



New metal-binding ethyldiamino- and dicarboxy-products from *Aspergillus niger* industrial wastes

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Abstract

The metal-binding ability of *Aspergillus niger* mycelial waste was improved by chemical modification. The latter was performed by introducing additional carboxy groups using oxidation methods or the introduction of the ethyldiamino group first by chlorination of *A. niger* using mesyl chloride and subsequent reaction of the product with ethylene diamine. Metal binding abilities of the products for Cd^{2+} , Co^{2+} , Ni^{2+} and Zn^{2+} were determined according to the Langmuir model, whereby pK_D^* -values of 3.88 up to 5.02 were revealed. Maximum capacities for the metals were found to be in the range 172 to 1064 mmol/kg.

Introduction

Heavy metal concentrations in waste waters released from industrial plants in Germany are restricted by law to values of 50–2000 $\mu\text{g/l}$ (Gemeins. Ministerialblatt I 1989), depending on the metal. For heavy metal removal, mainly used methods in waste water treatment are based on ion exchange or membrane techniques (Hartinger 1991). In ion exchange, organic polymers with metal binding functional groups, mainly weak acid or chelating groups, are applied to remove heavy metals from aqueous solutions (Dorfner 1970). During the last years a lot of research work has been done about the ability of microorganisms like bacteria or fungi to absorb heavy metals (Volesky 1987). Some microorganisms are known to be effective in metal sorption, especially at lower metal concentrations, because of metal binding groups on their surface or metabolism dependent procedures performing a metal uptake, the so called bioaccumulation (Gadd 1990). Furthermore the biosorption is also dealing with mechanisms of heavy metal removal by inactivated waste materials like mycelium of *A. niger* from citric acid plants (Luef *et al.* 1991). An increase in

metal sorption was found after chemical treatment of mycelial wastes (Muzzarelli & Tanfani 1982), but little work has been done on their chemical modification by introducing additional functional groups (Schuhmacher 1995; Naseem Akthar *et al.* 1996). Continuing the works of Schuhmacher (1995) we modified waste mycelium of *A. niger* by chemical methods, already successfully applied to wood (Gauer 1996) or chitosan (Matsumura *et al.* 1997). The purpose of this work was to create new compounds by introducing ethyldiamino- and dicarboxy-groups into the polysaccharide part of the cell wall of *A. niger* and to characterize their ability for removing heavy metals from aqueous solutions.

Materials and methods

Fungal material and chemicals

The *A. niger* waste material was kindly delivered by the S.A. Citrique Belge N.V. (Tienen, Belgium). It was washed thoroughly with distilled water and freeze-dried before use. The chemicals and solvents used

were of synthesis grade. The heavy metal salts used in batch-experiments were $\text{CdSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. All metal salts and standard substances were of analytical grade, their solutions were made in distilled water.

For the preparation of metal salt solutions with pH 6, aliquots from stock solutions (0.1 M) were diluted in 2(N-morpholino)-ethane sulfonic acid (MES, 0.01 M). For the investigation of metal-binding abilities with *A. niger* and its products derived from chemical modification, the adsorbents were sieved on standard sieves (pore size: 0.05 mm, 0.1 mm, 0.5 mm). The particles with a diameter of 0.1 to 0.5 mm were used in the adsorption tests.

Deoxychlorination of A. niger

According to Nakamura *et al.* (1992), 1.0 g of *A. niger* was stirred in 78 ml of DMF and heated to 40 °C. Methyl chloride (0.064–0.201 mol/g *A. niger*) was dropped into the mixture and further stirring was allowed at a temperature of 98 °C. After 4 h the solid was separated by centrifugation (4000 rpm, 3 min) and washed with distilled water and methanol. The product was treated with about 100 ml of sodium carbonate solution (5%) and afterwards washed with methanol and distilled water. The product (CD-Apg) was dried in an oven at 90 °C.

Reaction of CD-Apg with ethylene diamine

1.0 g CD-Apg was stirred in 10 ml of ethylene diamine (85%) for 3, 6, and 8 h at 92 °C (Tashiro & Shimura 1982). The product was separated by centrifugation (4000 rpm, 3 min), washed with distilled water and freeze-dried (ED-Apg).

Oxidation of A. niger with sodium periodate

1.0 g of *A. niger* was stirred in the dark in 50 ml of a sodium periodate solution (0.1 M in water) for 6 h at 60 °C. The product was separated by centrifugation (4000 rpm, 3 min), washed with distilled water and freeze-dried (Ox-Apg).

Oxidation of Ox-Apg with sodium chlorite

According to the method of Floor *et al.* (1989), 2.0 g of Ox-Apg were stirred in a mixture of 100 ml of distilled water, 50 mg Titriplex III and 1.6 ml hydrogen peroxide (30%). A mixture of 1.8 g sodium chlorite (80%) and 0.5 ml acetic acid (99–100%) was dropped

to the former mixture during 30 min while the pH was kept at 5 by adding small amounts of a sodium hydroxide solution (2.5 M). The mixture was stirred for 4, 6 and 8 h at room temperature. At the end of the reaction, the mixture was brought to pH 9 by adding sodium hydroxide solution. The products were separated by centrifugation (4000 rpm, 3 min), washed with distilled water and freeze-dried (DIC-Apg).

Adsorption of metal ions

In each case, three samples were treated in the following way: 25 mg of *A. niger* or product were added to 25 ml of a metal salt solution (0.05–3.0 M), buffered with MES (0.01 M) to pH 6 in a polyethylene tube (100 ml). The mixtures were shaken in the closed tubes at room temperature for 24 h.

Determination of metal ions

From each test tube, aliquots of the supernatants were added to a definite volume of nitric acid (6.5%). The metal ion concentrations of these solutions were determined by atomic absorption spectrometry either in the flame or by the graphite furnace mode (Ni).

Characterization of binding abilities to heavy metals

We used the Langmuir plot (Barrow 1984) for describing the adsorption behaviour of *A. niger* and the products by determination of maximal capacities (A_{max}) and the so-called ‘apparent dissociation constants’ (pK_{D}^*). In each case, three samples were shaken in buffered solutions of the metal salts described above and the remaining metal ion concentrations were determined by AAS. The data for the Langmuir isotherms were calculated from the average values of the equilibrium concentrations and the capacities found in the batch experiments. The values of A_{max} and pK_{D}^* were derived from the isotherms by their linearization.

Determination of Cl- and S-content

In each case, three samples were treated in the following way: About 100 mg of *A. niger*, CD-Apg or ED-Apg were burned in a Wickbold apparatus and the volatile products were absorbed in sodium hydroxide solution (0.1 M), which contained a small volume of 30% hydrogen peroxide in the case of S-determinations. Chlorine contents of the solutions

were determined by potentiometric titration. The S-contents of the solutions (as SO_4^{2-}) were determined by HPLC.

Determination of nitrogen content

Two samples of *A. niger*, DIC-Apg and ED-Apg (in each case about 200 mg) were treated by the Kjeldahl method (Merck 1964) for the determination of nitrogen content.

Determination of periodate consumption

After the reaction of *A. niger* with periodate, the mixture was centrifuged (4000 rpm, 3 min) and aliquots of the supernatants were mixed with a NaHCO_3 solution (sat.), a KI solution (10%) and distilled water, then titrated with a standard solution of Na_3AsO_3 (0.1 N). 2 ml of a starch solution (5%) were added to indicate the end of the titration. At least four aliquots were taken from each supernatant.

Determination of aldehyde groups

0.5 g of Ox-Apg or *A. niger* were mixed with 50 ml of an oxime reagent (Houben Weyl 1953) and shaken for 24 h in the dark. After centrifugation (4000 rpm, 3 min), aliquots of the supernatants were titrated with sodium hydroxide solution (0.5 N) until change of colour. The aldehyde contents of the substances were estimated from the consumption of the hydroxide solution and the amount of product. The determinations were repeated once in the case of Ox-Apg, twice in the case of *A. niger*.

Determination of carboxy groups

1.0 g of DIC-Apg or *A. niger* was brought into the H-Form by HCl treatment (0.1 M), washed with distilled water and freeze-dried. 0.5 g of the product were stirred in 50 ml of a calcium acetate solution (0.5 M) for 24 h. After centrifugation (4000 rpm, 3 min), aliquots of the supernatants were titrated with sodium hydroxide standard solution (0.1 M) against phenolphthalein. The content of carboxy groups was determined from the volume of hydroxide solution consumed. The determinations were performed twice.

Results

Analytical studies on A. niger

The content of functional groups and the nitrogen content of *A. niger* were investigated by the methods described above. The nitrogen content of *A. niger* was found to be 6.7%, no chlorine could be detected, while its sulphur content was determined to be about 0.5%. With *A. niger*, carboxy groups were detected in the range of 0.3 mmol/g. The determination of aldehyde groups failed with *A. niger*.

Oxidation of A. niger

We introduced carboxy groups into *A. niger* by first oxidation with periodate, followed by oxidation performed with sodium chlorite. While the periodate consumption increased during the oxidation at 60 °C with time, the aldehyde content of the products reached about 6 mmol/g product without further change even after 6 h of reaction. In this case, the yield of the product was about 73% of the amount of biomass applied. The aldehyde containing products were then oxidized with sodium chlorite at different reaction times. The resulting product (DIC-Apg) contained carboxy groups up to 0.6 mmol/g, which was twice the carboxy content formerly determined in *A. niger*. DIC-Apg was produced in 4 h and yielded about 72% of the periodate-oxidized primary product.

Introduction of ethyldiamino groups

We introduced the chlorine group into *A. niger* by reaction with methane sulfonyl chloride in DMF and the resulting product was treated with ethylene diamine to substitute the chlorine group (ED-Apg). The reaction of *A. niger* with mesyl chloride yielded brown products with chlorine contents up to 8%, while the sulphur contents in the products remained below 2% (Figure 1). During the reaction of CD-Apg with ethylene diamine, its former chlorine content was reduced from 8 to about 0.7% after 6 h of reaction (Figure 2). The nitrogen content of ED-Apg determined by the Kjeldahl method, increased from 6.7% in CD-Apg to 9.4% after 6 h of reaction.

Characterization of heavy metal binding

Metal binding studies were performed with *A. niger*, its carboxy derivative (DIC-Apg) and with its ethylene diamine substitute (ED-Apg) in the presence

Table 1. Maximal capacities (A_{\max}) and apparent dissociation constants (pK_D^*) and regressions (R) for some heavy metal complexes with DIC-Apg and ED-Apg derived from Langmuir-isotherms. Values derived from the plots by linearization

Metal	<i>A. niger</i>			DIC-Apg			ED-Apg		
	A_{\max} (mmol/kg)	pK_D^*	R	A_{\max} (mmol/kg)	pK_D^*	R	A_{\max} (mmol/kg)	pK_D^*	R
Cd	100	5.06	0.9861	274	5.02	0.9976	—	—	—
Co	—	—	—	—	—	—	172	4.49	0.9996
Ni	71	3.97	0.9901	182	3.92	0.9986	1064	4.13	0.9969
Zn	185	3.53	0.9788	303	3.88	0.9958	—	—	—

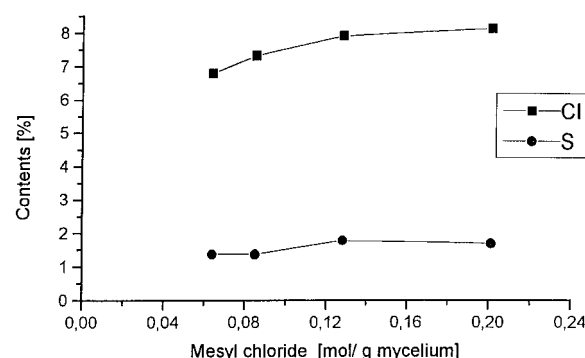


Figure 1. Deoxychlorination of *A. niger*: Cl- and S-contents after treatment with mesyl chloride. Conditions: 1.0 g of *A. niger*, 78 ml DMF, 98 °C, 4 h. Average values from three samples.

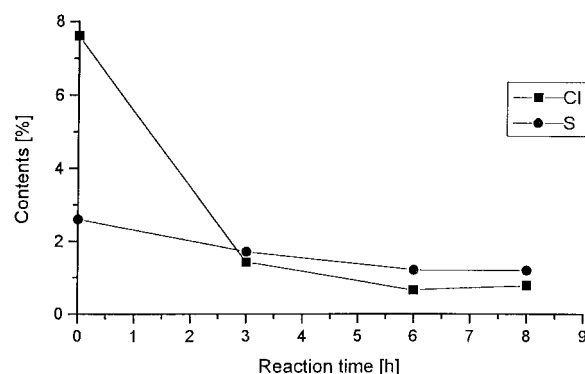


Figure 2. Reaction of CD-Apg with ethylene diamine: Time dependence of Cl- and S-contents. Conditions: 1.0 g CD-Apg, 10 ml ethylene diamine (85%), 92 °C. Average values from three samples.

of Cd^{2+} -, Co^{2+} -, Ni^{2+} - and Zn^{2+} -ions as described above. Apart from cobalt, all other metals were bound to *A. niger* according to the theory of Langmuir (Figure 3) with a bonding strength for cadmium ranging ten times higher than for nickel and zinc (Table 1). *A. niger* had its highest capacity for zinc (185 mmol/kg) compared to cadmium and nickel. In

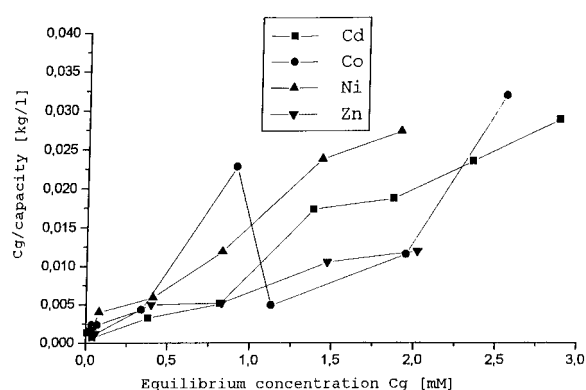


Figure 3. Langmuir-isotherms for *A. niger*. Conditions: 25 mg *A. niger*, 25 ml metal salt solution, buffered with MES (0.01 M) to pH 6, 24 h at room temperature. Average values for capacities and equilibrium concentrations from three samples were used for calculation.

the case of DIC-Apg, cadmium, nickel and zinc were bound according to the theory of Langmuir (Figure 4). Compared to *A. niger*, DIC-Apg revealed similar values of bonding strength but capacities double the size for cadmium and nickel (Table 1). With DIC-Apg the highest value of capacity was that for zinc. With ED-Apg however, only nickel and cobalt were found to correspond in that way. The binding of zinc and cadmium seemed to be related to a different mechanism (Figure 5). From the typical Langmuir plots (Figures 3–5), both maximal metal binding capacities and pK_D^* -values for *A. niger*, DIC-Apg and ED-Apg, respectively, were calculated (Table 1). Table 1 shows a preference of ED-Apg for nickel as compared with *A. niger* and DIC-Apg, although their pK_D^* -values are in the same range.

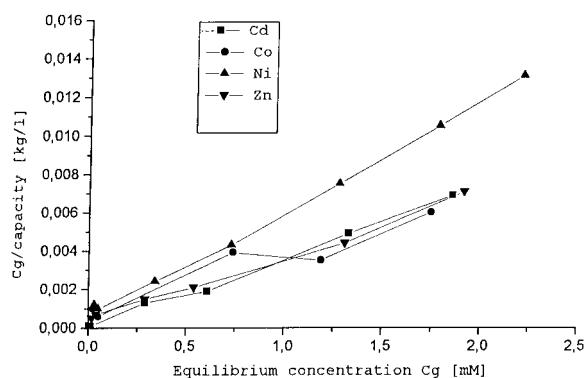


Figure 4. Langmuir-isotherms for DIC-Apg. Conditions: 25 mg DIC-Apg, 25 ml metal salt solution, buffered with MES (0.01 M) to pH 6, 24 h at room temperature. Average values for capacities and equilibrium concentrations from three samples were used for calculation.

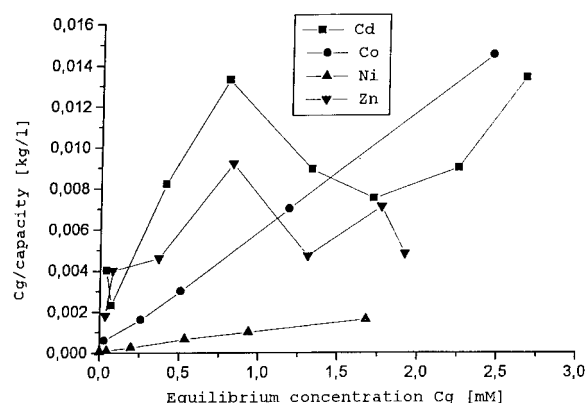


Figure 5. Langmuir-isotherms for ED-Apg. Conditions: 25 mg ED-Apg, 25 ml metal salt solution, buffered with MES (0.01 M) to pH 6, 24 h at room temperature. Average values for capacities and equilibrium concentrations from three samples were used for calculation.

Discussion

The purpose of our work was the chemical modification of *A. niger* mycelium in order to enhance its natural ability for metal sorption. The chemical nature of the cell wall in fungi and the availability of *A. niger* as a source for the industrial production of citric acid make it an interesting primary product for chemical modification. The cell wall of *A. niger* comprises 50–60% of alkali-insoluble chitin-glucan (Johnston 1965) suggesting the application of reactions known from the polysaccharide chemistry to achieve chemical modification of the mycelium.

For the introduction of carboxy groups into *A. niger* mycelium, we have chosen a subsequent oxidation with periodate and chlorite as it was applied

by Gauer (1996) in the oxidation of wood. This procedure prevented the mycelium from severe degradation and revealed good yields and products with optimized degree of functional groups. We assume a relation between the increase of carboxy groups and the raising of metal binding capacities in DIC-Apg. In order to investigate the behaviour of an ethylene diamine substituted *A. niger* towards metal binding, we first looked for a way to introduce ethylene diamine to the mycelial polysaccharide compartment. A lot of work has been done on the introduction of additional functional groups to cellulosic materials, using deoxy-chlorination (Polyakov & Rogovin 1963; Tashiro & Shimura 1982; Nakamura *et al.* 1992; Mocanu *et al.* 1996) as a first step in derivatization. The chlorine content found in CD-Apg may be a proof for chemically bound chlorine from the reaction with mesyl chloride. It is well known from the chlorination with sulphur containing acid chlorides, that small amounts of sulphur are found in the chlorinated products as well (Polyakov & Rogovin 1963). Thus the increase of sulphur content in CD-Apg may underline that chlorination took place in the reaction of *A. niger* with mesyl chloride. The reaction of CD-Apg with ethylene diamine caused a loss of chlorine and an increase of nitrogen in the product called ED-Apg. Since ED-Apg revealed a high affinity for nickel ions, the introduction of ethylene diamine groups into the matrix was assumed to be successful. Moreover, from the Irving–Williams series (Huheey 1988) ethylene diamine is known to form nickel-complexes of high stability.

Our investigations have shown that the application of chemical reactions well known from polysaccharide chemistry for the treatment of bulk biological material, like fungal mycelium, may create new products with increased abilities and preferences towards heavy metal binding. This has already successfully been performed with other biopolymers like wood or chitosan from crustacean shells (Schuhmacher 1995; Gauer 1996).

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References

- Barrow GM. 1984 *Physikalische Chemie*, Gesamtausgabe 6. Auflage, Braunschweig: Vieweg.

- Dorfner K. 1970 *Ionenaustauscher*, 3. Auflage, Berlin: Verlag de Gruyter.
- Fa. Merck 1964 *Die Untersuchung von Wasser – Eine Auswahl chemischer Methoden für die Praxis*, Darmstadt: Fa. Merck: 32–34.
- Floor M, Hofsteede LPM, Groenland WPT, Verhaar LAT, Kieboom APG, van Bekkum H. 1989 Preparation and calcium complexation of oxidized polysaccharides. II.: Hydrogen peroxide as co-reactant in the chlorite oxidation of dialdehyde glucans. *Rec Trav Chim Pays-Bas* **108**, 384–392.
- Gadd GM. 1990 Fungi and yeasts for metal accumulation. In: Ehrlich HL, Brierley CL, eds. *Microbial Mineral Recovery* New York: Macmillan, 249–275.
- Gauer J. 1996 Synthese kationenaktiver Derivate auf Holzbasis und Untersuchungen über ihren Einsatz zur Entfernung von Schwermetallen aus wäßrigen Lösungen. *Dissertation*, Universität des Saarlandes, Saarbrücken.
- Gemeinsames Ministerialblatt I* 1989 Anhang 40 zur allgemeinen Rahmen- und Verwaltungsvorschrift über Mindestanforderungen für das Einleiten von Abwasser in Gewässer, Bonn: 523–524.
- Hartinger L. 1991 *Handbuch der Abwasser und Recyclingtechnik für die metallverarbeitende Industrie*. 2. Auflage, München: Carl Hanser Verlag.
- Houben-Weyl 1953 *Methoden der organischen Chemie*, Band 2: Analytische Methoden, Stuttgart: Thieme Verlag.
- Huheey, J. E. 1988 *Anorganische Chemie, Prinzipien von Struktur und Reaktivität*, 3. Auflage, Berlin: Walter de Gruyter.
- Johnston, I. R. 1965 The composition of the cell wall of *Aspergillus niger*, *Biochem J* **96**, 651–658.
- Luef E, Prey T, Kubicek CP. 1991 Biosorption of zinc by fungal mycelial wastes, *Appl Microbiol Biotechnol* **34**, 688–692.
- Matsumura S, Yokochi E, Winursito I, Toshima, K. 1997 Preparation of novel biodegradable polyampholyte: Partially dicarboxylated chitosan. *Chem Lett* **3**, 215–216.
- Mocanu G, Constantin M, Carpov, A. 1996 Chemical reactions on polysaccharides, 5. Reaction of mesylchloride with pullulan. *Angew Makromol Chem* **214**, 1–10.
- Muzzarelli RAA, Tanfani F. 1982 The chelating ability of chitinous materials from *Aspergillus niger*, *Streptomyces*, *Mucor rouxii*, *Phycomyces blakesleeana* and *Choanephora cucurbitarum*. In: Hirano S, Tokura S, eds. *Chitin and Chitosan*, Proceedings of the Second International Conference on Chitin and Chitosan, July 12–14, Sapporo, Japan, 183–186.
- Nakamura S, Amano M, Saegusa Y, Sato T. 1992 Preparation of aminoalkyl celluloses and their adsorption and desorption of heavy metals. *J Appl Polym Sci* **45**, 265–271.
- Naseem Akthar M, Sivarama Sastry K, Maruhti Mohan P. 1996 Mechanism of metal ion biosorption by fungal biomass. *BioMetals* **9**, 21–28.
- Polyakov AI, Rogovin ZA. 1963 Synthesis of new cellulose derivatives -XXII. Synthesis of chlorocellulose and its conversion products. Preparation of amino- and nitrilocellulose. *Vysokomol. Soyed.* **5**: No 1 11–17; *Polym Sci USSR* **5**, 610–618.
- Schuhmacher C. 1995 Entfernung von Schwermetallen aus wäßrigen Medien durch Retention an chemisch modifizierter chitinhaltiger Abfallbiomasse. *Dissertation*, Universität des Saarlandes.
- Tashiro T, Shimura Y. 1982 Removal of mercuric ions by systems based on cellulose derivatives. *J Appl Polym Sci* **27**, 747–756.
- Volesky B. 1987 Biosorbents for metal recovery. *Trends Biotechnol* **5**, 96–101.