Mechanism of metal biosorption by *Cladosporium cladosporioides*
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The studies up to this stage were carried out from the process development point of view. However, from an academic point of view, it was felt that the mechanism of metal biosorption must be studied. We were interested in finding out the basis of gold and silver selectivity in case of *Cladosporium cladosporioides* #1. An attempt was made in this direction. These studies are discussed in this chapter.

Various mechanisms have been proposed for metal binding to fungal surfaces. These include physical adsorption on account of electrostatic interactions and ion-exchange, chemical binding, surface precipitation and metal transformations. Attempts have been made by different researchers to find out the functional groups involved in metal binding. Use of physical-chemical pretreatments to biomass for investigating the role of cell surface polymers has been demonstrated (Nakajima *et al.*, 1981). Some of the workers have analyzed the cell wall macromolecular composition of microorganisms to study their role in metal biosorption. A more direct approach has been the use of sensitive instrumental methods such as Transmission Electron Microscopy, Electron Spin Resonance Spectroscopy, Nuclear Magnetic Resonance Spectroscopy, Infra Red Spectroscopy, Neutron Activation Analysis, Energy Dispersive X-ray spectroscopy, Electron Spectroscopic and Chemical Analysis, etc. (Ashkenazy *et al.*, 1996; Beveridge *et al.*, 1997; Kuyucak and Volesky, 1988b; Majidi *et al.*, 1990).

Despite the efforts made so far as mentioned above, a definitive assignment of role to individual functional group in metal binding has not been possible because, the metals may be adsorbed by a combination of different mechanisms. In the present work, it was felt that the property of preferential metal uptake exhibited by *C. cladosporioides* #1 was worth investigating in
order to find out how gold and silver interact with fungal surface. This information might be useful in two aspects:
1. It might be possible to determine the basis of metal selectivity in C. cladosporioides #1.
2. It might suggest means to increase gold and silver biosorption by manipulation of surface features involved in biosorption.

To investigate these mechanistic aspects our studies involved a two prong approach. The first approach involved an analysis of fungal cell wall macromolecular composition, while the second one was targeted at studies on surface features of the biosorbent.

6.1. Cell wall isolation

The cell wall isolation procedure (Taylor and Cameron, 1973) was carried out at 4 °C. Mycelia (10 g wet weight) of C. cladosporioides grown in Sabouraud's medium were washed extensively with 1 liter of deionized distilled water. The mycelia were then resuspended in 50 mL distilled water to which 12.5 g glass beads (0.45 - 0.5 mm) were added. The suspension was homogenized (6 - 7 cycles) in B. Braun (Germany) mechanical cell homogenizer for 60 s. The fluid was then decanted and centrifuged at 1500 g for 10 min. Pellet was washed with cold distilled water 5 - 6 times until the supernatant appeared clear. At this stage, colorless cell wall pellet was obtained. The pellet was washed twice with decreasing concentrations of sodium chloride solution (5%, 2%, 1% w.v⁻¹). It was then washed with cold distilled water. Thin suspension of the cell wall material was observed under light microscope to ensure >90% cell disintegration. The material was lyophilized and stored at -10 °C until use. The cell wall yield was to the tune of 32% on a dry weight basis.
6.2. Biosorption of gold and silver by isolated cell wall preparation

The cell wall preparation was conditioned to pH 4 for gold uptake experiments. The cell wall (0.05 g) was added to gold solution (100 mg.L⁻¹, pH 4, 25 mL). After contacting for 30 minutes on a shaker at 120 rpm, the solutions were separated from cell wall and analyzed for residual gold. Addition of lyophilized mycelia to the gold solutions served as positive control, whereas, solutions without addition of cell wall or mycelia were kept as negative controls. It was observed that the specific gold uptake values were 96.6 mg.g⁻¹ for whole mycelium and 94.2 mg.g for the isolated cell wall preparation. Thus, cell wall was the site of gold deposition in inactivated C. cladosporioides #1. In a similar manner, it could be observed that specific uptake of silver was 44.5 and 42.8 mg.g⁻¹ for whole mycelia and isolated cell walls respectively. In this case, 0.05 mycelia/cell walls were contacted with 25 mL silver solution (100 mg.L⁻¹, pH 7).

The solutions of gold and silver after analysis of the residual metals were analyzed for increase in sodium, potassium, calcium, magnesium and manganese ions. It was observed that was no release of these ions from the biosorbent after metal contacting. This indicated that ion-exchange was not involved in the biosorption of gold and silver.

6.3. Determination of cell wall composition

6.3.1. Neutral sugars

Cell walls hydrolysis was carried out according to procedure used by Morrin and Ward (1989). Cell walls (10-20 mg dry wt.) were suspended in 0.7 mL of water and kept on ice. Then 1.8 mL of concentrated sulfuric acid (91% w.w⁻¹) was added dropwise with constant shaking and cooling on ice so as to avoid caramelization. After chilling the reaction mixture for 30 minutes on ice, it was
diluted to a final volume of 22.5 mL with distilled water. Tubes were evacuated and sealed, and incubated at 110°C in an oven for 10-12 hours. The hydrolysates were neutralized with BaCO₃ and deionized with amberlite IR-120 (H⁺) resin and vacuum evaporated. Neutral sugar content of sulfuric acid hydrolysate of cell wall was determined by the phenol - sulfuric acid method as used by Morrin and Ward (1989) with glucose as standard. Aliquots of sample in a final volume 1 mL were mixed with 1 mL of 5% phenol. 5 mL of concentrated sulfuric acid was rapidly added directly to the solution, and not along the walls of tubes. After cooling to room temperature, the color was measured at 490 nm. It was found that cell wall neutral sugar content was 12.8%. Neutral sugar content of whole mycelium was 23.7%.

6.3.2. Amino sugars

The procedure used for HCl - hydrolysis of cell walls was essentially the same as that used by Morrin and Ward (1989). Cell walls (10 - 20 mg dry wt.) were hydrolyzed with 6 N HCl (20 mL) at 110°C for 10 - 12 hours. Hydrochloric acid was removed by evaporation under reduced pressure and finally by keeping over NaOH pellets in a desiccator under vacuum. Amino sugar content was determined in an aliquot of HCl - hydrolysate of cell walls by using modified Elson-Morgan method (Good and Bessman, 1964), with glucosamine as standard. Aliquots of samples in a final volume of 1 mL were mixed with 1 mL of acetyl-acetone reagent in stoppered tubes and kept in a boiling water bath for 45 minutes. After cooling to room temperature, 4 mL of 95% ethanol was added to each tube and mixed thoroughly. Then, 1 mL dimethyl para-amino benzaldehyde (PDAB) reagent was added, mixed thoroughly and kept at room temperature for 1 hour. Color developed was measured at 540 nm.

Role of chitin and chitosan as metal binding sites has been highlighted earlier (Muzzarelli and Tanfani, 1982). In contrast, copper biosorption by *Ganoderma lucidium* (Muraleedharan and Venkobachar, 1990) was reported to be
unrelated to chitin content of the cell wall. In the present studies, hexosamine content in the cell wall of *C. cladosporioides* #1 was found to be 0.1%.

6.3.3. Proteins

Wall proteins were solubilized by boiling with 1 M NaOH for 30 minutes. Protein content was estimated by the method of Lowry *et al.*, (1951). It was observed that cell wall protein content was 2.72 mg% on dry weight basis.

6.3.4. Lipids

Cell walls preparation was treated with a 2 : 1 mixture of chloroform and methanol in order to extract the lipid components. The extracts were then evaporated and residual contents were weighed using a precision balance to find out the lipid content of *C. cladosporioides* #1 cell wall. The lipid content was found to be 4%.

6.4. Characterization of gold and silver biosorption on the basis of surface studies

The studies on characterization of metal biosorption mechanisms were carried out by using sensitive techniques such as Electron Spectroscopy for Chemical Analysis (ESCA) and Fourier Transform Infra - Red Spectroscopy (FTIR Spectroscopy). These techniques were used to get some information regarding the functional groups on biosorbent surface involved in biosorption. Biosorbent beads were loaded with gold and silver by repeatedly contacting with the metal solutions (100 mg l⁻¹). The beads were pre - conditioned to pH 2 for gold loading and pH 7 for silver loading. Conditioned beads without gold and silver loading were used as controls. For analytical work, biosorbent beads were crushed to a fine powder for use.
6.4.1. Studies using X-ray Photoelectron spectroscopy (XPS)

The studies were carried out using ESCALAB MK II Spectrometer (V. G. Scientific Co. UK). XPS spectra before and after metal biosorption were taken to identify groups participating in the biosorption. The peak for gold was not clearly identifiable. Analysis of other elements such as carbon, oxygen, sulfur and nitrogen was carried out to find out whether, binding of gold affected these components on the biosorbent surface. Sulfur was not detected in the biosorbent preparation. It was observed that the binding energies corresponding to carbon (285 eV) and oxygen (533 eV) did not change after gold biosorption. The content of carbon reduced marginally from 76.2 to 71.7% (Figure 6.1), while that of oxygen increased slightly from 16.3% to 18.9% (Figure 6.2). Biosorbent that was not loaded with gold did not show the presence of nitrogen; whereas, gold loaded biosorbent showed 1.3% nitrogen. Thus, nitrogen content being very low, it might be said that nitrogen may not be very important in the biosorption of gold. It might be conjectured therefore, that gold biosorption was not associated with chemical reactions with surface carbon or oxygen groups per se. Acidic conditions probably made conditions favorable for gold binding on account of protonation of surface groups. The gold anion was then able to bind protons by electrostatic interactions. Biosorbent conditioned to pH 2 showed presence of additional band corresponding to silicon (binding energy 101.5 eV) in both gold loaded and non-loaded samples. It is not clear whether silicon contamination occurred as a result of leaching from the glassware used in experiments. Sticking tape used for sample holding might also have contributed to the silicon peak observed in the samples.

Biomass conditioned to pH 7 showed presence of carbon at 285 eV (73.3%), oxygen at 533 eV (19.7%) and nitrogen at 400 eV (2.0%). Sulfur was not detected. Upon silver binding, the carbon content reduced to 59.6% indicating its involvement in biosorption (Figure 6.4). The increase in oxygen content to 35.0% also indicated its role in silver biosorption (Figure 6.5). Although
Figure 6.1. XPS analysis of *C. cladosporioides* #1 conditioned to pH 2 showing carbon peak at 285 eV. a) unloaded biomass (carbon content = 76.1%), b) biomass loaded with gold (carbon content = 71.7%).

![Figure 6.1 XPS Carbon Analysis](image1.png)

Figure 6.2. XPS analysis of *C. cladosporioides* #1 conditioned to pH 2 showing oxygen peak at 533 eV. a) unloaded biomass (oxygen content = 16.3%), b) biomass loaded with gold (oxygen content = 18.9%).

![Figure 6.2 XPS Oxygen Analysis](image2.png)
Figure 6.3. FTIR spectra of *C. cladosporioides* #1 conditioned to pH 2. a) unloaded biomass, b) biomass loaded with gold.
Figure 6.4. XPS analysis of C. cladosporioides #1 conditioned to pH 7 showing carbon peak at 285 eV. a) unloaded biomass (carbon content = 73.3%), b) biomass loaded with silver (carbon content = 59.6%).
Figure 6.5. XPS analysis of *C. cladosporioides* #1 conditioned to pH 7 showing oxygen peak at 533 eV. a) unloaded biomass (oxygen content = 19.7%), b) biomass loaded with gold (oxygen content = 35.0%).

Figure 6.6. XPS analysis of *C. cladosporioides* #1 conditioned to pH 7 showing nitrogen peak at 400 eV. a) unloaded biomass (nitrogen content = 7%), b) biomass loaded with gold (nitrogen content = 0.3%).
nitrogen content was lower (2%), reduction in nitrogen to 0.3% indicated possible involvement of nitrogen in silver sorption (Figure 6.6). Silver was detected (5.1%) at 369 eV and 375 eV in the silver loaded sample (Figure 6.7), whereas, it was not detected in the non-loaded or control biosorbent.

6.4.2. Studies using Fourier Transform Infrared Spectroscopy (FTIR)

A Perkin Elmer FT-IR Spectrum 2000 (England) Spectrometer was used in FTIR analysis. Powdered samples were pelleted using potassium bromide (KBr). It could be seen that conditioning the biomass of C. cladosporioides #1 to pH 2 resulted in elongation of the 875 - 1750 cm⁻¹ region. This indicated that the oxygenated forms of carbon such as carbonyl and carboxyl groups were more exposed at acidic pH that could probably favor gold biosorption. When the biomass conditioned to pH 2 was loaded with gold, no changes were seen in the IR spectrum (Figure 6.3). This indicated that chemical reactions might not be involved in biosorption of gold. This supports the results of XPS analysis indicating that gold anions (AuCl₄⁻) probably bind to protonated carbonyl and carboxyl groups by electrostatic interactions. FTIR analysis used in studies on gold biosorption by the alga Sargassum natans (Kuyucak and Volesky, 1989b) revealed that carbonyl groups were involved in binding gold with less involvement of N-containing groups.

FTIR analysis of silver loaded biosorbent (pH 7) showed changes affecting certain bands in the 875 - 1750 cm⁻¹ region as compared to the non-loaded conditioned biomass (Figure 6.8). The 1040 - 1070 cm⁻¹ and 1743.6 cm⁻¹ bands in non-loaded biomass were due to C-O and C=O stretching in carbonyl and carboxyl groups. These band elongated after silver biosorption indicating role of these groups in silver biosorption. Changes in 1370 - 1500 cm⁻¹ region corresponded to sorption of silver by various oxygenated forms of carbon. The strong absorption bands at 1650.9 cm⁻¹ and 1743.6 cm⁻¹ in non-loaded biomass were due to C=O stretching of carboxyl group. The 1650.9 cm⁻¹ band shifted to 1655.6 cm⁻¹ while the 1743.6 cm⁻¹ band became
elongated after silver biosorption indicating involvement of carboxyl groups in silver biosorption.

6.5. Comparative account of gold and silver biosorption using the non-selective strain *C. cladosporioides* #2

In order to find out the basis of selectivity in precious metal biosorption, the non-selective culture *C. cladosporioides* #2 was analyzed using XPS and FTIR and the results were compared with the preferential metal binding strain #1. Carbon peak at 285 eV remained unaffected (Figure 6.9). Although content of oxygen did not change significantly (19.2% and 18.4% in gold loaded and non-loaded biomass, respectively), the peak shifted from 532.3 eV to 534.4 eV (Figure 6.10). Thus, it could be said that, gold biosorption in case of the strain #2 involved chemical interactions with oxygenated species of carbon and nitrogen. Again in this case sulfur was not detected, but, higher content of nitrogen (6%) was detected (Figure 6.11). The nitrogen peak at 400.4 eV shifted to 402.3 eV after gold binding. The peak at 84.7 eV corresponding to gold was clearly observed (Figure 6.12). This peak was indicative of gold complex formation with carbon-nitrogen species.

It could be seen from FTIR analysis (Figure 6.13) that conditioning to acidic pH resulted in shifting of the 1372 cm\(^{-1}\) band to 1382 cm\(^{-1}\). This band is due to C-N stretching vibration. The 1031.7 cm\(^{-1}\) band due to C-N and C-O stretching shifted to 1027.7 cm\(^{-1}\). These result indicated that C-N and C-O species might be involved in the biosorption of gold.

When the biomass loaded with silver was analyzed, it was observed that there were no changes in positions of carbon, oxygen or nitrogen peaks in the XPS spectra (Figures 6.14, 6.15 and 6.16, respectively). Reduction in nitrogen peak from 4.4% to 0.2% was evident and it indicated involvement of nitrogen in silver biosorption (Figure 6.16), while there was marginal increase in the oxygen peak from 17.3 to 19.3% (Figure 6.15). Figure 6.16 also shows the
The author is aware about the limitations of this work. Further experimentation is required to gather more evidence regarding metal-selectivity in biosorption. A detail analysis of cell wall composition of the two strains of *C. cladosporioides* needs to be carried out. Use of enzymes to remove particular cell wall constituents and evaluating the effect of such treatments on metal biosorption would be further helpful in assigning roles to these individual constituents.

Another approach could be the use of mutant strains of *Cladosporium cladosporioides*. Mutants lacking in particular cell wall constituents could be obtained by treating the cultures with mutagenic agents. It would be worthwhile to study metal biosorption by such mutant strains in order to understand the mechanism of biosorption. Such studies are being carried out in the laboratory.