms of a number of adsorbents. Two fungal species, *A. oryzae* and *R. oryzae*, are included in this figure. As can be seeen in this comparison, acid-washed *A. oryzae* mycelia exhibit a clear advantage in Cu(II) removal compared with other

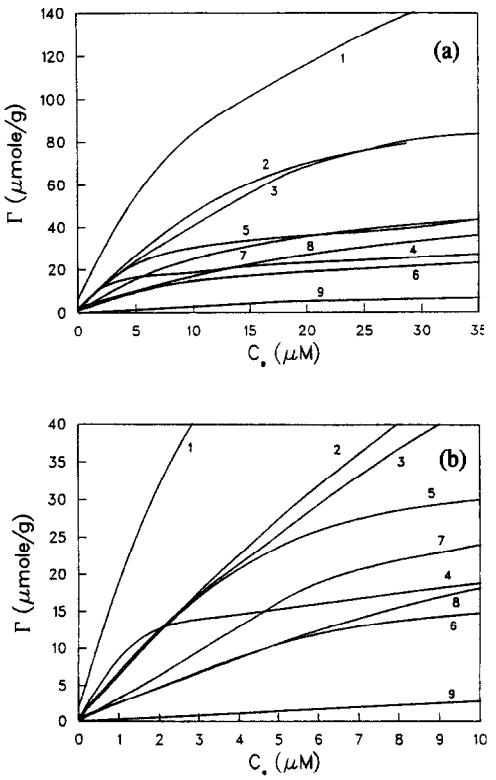

Fig. 1. Comparing the Cu(II) adsorption isotherm with various adsorbents. $pH_{final} = 5.00$, $I = 5 \times 10^{-2}$ M $NaClO_4$: (a) high conc. range; (b) low conc. range. Adsorbents include (1) *A. oryzae*, (2) activated sludge[1], (3) *R. oryzae*, (4) *S. cerevisiae* (yeast)[2], (5) *S. quadricauda* (algae)[3], (6) *C. vulgaris* (algae)[3], (7) chitin (crab shell)[4], (8) activated carbon (Nuchar SA)[5], and (9) activated carbon (Filtrasorb 400)[5]. [1]Tein and Huang (1987); [2]Huang *et al.* (1989); [3]Harris and Ramelow (1990); [4]Su (1989); [5]Corapcioglu (1984).

Table 1. The effect of acid pretreatment on the fungal biomass

Fungal species	Treatment condition: Acid conc.	Treatment condition: Treatment time	Biomass loss (%)*	Cu(II) adsorption capacity (μmol/g)†
A. oryzae	0	0	19.6	12.10
	0.005 N	5 min	19.4	25.57
	0.005 N	20 min	22.4	38.16
	0.001 N	20 min × 2	24.5	42.84
	0.005 N	20 min × 2	30.6	50.73
	0.005 N	20 min × 4	30.4	50.55
	0.050 N	20 min × 2	30.0	54.06
	0.100 N	20 min × 2	30.0	55.57
R. oryzae	0	0	2	45.5
	0.005 N	20 min × 2	18	45.5

*Comparing to native biomass after treated biomass react with Cu(II).

†Initial Cu(II) conc. = 5×10^{-5} M; initial mycelia conc. = 0.98 g/l; final pH = 5.0; $I = 5 \times 10^{-2}$ M $NaClO_4$.

adsorbents. Acid-washed filamentous fungi (*Aspergillus* and *Rhizopus* sp.) as well as the mixed culture of bacteria (activated sludge) show a higher Cu(II) adsorption capacity than the single cells (yeast and algae). Generally, the biosorbents compare favorably with two types of common activated carbon. Figure 1(b) clearly indicates that the removal of Cu(II) by the various solids in dilute solutions follows the order: acid-washed *A. oryzae* > acid-washed *S. cerevisiae* > *R. oryzae* ≈ activated sludge ≈ *S. quadricauda* (algae cell) > protein-chitin > Nuchar SA ≥ *C. vulgaris* > Filtrasorb 400. However, in the higher concentration range, the effectiveness of Cu(II) removal appears to follow the order: acid-washed *A. oryzae* > *R. oryzae* ≈ activated sludge > *S. quadricauda* (algae cell) ≈ protein-chitin > Nuchar SA > acid-washed *S. cerevisiae* > *C. vulgaris* > Filtrasorb 400.

Conditions of acid treatment

Table 1 lists the effect of acid treatment on the Cu(II) adsorption capacity of fungal biomass. It indicates that the weight of the fungal biomass was decreased after acid treatment, and its loss is dependent on the condition of acid treatment. Generally, the residual weight of acid-washed biomass becomes stable when the concentration of acid solution used to treat the biomass is greater than 5×10^{-2} M, and the washing time is two times 20 min or longer. Under the same condition of acid-washing, the weight loss of *A. oryzae* is more than *R. oryzae*. The capacity of metal adsorption is increased by increasing the concentration of acid solution. This implies that the acid treatment on the fungal mycelia results in not only a physical cleaning or washing-out, but also some chemical transformation, such as denaturation of the protein molecules.

Acid treatment effect

It has been shown in a previous study of Huang *et al.* (1991) that the acid-washing process resulted in a better metal removal capacity of *A. oryzae* mycelia than other methods over the whole range of pH tested, especially in the acidic range. According to the experimental result, it has been found that *R. oryzae* undergoing acid-washing is still viable, while acid-washed *A. oryzae* is non-viable when placed on SDA. A typical diagram of pH effect on the removal of metal by untreated and acid-washed mycelia is shown in Fig. 2. It clearly indicates that, unlike *A. oryzae*, acid-washing does not alter the metal adsorption capacity of *R. oryzae* mycelia, or their bio-activity. From the X-ray EDA spectra as shown in Figs 3 and 4, *A. oryzae* mycelium after being acid-washed contains a higher percentage of surface nitrogen. This indicates that acid treatment may dissolve polysaccharide compounds in the outer layer of the fungal wall (e.g. around 30% weight loss during acid-washing listed in Table 2), and therefore produce additional binding sites (amino group). However, the surface components of *R. oryzae* remain unchanged after being acid-washed. This evidence provides an explanation for the unchanged metal adsorption capacity of *R. oryzae* mycelia. In conclusion, the acid-washing process results not only in a clean-up of the surface impurities (e.g. K^+), stabilization of the surface compounds, and reduction of enzyme activity, but also opens the available site for metal adsorption. Hence, acid-washed *A. oryzae* mycelia shows a much higher metal removal capacity than untreated mycelia over the pH range 2–9.

Regeneration and reuse

In order to evaluate the efficiency of using mineral acid (5×10^{-5} M $HClO_4$) to regenerate spent fungal mycelia, a series of batch adsorption and desorption experiments were performed. Typical results of the relation between the untreated, acid-treated, and

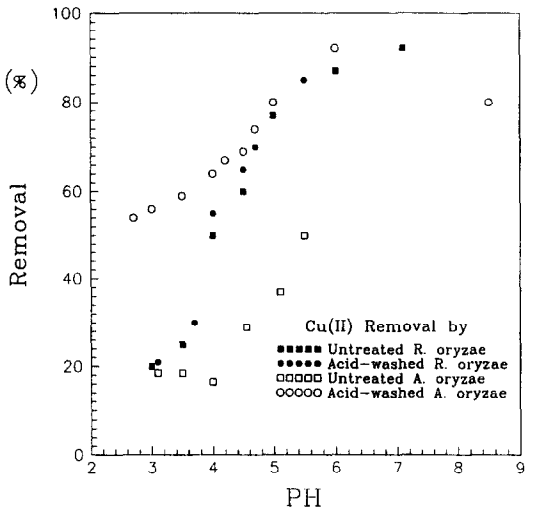

Fig. 2. Cu(II) removal by two fungal species without pretreatment or with acid-pretreatment under various pH conditions. Species includes *Rhizopus oryzae* and *Aspergillus oryzae* Cu(II) conc. = 5×10^{-5} M, mycelia conc. = 0.82 + 0.04 g/l, and $I = 5 \times 10^{-2}$ M $NaClO_4$.

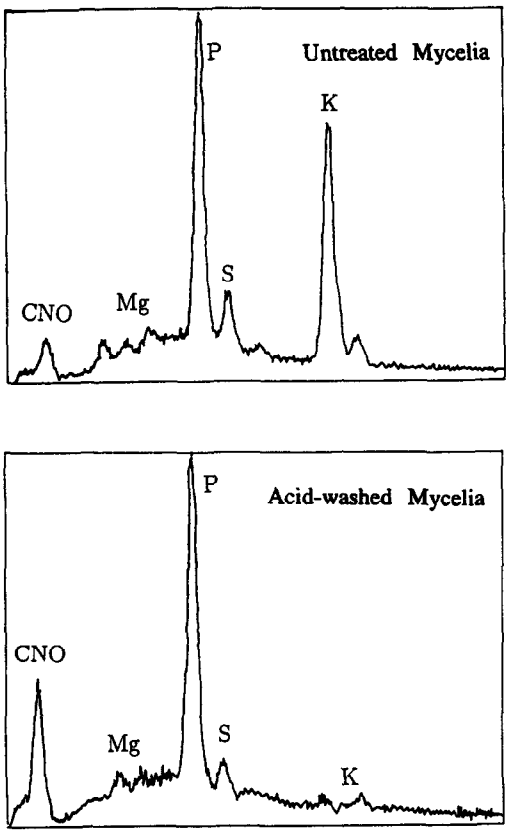

Fig. 3. X-ray energy dispersion analysis spectra of untreated and acid-washed *A. oryzae* mycelia.

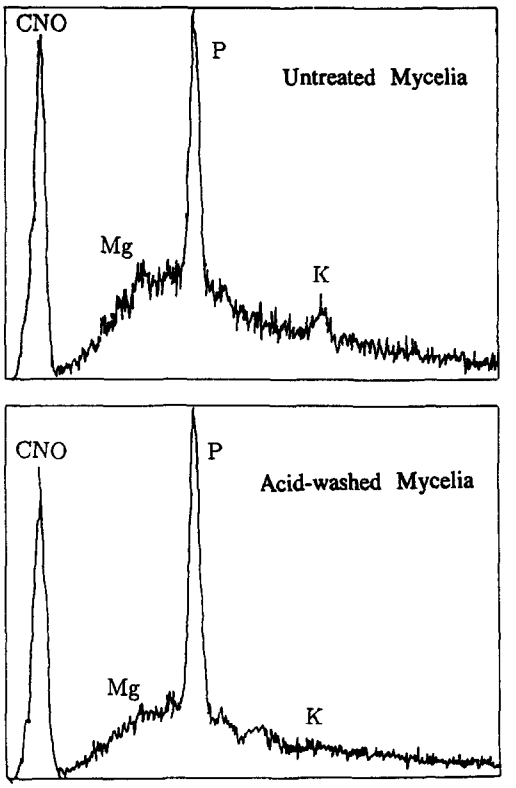

Fig. 4. X-ray energy dispersion analysis spectra of untreated and acid-washed *R. oryzae* mycelia.

reused *A. oryzae* mycelia undergoing adsorption and desorption are shown in Fig. 5. Cu(II)-laden untreated mycelia could attain 100% regeneration after the first batch of acid-washing. The metal uptake capacity of the regenerated mycelia was higher than that of either the untreated or the acid-washed mycelia. The stability of biomass for binding with Cu(II) was conducted over a number of cycles involving sequential adsorption and regeneration operations. The results shown in Fig. 6 clearly indicate that the Cu(II) binding capacity of biomass remains constant from the second regeneration cycle up to the 12th regeneration cycle. As no mechanical damage was observed during the regeneration, further regeneration cycles would have been possible. This implies that the acid-washed fungal biosorbent used to remove metal ions can be reused by acid regeneration for a number of times without losing or creating binding sites. It is suggested that acid-washing take place, not only as a pretreatment step, but also as a regeneration step in the heavy metal removal process. These dual purposes, therefore, give the acid-washing biomass an indication of successful prospect. Norberg and Persson (1984) have reported that acid treatment does not affect the metal uptake values of the biomass when it is reexposed to a metal solution after regeneration.

Settling characteristics

The settling characteristics of a fungal mycelia suspension can be monitored by using either the sludge volume index (SVI), or by measuring the zone settling rate. The SVI is the volume in milliliters occupied by 1 g of suspension after 30 min of settling.

For good settling in conventional activated sludge plants, a typical SVI is under 300. Table 2 lists the sludge volume index of the untreated and the acid-washed *A. oryzae* biomass. The SVIs of other fungal species are also listed for comparison. Treating

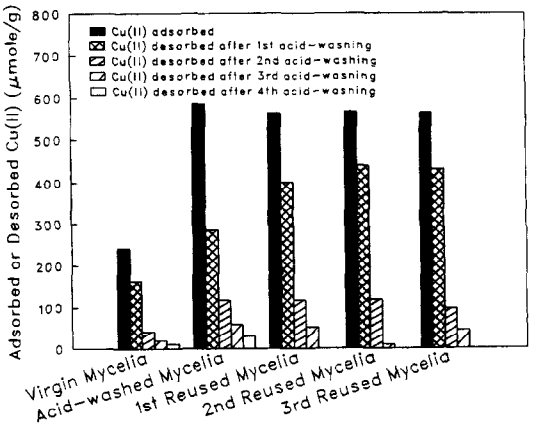

Fig. 5. Successive Cu(II) adsorption and desorption in the untreated and reused *A. oryzae* suspension. Regenerant: 5×10^{-3} M $HClO_4$.

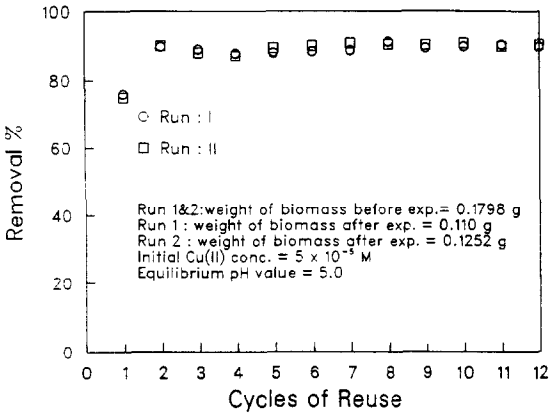

Fig. 6. The stability of regeneration and reuse of *A. oryzae* mycelia for Cu(II) removal.

the mycelia with either $HClO_4$ or H_2SO_4 can lower their SVIs, and hence, enhance their settling efficiency. Untreated *Rhizopus oryzae* mycelia and *Aspergillus nidulans* mycelia have a low SVI, while *Fusarium solani* mycelia possess a very high SVI that can cause a serious problem in solid–liquid separation, regardless of the fact that *Fusarium solani* has been found to be the most powerful species for Cd(II) removal from solutions among several fungal species (Huang *et al.*, 1988). It is concluded that acid-washed mycelia have a priority over the untreated mycelia in the degree of compression sedimentation, which will be effective in solid–liquid separation when the completely mixing reactor is available for heavy metal removal from solutions.

Column studies

The fungal mycelia and conidia were transferred to 200 ml of growth media that was shaken (200 rpm) at room temperature for precultivation. After a given number of days, filamentous fungi were grown as discrete and nearly spherical pellets with a size of 2–3 mm. These pellets were then transferred to a stirred chemostat reactor of 1 l working volume to produce large volumes of mycelia.

A typical set of results obtained from the operation of a mycelial pellet column of *A. oryzae* to remove Cu(II) from the solution is given in Fig. 7. Over the

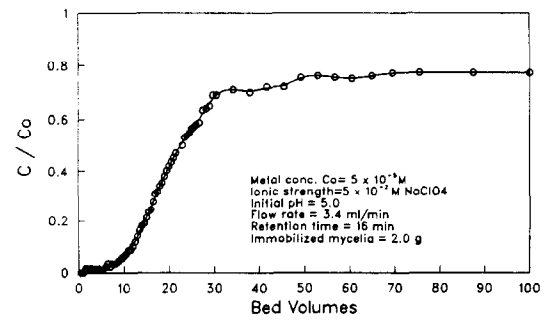

Fig. 7. Breakthrough curve for Cu(II) removal by *A. oryzae* pellet bed.

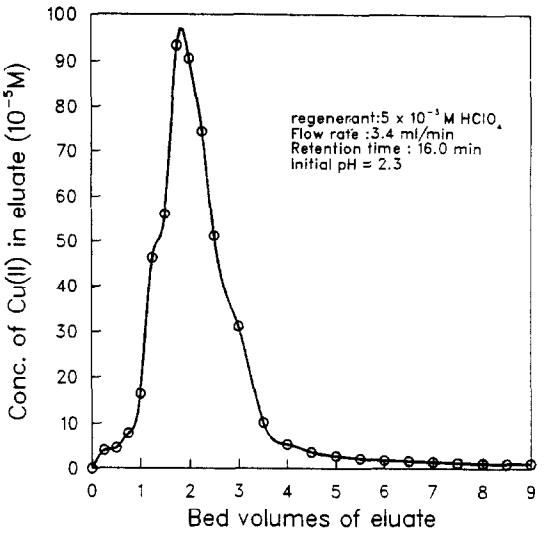

Fig. 8. Regeneration curve for Cu(II)-laden *A. oryzae* column.

first 10 bed volumes of operation, the column works at maximum efficiency. With this reactor configuration, Cu(II) ion removal took place successively as it progressed up the column. After 30 bed volumes, Cu(II) removal was still significant and the efficiency remains constant up to more than 100 bed volumes. This phenomenon was attributed to the intracellular uptake of Cu(II), which is similar to the result for yeast as indicated by Huang *et al.* (1989).

When the adsorbent became saturated, it was reconditioned with acid treatment and put back into service. A strong acid solution, 5×10^{-3} M $HClO_4$, identical to the acid-washing reagent for the treatment of biomass, was used as the regenerant to desorb metals from the metal-laden column (Fig. 8). According to the result (Fig. 6), acid treatment does not affect the metal uptake capacity of the biomass when it is re-exposed to a metal solution after regeneration. In our experiment, acid treatment of the column did not alter the surface characteristics except by releasing bound metals from the pellets. This is because we have washed fungal pellets with a strong acid (the same as we used in regeneration). The result shows that a Cu(II)-laden column can be stripped of the adsorbed Cu(II) using one volume of acid (5×10^{-3} M $HClO_4$) per 20 volumes of Cu(II) (5×10^{-5} M) containing solution. Whether the metal is accumulated predominantly by surface binding or by intracellular uptake will affect the economics of

Table 2. The sludge volume index (CVI) of various types of fungal mycelia

Type of biomass	SVI
Untreated *Aspergillus oryzae*	396
Untreated *Rhizopus oryzae*	250
Acid-washed *Aspergillus oryzae*	195
Acid-washed *Rhizopus oryzae*	250
Untreated *Aspergillus nidulans*	270
Untreated *F. solani*	> 800

the recovery process. While surface-bound metal might be removed easily by acid-washing, intracellular metal could only be released by destructive treatment. It is clear that the removal of Cu(II) by the intracellular uptake of mycelia from the breakpoint to complete exhaustion is significant. Unless the metal-loading biosorbents are to be discarded after exhaustion, the operation should be terminated prior to the breakpoint, in order to extend the operation cycles of the column for heavy metal removal.

SUMMARY

In general, filamentous fungi possess higher metal adsorption capacities than either yeast or algae, and their high adsorption constant implies that they have a preference for heavy metal removal. Acid washing is the most promising method for the enhancement of metal adsorption capacity of *A. oryzae* mycelia. However, the metal adsorption capacity of *R. oryzae* remained unchanged after being acid-washed. Generally, untreated biomass was not an effective adsorbent for heavy metal removal. The acid-washed fungal biosorbent, used to remove metal ions, can be reused after acid regeneration for a number of times without losing or creating binding sites. In addition, acid-washing of fungal biomass can improve their settling efficiency. Cultivating fungal mycelia in a pellet form is an effective means of immobilizing fungal mycelia. The method established in this study provides the high-yield, uniformly-sized particles (2–3 mm in diameter) that are effective for biomass–liquid separation. These biosorption columns can completely remove the heavy metal before the breakpoint. After the breakpoint, a significant amount of Cu(II) removal over a long period was observed. This is thought to be the result of intracellular uptake.

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