Heavy Metal Biosorption sites in *Penicillium cyclopium*

*[a] KOLISHKA TSEKOVA; [b]DARINKA CHRISTOVA; [a]MARIA IANIS

[a] - *Department of Microbial ecology, Institute of Microbiology, Bulgarian Academy of Sciences, Acad.G. Bonchev str., bl. 26, 1113 Sofia, Bulgaria*
[b] – *Laboratory of Amphiphilic and Ionogenic Polymers, Institute of Polymers, Bulgarian Academy of Sciences, Acad.G. Bonchev str., bl. 103A , 1113 Sofia, Bulgaria*

ABSTRACT: The biomass of *Penicillium cyclopium* was subjected to chemical treatment to study the role of the functional groups in the biosorption of heavy metal ions. The modifications of the functional groups were examined with infrared spectroscopy. Hydroxyl groups were identified as providing the major sites of heavy metal deposition, augmented by secondary binding by amides and carboxylates. Phosphate groups and the lipids fraction of the biomass did not play significant role in biosorption of the metal ions in the studied biomass. Biosorption of copper and cobalt ions displaced K^+, Mg^{2+} and Ca^{2+} present on the biosorbents indicating that biosorption took place as a result of an ion-exchange process. @JASEM

Utilization of biomaterials for the uptake and concentration of heavy metal ions from dilute aqueous solution has been proposed by many researchers as an alternative and relatively inexpensive method for water treatment in comparison to other metal recovering processes (Saitoh *et al*., 2001; Breierova *et al*., 2002; Franco *et al*., 2004; El-Sayed and El-Morsy 2004). Processes using dead cells biomass can be of great interest, because of the large variety and the low cost of these biological materials (Kratchovil and Volesky, 1998]) Filamentous fungi are used in fermentation industries to produce varied metabolites and thousands of tons of residual biomass are produced each year, containing poorly biodegradable biopolymers and make bad fertilizers for agricultural use. On the other hand, fungi frequently display a higher affinity for metal ions compared to other microbial groups and can accumulate metals from their external environment by means of physicochemical and biological mechanisms (Khoo and Ting, 2000; Cabuk *et al*., 2004; Preetha and Viruthagiri, 2005).

Biosorption of heavy metals by microorganisms often occurs as a result of complexation mechanism involving sites in the biomass containing functional binding groups found in carbohydrates, lipids, proteins and other biopolymers in microbial cell envelop (Gazso, 2001; Franco *et al*., 2004). Uptake may be enhanced by electrostatic attraction to negatively changed functional groups on the cell surface, resulting in passive attraction of various metal cations, but this is a secondary mechanism (Tobin *et al*., 1990; Kapoor and Viraraghavan, 1997). Few studies have been published dealing with the contribution of carboxyl, amine and phosphate groups present in fungal biomass to the metal uptake (Tobin *et al*., 1990; Akthar *et al*., 1996; Kapoor and Viraraghavan, 1997; Sarret *et al.*, 1998). The role

played by the hydroxyl groups, however, has not been studied.

The objective of the present study was to investigate the participation of some functional groups, namely carboxyl, amino, phosphate and hydroxyl, as well as lipids present in the waste biomass of *Penicillium cyclopium* in the biosorption of copper and cobalt ions from aqueous solutions.

MATERIALS AND METHODS
The fungus used in this study, *Penicillium cyclopium*, is deposited at the Collection of the Institute of Microbiology at the Bulgarian Academy of Sciences. The conditions for production of biomass have been described previously (Tsekova, K. et al 2001). The biomass, given from the stationary growth phase, was separated by filtration, washed with deionized distilled water and dried for 24 h at $60^{\circ}C$. The dried biomass was powdered using a mortar and pestle. Hereafter the biomass thus prepared will be referred to as raw biomass (control).

In order to understand the role of functional groups in biosorption of copper and cobalt ions, portions of the raw biomass were chemically treated in different ways. The chemical treatments applied to the biomass were as follow: Esterification of the carboxylic groups present on raw biomass by treating with anhydrous methanol and concentrated hydrochloric acid as described by Kapoor and Viraraghavan (1997). The resulted biomass sample will be referred to as B1.

Methylation of amines by biomass treatment with formaldehyde and formic acid (Kapoor and Viraraghavan, 1997). The modified biomass thus obtained will be referred to as B2.

Esterification of the phosphate groups using triethyl phosphite and nitromethan under reflux conditions as

described by Tobin et al. (1990). The resulted biomass will be referred to as B3.

Esterification of the hydroxyl groups present on the biomass by specific reaction widely applied for determination of hydroxyl content of carboxylic acids, which was performed by the follow way: 0.2g of raw biomass was suspended in 10ml mixture of pyridine and acetic anhydride (12 to 88 volume ratio) and heated for 2 h at 60°C. The biomass thus obtained will be referred to as B4.

Extraction of the lipid fraction of the biomass by acetone under reflux conditions as applied by Tobin et al. (1990) to investigate the mechanism of metal uptake by denatured *Rhizopus arrhizus* biomass. The extracted biomass obtained will be referred to as B5.

Infrared (IR) spectra of raw biomass and chemically modified biomass were recorded on Vector 22 (Bruker) spectrometer in KBr pellets.

Stock solutions (10 mM) of copper and cobalt were prepared in deionized distilled water using the corresponding salts ($CuSO_4.5H_2O$, $CoCl_2.6H_2O$). A known amount of each biomass sample (1mg/ml) was suspended in separate solution (pH 5.0) containing 1 mM of copper or cobalt ions, and shaken at 220 rpm on a rotary shaker at 30°C until the equilibrium in the solutions was reached. Then the reaction mixtures were filtered using Whatman №1 filter paper. The concentration of copper and cobalt ions in the filtrates was determined using an atomic absorption spectrophotometer (model 2380; Perkin Elmer, Uberlingen, Germany). Sorption experiments were also conducted to study the release of calcium, magnesium and potassium ions from the raw biomass as a result of biosorption of copper and cobalt using the same procedure. Concentration of copper, cobalt, calcium, magnesium and potassium ions in the filtrates was analyzed. All the sorption experiments were conducted twice and the average of two parallel experiments was used in the data analyses. The amount of heavy metal ions (mg or mmoles) biosorbed per g of biomass (q) was calculated as described previously (Ahmad *et al.*, 2005; Tsekova *et al.*, 2001).

RESULTS AND DISCUSSION

IR spectra of raw and chemically modified biomass were obtained to evaluate the effects of the chemical treatment on the functional groups, which may be involved in heavy metal biosorption IR spectrum of raw biomass is shown on Fig. 1. The broad peak at 3385 cm^{-1} is indicative for the presence of both stretch amine (v NH) bands and stretch vibrations of hydrogen bonded OH-groups (Pouchert, 1981). The strong band at 1653 cm^{-1} (vC=O, amide I) together with the band at 1554 cm^{-1} (δNH, amide II) confirms the presence of amide functions in the biomass of *Penicillium cyclopium.* The strong band confirms the presence of hydroxyl groups with maximum at 1040 cm^{-1} (C-O stretch vibrations). No clear evidences for the presence of carboxyl functions in the sample were found in the IR spectrum, as the characteristic carboxyl bands between 1725-1695 cm^{-1} was not observed. IR spectrum of the sample **B1** obtained by treatment with methanol in acidic conditions aiming to block the carboxyl functions eventually present in the biomass was found to be almost identical to those of the raw biomass.

The spectrum of biomass sample **B2** displays only slight changes in the broad band at 3385 cm^{-1} assigned for stretch amine and hydroxyl vibrations. It is obvious that the fraction of primary and secondary amine groups capable to undergo methylation is very low (data not shown). The major part of amine groups in *Penicillium cyclopium* are bound in amide linkages which is proved by the strong absorption at 1653 cm^{-1} (vC=O, amide I) and the band at 1554 cm^{-1} (δNH, amide II). In the spectrum of the raw biomass, the small band at 1230 cm can be considered as arisen from the stretch P=O vibration (Fig.1). The treatment of the biomass with triethyl phosphite (sample B3) resulted in a change in the relative intensity of the bands at 1230 and 1036 cm^{-1} (data not shown). This can be assigned to the esterification of primary hydroxyl groups rather than to the reaction with phosphate as proposed by Kapoor and Viraraghavan (1997).

The most considerable changes in the IR spectrum showed sample **B4** representing the acetylated hydroxyl groups in the biomass (Fig. 2). The strong new absorptions appeared at 1747 cm^{-1} (carbonyl stretch band) and 1239 cm^{-1} (carbon-oxygen single bond stretch vibration) proving the high content of OH groups in the *Penicillium cyclopium* biomass successfully esterified. The spectrum of sample **B5** obtained after extraction with acetone in order to eliminate the lipid fractions shows no significant changes in the quality and quantity of the functional groups present in the biomass in comparison to the control.
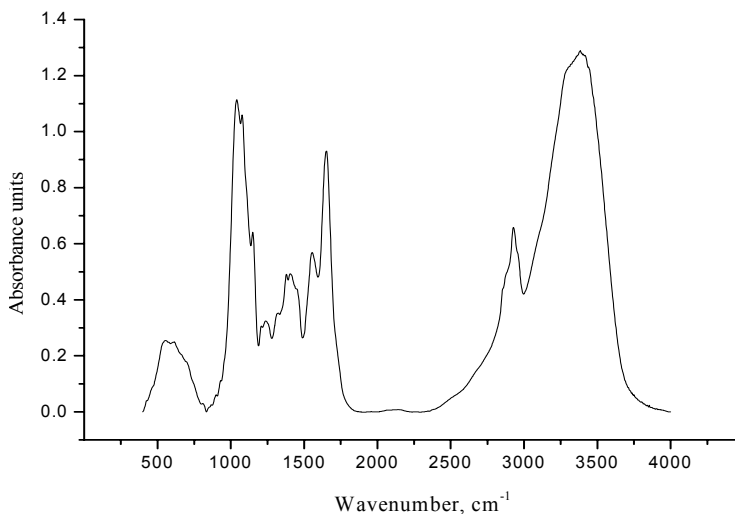
Kolishka Tsekova; Darinka Christova; Maria Ianis

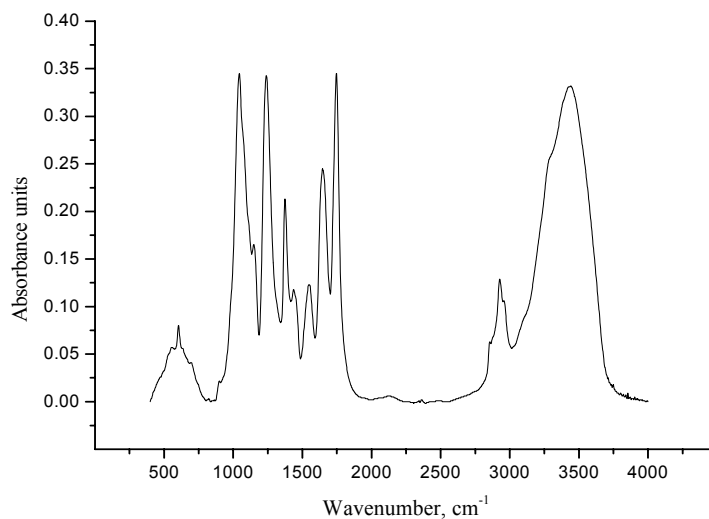**Fig.1**. Infrared spectrum of raw biomass (control)

Fig.2. Infrared spectrum of biomass sample (**B4)** after pyridine and acetic anhydride treatment

Results of metal binding studies for raw and chemically treated *Penicillium cyclopium* biomass are shown in Fig. 3. Biosorption of copper and cobalt was observed to be strongly inhibited when hydroxyl groups were acetylated (B4), indicating the important role of these groups in the biosorption of heavy metal ions. It can be concluded that hydroxyl groups represent the major fraction of reactive groups in *Penicillium cyclopium,* which is confirmed by the results shown in Fig 2. Significant reductions in metal ion biosorption were obtained for biomass in which the amino groups were blocked (B2), more clearly expressed in the case of copper ions. Cobalt ions biosorption was observed to be more sensitive to

modifications of the biomass treated with methanol and hydrochloric acid (B1) in comparison to copper ions. On the other hand, the participation of amine as well as carboxyl groups seems to be the same in the case of cobalt ions biosorption, where the observed reduction of the uptake was found to be about 30 % to the control. In opposite, the process of copper biosorption by *Penicillium cyclopium* seems to perform by the main participation of hydroxyl groups, following by amino groups, where the reduction of the uptake was found to be about 55 % and 40 % respectively. Slight decrease in the heavy metal uptake was observed in samples treated with triethyl phosphite, more clearly expressed in the case of

Kolishka Tsekova; Darinka Christova; Maria Ianis

cobalt ions biosorption. The extraction of the biomass with acetone (B5) leads to slight decrease in copper biosorption, but has insignificant effect on cobalt biosorption. The differences in the uptake values could be due to some structural changes of the binding sites, provoked by the conditions of the treatment, more clearly expressed in the case of the copper ions.
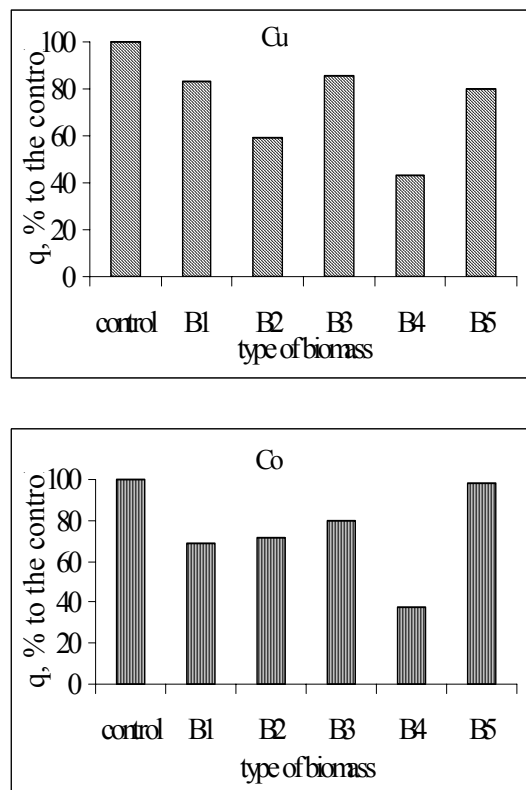

Fig.3. Heavy metal uptake by raw biomass (control) and chemically modified biomass samples of *Penicillium cyclopium* (0.100 mg of each type of biomass was contacted with 100 ml 1mM solution of Cu or Co ions at pH 5.0)

In general, the exact mechanisms responsible for heavy metal biosorption on fungal biomass are unclear. To the best of our knowledge the functional groups in *Penicillium cyclopium* biomass and their participation in the metal ion absorption are not investigated. Tobin et al. (1990) by different chemical treatments to *Rhizopus arrhizus* reported that phosphate and carboxyl groups were the most important in heavy metal biosorption while the amine groups did not play any significant role in this process. The other studies conducted on the mechanism of biosorption indicated that carboxyl groups were important in copper biosorption (Akthar et al. 1996) as well as both carboxyl and amine groups are found to participate mainly in biosorption

of lead, cadmium and copper on *Aspergillus niger* biomass (Kapoor and Viraraghavan, 1997). The current results suggest that copper shows preference to amine sites in comparison with carboxylates and these results are in agreement to those obtained by other investigations (Kapoor and Viraraghavan, 1997; Saitoh *et al.*, 2001). Fig. 3 also shows that, when OH groups were modified biosorption of copper and cobalt was reduced by a much degree than the reduction in biosorption, caused by modifications of both amine and carboxyl groups. Thus, it can be concluded that the major functional groups involved in heavy metal biosorption are the hydroxyl groups, augmented by secondary binding groups such as amino and carboxyl. On the other hand, Sarret et al. (1998) showed that Zn and Pb bind to the predominant phosphate ($\approx$95%) and minor carboxyl groups ($\approx$5%), presenting binding sites in *Penicillium chrysogenum* cell walls. In our investigation, the treatment with triethyl phosphite resulted in 15 and 20% reduction of the copper and cobalt uptake, respectively, and was assigned to the blocked primary hydroxyl groups. Therefore it needs to be emphasized that mechanism of biosorption of heavy metals on different fungi may involve different functional groups to varying extents.

Biosorption of copper and cobalt on raw biomass was accompanied with release of Mg, Ca and K ions into the reaction mixture, suggesting that the mechanism of biosorption is similar to metal removal by ion-exchange resins. Fig. 4 shows the amounts of copper and cobalt biosorbed and Mg, Ca and K ions released during the biosorption. Non-stechiometric release of Mg, Ca and K ions for both heavy metals ions was observed. The ratio of mmoles of metal ion biosorbed to the sum of mmoles of Mg, Ca and K ions released from the raw biomass was 0.74 for copper and 1.6 for cobalt. Non-stechiometric exchange of ions could have resulted to the different positive charge of the ions. The potassium ions carries a single positive charge while copper and cobalt ions carry a double charge, therefore two mmoles of potassium ions will be exchanged for one mmol of copper and cobalt ions. On the other hand, it is also possible that some amount of copper and cobalt may not be carrying a double charge at pH 5.0 and thus may not result in one to one exchange with Mg and Ca ions and exchange of ions may not take place as in balanced chemical equations. Recent studies have shown that Mg, Ca, K and hydrogen ions were also released from biomass as a result of biosorption (Kapoor and Viraraghavan, 1997; Akthar *et al.*, 1996).

The present investigation shows that metal binding mechanisms of investigated *Penicillium cyclopium* biomass involve weakly acid exchange in addition to complexation of metal ions with some functional groups of the biosorbents. It appears that hydroxyl

Kolishka Tsekova; Darinka Christova; Maria Ianis

groups of the biomass present the major sites for heavy metal deposition and minor binding sites, involving mainly amides and carboxylates, augmented the uptake.
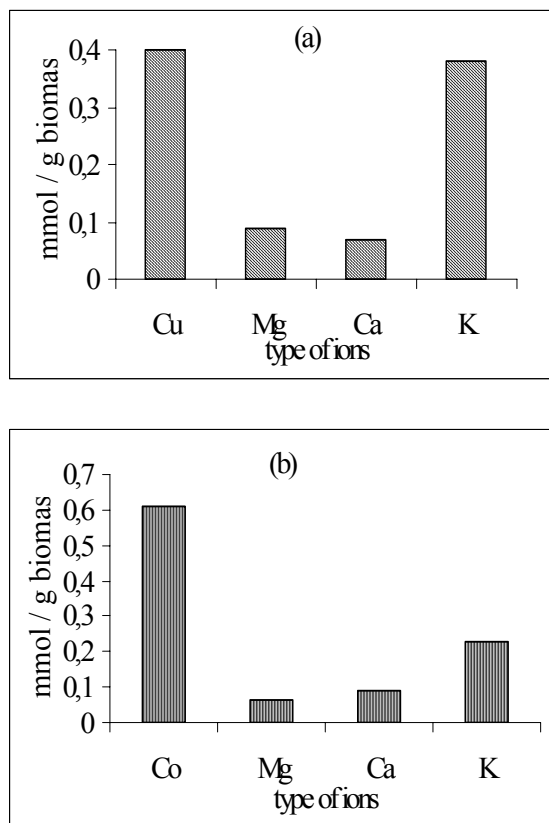

Fig 4. Amounts of copper (a) and cobalt (b) ions biosorbed and Mg, Ca and K ions released upon biosorption (0.100 mg of raw biomass was contacted with 100 ml 1mM solution of Cu or Co ions at pH 5.0)

REFERENCES

Ahmad, I; Zafar, S; Ahmad, F (2005) Heavy metal biosorption potential of Aspergillus and Rhizopus sp. isolated from wastewater treated soil. J Appl Sci Environ Mgt 9 (1): 123-126.

Akthar, N Md; Sastry, K S; Mohan, P M (1996) Mechanism of metal ion biosorption by fungal biomass. Biometals 9: 21-28.

Breierova, E; Vajczikova, I; Sasinkova, V; Stratilova, E; Fisera, M; Gregor, T; Sajbidor, J (2002) Biosorption of cadmium ions by different species. Z. Naturforch 57c: 634-639.

Cabuk, A; Ilhan, S; Filik, C; Caliskan F (2004) Pb^{2+} biosorption by pretreated fungal biomass. Turk. Journal of Biol 29: 23-28.

El-Sayed, M; El-Morsy, I (2004) Cunninghamella echinulata a new biosorbents of metal ions from polluted water in Egypt. Mycologia 96 (6): 1183-1189.

Franco, L; Maia, R; Porto, A; Messias, A; Fukushima, K;de Campos-Takaki, G (2004) Heavy metal biosorption by chitin and chitosan isolated from Cunninghamella elegans (IFM 46109). Braz. J. of Microbiol 35: 243-247.

Gazso, L G (2001) The key microbial processes in the removal of toxic metals and radionuclides from the environment. CEJOEM **7** (3-4): 178-185.

Kapoor, A; Viraraghavan, T (1997) Heavy metal biosorption sites in Aspergillus niger. Bioresource Technology 61: 221-227.

Khoo, K M; Ting, Y P (2000) Biosorption of gold by immobilized fungal biomass. Biochem. Engineering J 8: 51-59.

Kratchovil, D; Volesky, B (1998) Advances in the biosorption of heavy metals. TIBTECH 16: 291-300.

Pouchert, C J (1981) The Aldrich Library of Infrared Spectra, Edition 3, Ed., Aldrich Chemical Company, Inc.

Preetha, B; Viruthagiri, T (2005) Biosortion of zinc (II) by Rhizopus arrhizus: equilibrium and kinetic modeling. African J of Biotech 4 (6): 506-508.

Saitoh, T; Nakagaki, N; Uchida, Y; Hiraide, M; Matsubara C (2001) Spectrophotometric determination of some functional groups on Chlorella for the evaluation of their contribution to metal uptake. Analytical Sci 17: 793-795.

Sarret, G; Manceau, A; Spadini, L. (1998) Structural determination of Zn and Pb binding sites in Penicillium chrysogenum cell walls by EXAFS spectroscopy. Environmen Sci and Technol 32 (11): 1648-1655.

Tobin, J M; Cooper, D G; Neufeld, R J (1990) Investigation of the mechanism of metal uptake by denatured Rhizopus arrhizus biomass. Enzyme Microb Technol 12: 591-595.

Tsekova, K; Dencheva, V; Ianis, M (2001) Copper uptake by Penicillium brevicompactum. Z Naturforsch 56c: 803-805.

Kolishka Tsekova; Darinka Christova; Maria Ianis