

## Biosorption of Cr(III) by the Cell Wall of *Mucor hiemalis*

C. Ebner\*, T. Pümpel, M. Gamper

*Institute of Microbiology, Technikerstraße 25, University of Innsbruck, A-6020 Innsbruck, Austria*

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### ABSTRACT

Biosorption of chromium(III) by whole cells and isolated cell walls of *Mucor hiemalis* was investigated. A fast initial sorption of Cr(III) on the cell wall was found, reaching 80% of the calculated maximum load after 30min contact time. However, the final biosorption maximum could not be reached after two hours, indicating a complex binding mechanism comprising more than one sub-process. From the Langmuir-fitted biosorption isotherms theoretical maximum biosorption capacities of 132 and 22mg Cr(III)/g d.w. were calculated for cell wall and whole cells, respectively. The composition of isolated cell walls was studied. The major components were chitosan (32%) and chitin (11%). The type of nitrogen source in the cultivation medium (NaNO<sub>3</sub> and peptone from casein) strongly influenced the cell wall composition. The contents of chitosan and phosphorus in the cell wall were significantly higher with NaNO<sub>3</sub> in comparison to peptone from casein, whereas a higher protein content was found using peptone. The biomass and extracted cell walls with higher contents of chitosan and phosphorus, deriving from the cultivation with NaNO<sub>3</sub>, showed an increased biosorption capacity for Cr(III). These results indicate that chitosan and phosphorus containing functional groups are the most probable binding sites for trivalent chromium in the cell wall, whereas proteins do not play a role in the biosorption of Cr(III) by *Mucor hiemalis*. © 2002 SDU. All rights reserved.

Keywords: Biosorption; Chromium; Cell wall; Fungi; *Mucor hiemalis*

### 1. INTRODUCTION

Chromium is a transition metal located in group VI-B of the periodic table. Although it is able to exist in several oxidation states, the most stable and common forms are the trivalent Cr(III) and the hexavalent Cr(VI) species, which display quite different chemical properties (McGeath and Smith, 1990).

The world production of Cr is in the order of 10<sup>7</sup> tons per year; 60-70% is used in alloys, including stainless steel, and 15% is used in chemical industrial processes, mainly leather tanning, pigments and electroplating (McGeath and Smith, 1990; Stern, 1982; Papp, 1985). The widespread use has converted Cr into a serious pollutant of air, soil and water (Khasim *et al.*, 1989; Armienta-Hernandez and Rodriguez-Castillo, 1995).

Beside conventional treatment techniques for Cr bearing waste waters like chemical precipitation, ion exchange, evaporation, cementation, electrolysis and reversed osmosis, the use of biosorption as alternative treatment method is becoming an attractive technique. Living and dead cells of fungi are able to remove heavy metals from aqueous solutions (Volesky, 1990; Kapoor and Viraraghavan, 1995; Tobin *et al.*, 1994), and several papers deal with the biosorption of chromium by fungi. Bosecker (1993) compared the Cr biosorption capacity of 80 fungal strains. Sekhar *et al.* (1998) checked the potential of fungal waste biomass from industrial fermentation for Cr(III) biosorption. The biosorption capacity of *Aspergillus niger* for Cr(III) was compared with a conventional ion exchange resin in a study carried out by Akthar and Mohan (1995), who found a higher biosorption potential of the fungal biomass in the lower concentration range in comparison to the synthetic ion exchanger.

\* Corresponding author. E-mail: c.ebner@uibk.ac.at

Up to now no efforts were done to elucidate the mechanism of biosorption of Cr(III) by fungal cell walls. Effective binding of chromium to pure chitin (Chui *et al.*, 1996) and chitosan (Bailey *et al.*, 1999) has already been reported and it can be supposed that these fungal wall-polymers also play a role in the biosorption of chromium by the intact cell wall of fungi.

The objective of the present study was to characterise Cr(III) biosorption by *Mucor hiemalis* cell walls and the identification of potential binding sites. The kinetics of the biosorption process was studied and the binding of Cr(III) at different equilibrium concentrations was measured. The composition of the cell wall was analysed for potential metal complexing compounds. The contents of such components in the cell wall were varied by changing the composition of the nutrient medium, and the influence on metal biosorption was investigated.

## 2. EXPERIMENTAL PROCEDURE

### 2.1. Organism and culture conditions

The fungal strain *Mucor hiemalis* MP/92/3/4 was isolated from the upper 2cm of farmland soil near Innsbruck, Austria. The strain was selected from several Cr(III) resistant fungi due to its predominant biosorption potential for Cr(III) (Pillichshammer, 1993). The fungus was maintained on Czapek Dox agar plates (in g/l: sucrose, 30; NaNO<sub>3</sub>, 3; K<sub>2</sub>HPO<sub>4</sub>, 1; MgSO<sub>4</sub> × 7H<sub>2</sub>O, 0.5; FeSO<sub>4</sub> × 7H<sub>2</sub>O, 0.01; agar, 16; pH 5.5). In order to keep its resistance for Cr(III), a sterile solution of Cr(NO<sub>3</sub>)<sub>3</sub> × 9H<sub>2</sub>O was added to the medium after autoclaving and cooling to give a final concentration of 100mg/l Cr(III). Biomass production was carried out by liquid cultivation in conical flasks on a gyratory shaker (5 days of cultivation, 150rpm, 30°C) using Czapek Dox medium. For the production of fungal biomasses with different biosorption potentials, the nitrogen sources NaNO<sub>3</sub> (3g/l) and peptone from casein (3.43g/l) were used.

### 2.2. Isolation of cell walls

Biomass of *Mucor hiemalis* was harvested after liquid cultivation by vacuum filtration through nylon mesh. The biomass was washed several times with deionised water. The cells were mechanically disintegrated using a bead beater with glass beads of 0.5mm diameter in 50mM Tris buffer, pH 7.8. After disintegration of the fungal biomass, glass beads and cell wall fragments were separated by vacuum filtration through a metal sieve (250µm). The filtrate containing the cell wall fraction was centrifuged (10min, 8000rpm) and the supernatant discarded. The pellet was washed twice with 1% Na-desoxycholin solution and twice with distilled water (double volume of the pellet volume each) by shaking on a head over head shaker for 1 hour, in order to remove cytoplasmatic residues. The wash solutions were removed after each step by centrifugation (15min, 10000rpm). The isolated cell wall material was then dried overnight at 80°C and ground to a fine powder using a pestle and a mortar. The cell wall preparations were microscopically pure and devoid of cytoplasmatic contaminations.

### 2.3. Metal biosorption tests

#### 2.3.1. Metal biosorption by fungal biomass

Cr(III) solution in distilled water (1mM, 30ml), prepared from Cr(NO<sub>3</sub>)<sub>3</sub> × 9H<sub>2</sub>O and adjusted to pH 5, was filled into 100ml Erlenmeyer flasks. After saturation of the glass surface with Cr(III) by shaking the flasks (25°C, 200rpm, 0.5h) the initial metal concentration was determined. Appropriate amounts of fungal biomass were added to the metal solution and the flasks were shaken for 1h at 25°C, 200rpm. Final metal concentrations were analysed after separating the biomass by vacuum filtration through paper filter. The biomass dry weight was determined gravimetrically after drying overnight at 80°C. The Cr-loaded biomass was digested in boiling, concentrated HNO<sub>3</sub>, and the concentration of Cr(III) was analysed in the 1:10 diluted digest.

Cr(III) biosorption was calculated from the difference between initial and final concentration of chromium in the biosorption test (indirect method) and the chromium concentration of the biomass digest (direct method), respectively. Both analyses gave the same results.

All metal analyses were done by a Perkin Elmer 2380 flame atomic absorption spectrometer (FAAS). Glass and plastic ware for the experiments was washed with 10% HNO<sub>3</sub> and deionised water prior to use.

### 2.3.2. Metal biosorption by cell walls

#### 2.3.2.1. Kinetic of Cr(III) biosorption by cell walls

A set of 100ml Erlenmeyer flasks each containing 20ml of metal solution as above and 5mg of the biosorbent was shaken on a gyratory shaker at 30°C, 150rpm. Liquid samples (1ml) were taken from the flasks after 7.5, 15, 30, 60 and 120 minutes. The cell walls were then separated from the solution by centrifugation, the supernatant was diluted 1:10 with 1% HNO<sub>3</sub> and the residual metal concentration was determined by FAAS.

#### 2.3.2.2. Isotherm of Cr(III) biosorption by cell walls

The following chromium solutions (prepared as above) were filled into appropriate flasks (in triplicate tests): 0.01mM (500ml), 0.03mM (400ml), 0.1mM (250ml), 0.3mM (50ml), 1mM (20ml), 3mM (10ml), and 10mM (5ml). 5mg of dried cell wall material was added to each vessel, which was then shaken at 150rpm, 30°C for 1h. The cell walls were removed by filtration (0.2µm) and the filtrate was subjected to metal analysis by FAAS. Cr(III) biosorption was calculated from the difference between initial and final concentration of chromium as above.

### 2.4. Analysis of cell wall composition

#### 2.4.1. Dry weight and ash

The dry matter of the cell wall was determined gravimetrically as the residue remaining after drying overnight at 80°C. The ash content of the cell wall was determined as described by Bartnicki-Garcia and Nickerson (1962).

#### 2.4.2. Chitin and chitosan

For the determination of the cell wall polymers chitin and chitosan a new and sensitive method was developed. Chitin and chitosan both are polymers of glucosamine; chitin is poly(N-acetylglucosamine), chitosan is poly(D-glucosamine). The total amount of glucosamine was measured colorimetrically after acidic digestion, the concentration of acetate, split from the chitin by alkaline digestion, was analysed by HPLC. The molar amount of glucosamine is equivalent to the total (molar) amount of chitin and chitosan. The molar amount of acetate represents the molar amount of chitin, the difference between glucosamine and acetate equals the molar amount of chitosan.

Cell wall material (in triplicate tests of 20mg) was digested either in acid (2.5ml 6M HCl, 130°C for 1.5h) or in base (3ml saturated KOH, 130°C, 1h) in high pressure teflon bombs (Berghof BTU 842). The acidic hydrolysates were transferred into 500ml volumetric flasks and made up to volume with distilled water. The colorimetric determination of glucosamine was done according to Ride and Drysdale (1972). The alkaline hydrolysates were transferred into 50ml volumetric flasks, made up to volume with distilled water and further diluted 1:10 with distilled water. The concentration of acetate was determined by HPLC (devices: Pharmacia-LKB pump, auto-injector and UV detector (213nm); column: Aminex HPX 87H, 30°C; solvent: 2.5mM H<sub>2</sub>SO<sub>4</sub>; flow rate: 0.5ml/min).

#### 2.4.3. Phosphorus and protein

Sequential extraction and hydrolysis of the cell wall material was carried out according to Bartnicki-Garcia and Nickerson (1962). Colorimetric determination of phosphorus and protein in the fractions was done as described by Olsen and Sommers (1982) and Bradford (1976), respectively.

#### 2.4.4. Sulphur

The total sulphur content of the cell walls was determined using the method described by Tabatabai (1982) where all sulphur is oxidised to sulphate. The concentration of sulphate was determined by HPLC (devices: Pharmacia-LKB pump and auto-injector, Biorad conductivity monitor; column: ICPak Anion HC, 35°C; solvent: stock solution (g/l) Na-gluconate, 17.4; boric acid, 18; Na-tetraborate x H<sub>2</sub>O, 25; (ml/l) glycerine, 250; distilled water, 750; final solvent (ml/l): stock solution, 20; n-butanol, 20; acetonitrile, 100; distilled water 860; flow rate: 1.5ml/min).

#### 2.4.5. Amino acids

A qualitative analysis of amino acids was done by thin layer chromatography according to the method described by Brenner *et al.* (1967).

#### 2.4.6. Metal and phosphorus content of ash

The cell wall ash was digested with 2ml of 65% HNO<sub>3</sub>. After evaporation of the acid to near dryness, the digestion was repeated, and the residue dissolved in 1% HNO<sub>3</sub>. The solution was transferred to a 25ml volumetric flask and made up to volume with distilled water.

The metal analysis was carried out by FAES (Na and K) and FAAS (other metals). The selection of metals for analysis was done on the basis of the cell wall composition of *Mucor rouxii* described by Bartnicki-Garcia and Nickerson (1962). The phosphorus content of the ash was analysed as described above.

### 3. RESULTS

#### 3.1. Biosorption of Cr(III) by cell walls

The biosorption of Cr(III) by isolated cell walls was characterised by the kinetics of the process and the metal biosorption at different equilibrium concentrations. Results depicted in Figure 1 clearly show a fast biosorptive process, reaching 80% and 90% of the estimated maximum uptake after 30 and 60min, respectively. However, the final equilibrium of biosorption could not be reached within 2 hours of contact, an indication of additional, slow metal immobilisation processes. Based on these results, an equilibration time of 60min, well distinguishing the fast biosorptive from other, slow processes, was chosen for further experiments.

Figure 2 presents the biosorption isotherms of Cr(III) by intact cells and by isolated cell walls of *Mucor hiemalis*. Both curves show the same pattern with a very steep rise in the adsorption density with increasing metal concentration, particularly in the lower concentration range, indicating a high affinity binding system.

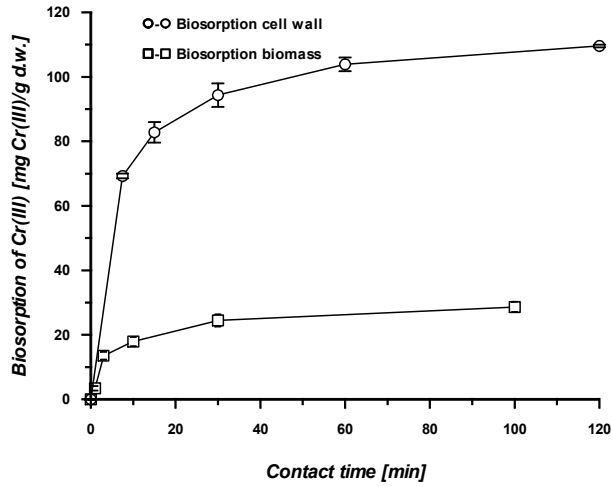


Figure 1. Kinetics of Cr(III) biosorption by cell walls and biomass of *Mucor hiemalis*. (Biomass data from Pillichshammer (1993), with permission)

For the mathematical description of the resulting data the adsorption model of Langmuir was used; correlation coefficients ( $r^2$ ) were 0.94 and 0.97 for cell wall and biomass, respectively. The Freundlich model was not applicable.

$$Q = \frac{Q_{\max} \cdot b \cdot C}{1 + b \cdot C}$$

- Q = Metal biosorption (mg Cr(III)/g d.w.)
- $Q_{\max}$  = Maximum biosorption capacity (mg Cr(III)/g d.w.)
- b = Langmuir constant ( $\text{mM}^{-1}$ )
- C = Equilibrium concentration (mM)

With this model a maximum biosorption capacity ( $Q_{\max}$ ) of  $132 \pm 7$  and  $21 \pm 1$  mg Cr(III)/g d.w. was calculated for the cell wall and for whole cells, respectively. The corresponding Langmuir constants (b) are  $20 \pm 6$  and  $17 \pm 6 \text{mM}^{-1}$ .

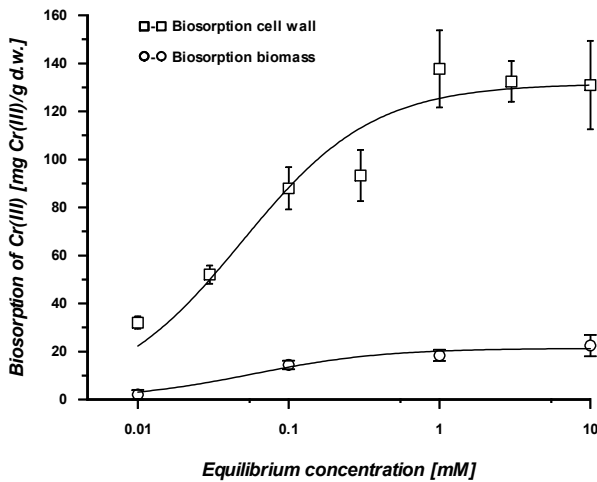


Figure 2. Isotherms of Cr(III) biosorption by cell walls and biomass of *Mucor hiemalis*. (Biomass data from Pillichshammer (1993), with permission)

### 3.2. Composition of the cell wall

Table 1 shows that more than 80% of the total cell wall mass could be attributed to defined elements and compounds. From all substances analysed, chitin and chitosan represented the most prominent compounds, contributing 11% and 32%, respectively. Beside these major structural cell wall polymers, protein, phosphorus, sulphur, and a series of metals were quantified in the cell wall.

Table 1  
 Composition of the cell wall of *Mucor hiemalis*

Fraction or Compound	Sub-Fraction	% in fraction	% in total
Water	total		7.6
Ash	total		25
	phosphorus in ash	22.3	
	Ca in ash	0.415	
	Mg in ash	5.7	
	Cu in ash	0.145	
	Cr in ash	0.024	
	Fe in ash	0.895	
	Mn in ash	0.008	
	Co	n.d.	
	Na in ash	2.15	
	K in ash	2.67	
Chitin	total		11
Chitosan	total		32
Protein	total		5
	soluble in cold NaOH		2.71
	soluble in hot NaOH		2.27
	soluble in cold acetic acid		n.d.
	soluble in hot acetic acid		n.d.
Amino acids	aspartic acid, glutamic acid		+
	threonine, lysine, tyrosine		+
	leucine, isoleucine, glycine		+/-
	arginine, histidine, alanine		+/-
	threonine, methionine		+/-
	cysteine, asparagine		-
	glutamine, serine		-
	tryptophan, proline		-
	phenylalanine		-
Phosphorus	total		1
	soluble in cold NaOH		0.6
	soluble in hot NaOH		0.23
	soluble in cold acetic acid		0.11
	soluble in hot acetic acid		0.043
Sulphur	total		0.5
Sum			82.1

n.d.: below detection limit

### 3.3. Nitrogen source, cell wall composition and biosorption of Cr(III)

Cultivation of *Mucor hiemalis* in medium with the defined nitrogen source nitrate led to an increased biosorption capacity of the fungal biomass in comparison to the use of peptone. The biosorption capacity of intact cells from the nitrate medium was almost twice the capacity of

the biomass deriving from the peptone cultivation. Also the corresponding, isolated cell walls showed a significant difference in biosorption capacity for chromium, but the difference was not as pronounced as in the case of intact cells (Figure 3).

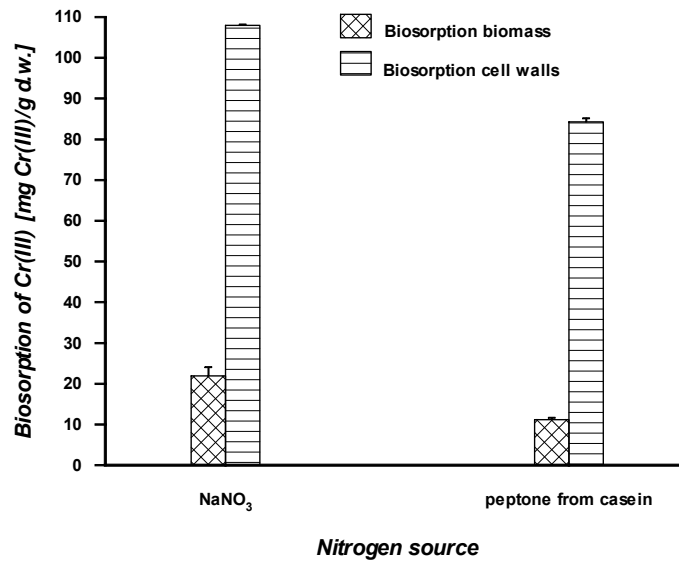


Figure 3. Biosorption of Cr(III) by biomass and cell walls of *Mucor hiemalis* cultivated with different nitrogen sources

Figures 4 and 5 show the variations in the content of major cell wall ingredients after cultivation of the fungus on the different nitrogen sources. The use of peptone from casein led to significantly decreased contents of chitosan and phosphorus. The chitin content remained unchanged, whereas the content of protein only was increased in the cell walls, compared to the cultivation on nitrate.

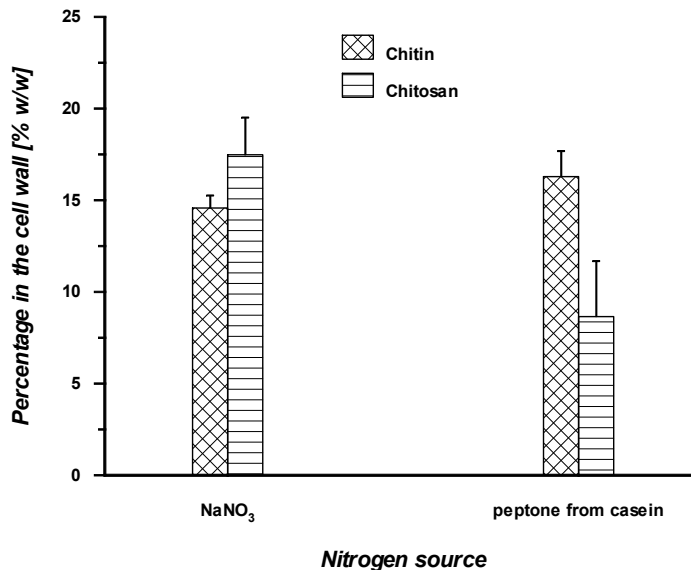


Figure 4. Chitin and chitosan content of cell walls of *Mucor hiemalis* cultivated with different nitrogen sources

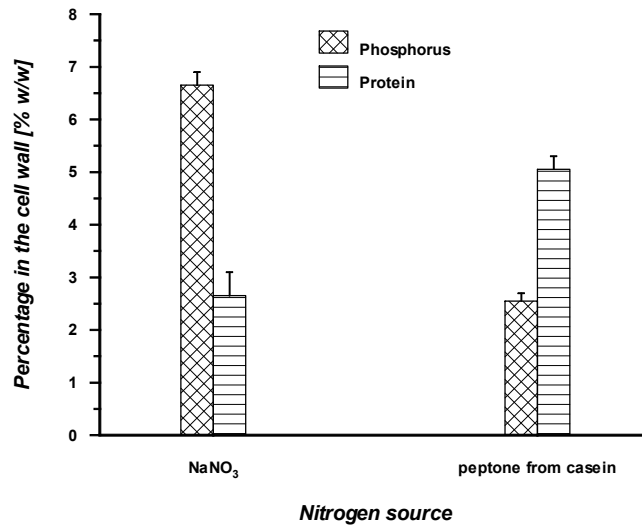


Figure 5. Phosphorus and protein content of cell walls of *Mucor hiemalis* cultivated with different nitrogen sources

#### 4. DISCUSSION AND CONCLUSIONS

Isolation of *Mucor hiemalis* cell walls and characterisation of the Cr(III) biosorption by the cell walls were the first objectives of the present work. It is commonly known that disintegration of fungal cells is difficult due to the good mechanical stability of fungal cell walls. Mechanical disintegration using a bead beater was the finally selected method to efficiently break open the fungal hyphae and to separate cell wall fragments from the cytoplasm. The advantage of mechanical disintegration in comparison to chemical extraction methods of cell walls carried out in other studies (Baik *et al.*, 2002) is the minimal impact on cell wall constituents, including functional groups responsible for biosorption.

The isolated cell walls were used for biosorption studies with trivalent chromium. The kinetics of biosorption suggested that at least two different processes were involved in the immobilisation of the metal on the cell wall. Tsezos and Volesky (1982) and Volesky and Tsezos (1982) have shown that biosorption of uranium by cell walls of *Rhizopus oryzae* comprises a fast biosorptive process followed by a much slower precipitation process of already bound metal. Additional metal ions can be immobilised due to this mechanism rather than by a simple biosorption process. A comparable biosorption kinetic in the present study would seem to suggest that Cr(III) removal by the closely related fungus *Mucor hiemalis* followed a similar mechanism of successive biosorption and bioprecipitation. This hypothesis is also supported by the work of Eiden *et al.* (1980) who showed this sequential process with Cr(III) binding by chitosan, a major cell wall component of *Mucor hiemalis* (see below).

The biosorption of Cr(III) at different equilibrium concentrations was measured in order to get more details about this part of metal binding to the cell wall. The resulting data pairs showed a typical saturation curve which could well be described by the Langmuir equation, which points to the predominance of a well-defined binding site. (The Langmuir equation corresponds to the mass law and mathematically describes sorption processes to surface binding sites with constant standard free energy of adsorption. (Morel, 1983)). The Freundlich model, a more empirical approach to characterise sorption processes, was not suitable to describe the experimental biosorption data. A theoretical maximum biosorption capacity of  $Q_{\max}=132\text{mg Cr(III)/g d.w.}$  was calculated for the cell wall; in contrast, whole cells reached a maximum of  $21\text{mg Cr(III)/g d.w.}$  Taking into consideration the contribution of cell wall to the total fungal dry weight ranging from 14 to 18% (Bartnicki-Garcia and Nickerson, 1962) the suggestion of Pillichshammer *et al.* (1995) saying that most of the chromium is bound within the cell wall of



this strain, was confirmed by the present experiments. From our data it can also be deduced that no additional binding sites (e.g. on the inside of the cell wall) became available due to the cell wall preparation procedure. The more or less identical Langmuir constants 'b' (expressing binding affinity), calculated from the Langmuir-fitted biosorption isotherms of both intact cells and isolated cell walls, indicate a similar biosorption equilibrium for both biosorbents: the cell wall preparation procedure did not seem to influence the chemical characteristics of the binding sites.

The chemical composition of the *Mucor hiemalis* cell wall was also studied, with particular emphasis placed on elements and compounds with well known metal binding potential. Phosphate-, sulphur-, amine-, carboxyl- and aminoacetate groups on proteins and polysaccharides are the predominant functional groups in fungal cell walls (Siegel *et al.*, 1990). Chitosan offering amine groups represented the major component of the *Mucor hiemalis* cell wall (32% of the cell wall dry weight), chitin carrying aminoacetate groups amounted to 11%. Together, these closely tied and related polysaccharides made up approximately half of the cell wall. These results correspond well with the findings of Bartnicki-Garcia and co-workers (Bartnicki-Garcia and Nickerson, 1962; Bartnicki-Garcia and Reyes, 1968) about the cell wall of *Mucor rouxii*.

Chitin and chitosan are well known for their metal binding potential shown by biosorption studies with crab shells containing high amounts of chitin (An *et al.*, 2001) and purified chitin and chitosan (Muzzarelli and Tubertini, 1969; Guibal *et al.*, 1999; Ruiz *et al.*, 2000; Benguella and Benaissa, 2002). More detailed studies have shown that the above mentioned N-containing groups of chitin and chitosan are responsible for the immobilisation of heavy metals (Kapoor and Viraraghavan, 1997; Dambies *et al.*, 2001; Jianlong, 2002). Depending on the metal and on the pH in solution, the immobilisation of metals by these groups is either based on ion-exchange or chelation (Ruiz and Guibal, 2000).

Concerning Cr(III), Chui *et al.* (1996) found effective binding by chitin containing shells of crustaceans, and Bailey *et al.* (1999) reported a high biosorption potential of chitosan. Eiden *et al.* (1980) already described the process of Cr(III) immobilisation on chitosan as a sequence of biosorption and bioprecipitation; SEM photomicrographs showed micro-precipitates on the chitosan surface after exposure to a Cr(III) containing solution. EDAX analysis showed an intensive Cr signal of the formed precipitates. A similar process can be hypothesised for Cr(III) immobilisation within the cell wall of *Mucor hiemalis*, as already discussed above in the context of biosorption kinetics. Investigations of Pillichshammer (1993) with the same strain strongly support this hypothesis: the longer the cells were exposed to Cr(III), the less Cr(III) could be desorbed from biomass with acid.

Not much is found in literature about the influence of culture conditions on the content of chitin and chitosan in fungal cell walls. Tan *et al.* (1996) investigated the development of chitosan during the cultivation of various Zygomycetes (to which *Mucor* sp. and *Rhizopus* sp. belong), reporting the highest chitosan content in the late exponential growth phase. Chitin and chitosan both are polymers containing nitrogen; in the present study therefore the influence of the nitrogen source in the cultivation medium on the respective composition of the cell wall of *Mucor hiemalis* was studied: The cell wall of fungi grown on the complex nitrogen source peptone had a significantly lower chitosan content than the cell wall of fungi grown on the chemically defined nitrate (cells were harvested in the same growth phase). Arcidiacono and Kaplan (1992) detected another effect of complex and defined growth sources on chitosan. They found chitosan molecules with lower molecular weight when *Mucor rouxii* was cultivated on complex rather than on defined nutrients. Not only chitosan but also the contents of protein and phosphorus in the cell wall were affected by growth of *Mucor hiemalis* on different nitrogen sources. Cultivation on peptone halved the phosphorus content and doubled the protein content of the cell wall, compared to cultivation on nitrate.

The Cr(III) biosorption capacities of whole cells and cell wall of *Mucor hiemalis* cultivated either on peptone or nitrate were compared. Biomass with the higher chitosan and phosphorus content (grown on nitrate) also exhibited a significantly higher Cr(III) biosorption capacity. The excellent metal binding properties shown for pure chitosan can now also be confirmed for the compound within its natural matrix, i.e. the fungal cell wall.

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