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HEXAVALENT CHROMIUM ACCUMULATION BY MICROSCOPIC FUNGI

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Abstract: The purpose of the study was to optimize the removal of Cr(VI) by means of the *Trichoderma viride* strain isolated from chromium mud samples a well as the *Aspergillus niger* and *Penicillium citrinum* strains from other environments. The growth of organism and removal of chromium(VI) was carried out in water solution of various chromium(VI) contents. The research was carried out at optimal pH for each fungus i.e. *Aspergillus niger* 4.0, *Penicillium citrinum* 5.0 and *Trichoderma viride* 4.5. During 14 days of incubation, samples of 5 ml each were collected every day in order to determine chromium(VI) content in the solution and the efficiency of bioaccumulation of this element was then specified. Furthermore, chromium contents in filtrate and mycelium were checked to verify this type of biological activity of microorganisms. The fungi culture investigated in this study could grow at 10–125 mg/l chromium concentration which indicated that it was characterized by high tolerance to various concentrations of chromium. At 125 mg/l chromium, these organisms could accumulate successfully about 90% of chromium. High tolerance of this culture can make it a potential candidate to be a heavy metal scavenger of chromium.

INTRODUCTION

Chromium is a geochemical component of anthropogenic origin. Cr occurs widely in the earth's crust as a natural component of rocks, minerals, soils and water. This is the element widely applied in many industrial branches, such as chemical, metallurgical and tannery industries, hence its high concentrations occur in sewages from tanneries and dye-houses. Its biological and chemical properties depend upon its properties. In the natural environment, the most often occurring valences of chromium are Cr(III) and Cr(VI). Cr(VI) is more toxic and harmful, both for the natural environment and for people. Because of its toxic properties and high mobility, effluents and waste containing this element are treated as highly dangerous. An additional problem is constituted by growing costs of their storage, dump preservation and transport of waste [1–4].

In the recent years, the issues of environmental protection and rational management of mineral resources influenced the water, air and soil protection. These changes are aimed at the requirements for the human activity effects and make crucial the entire analysis of applied technologies in order to lower the losses of mineral raw materials and metals recovery as well as their recycling. The currently applied chemical methods

of treatment of the effluents containing chromium, whose results are not satisfying require significant financial costs [5]. Biotechnological processes may be an alternative [6, 7, 8]. The application of selectively chosen microorganisms may significantly limit the amount of chromium introduced to the natural environment. The main advantage of biotechnological methods is the fact that these methods are economical and environmentally friendly. Chromium is removed by cellular metabolism of microorganisms, mainly by bioaccumulation, biosorption and biotransformation.

The previous investigations describe the applications of living and dead microorganism cells to remove Cr(VI) from water solutions by biosorption [9–14] and bioaccumulation [9, 15–25]. Each of these methods has its advantages and disadvantages. The application of dead biomass removes the problems connected with the toxic metal concentration in the studied solution and requirements connected with the growth environment, i.e. nourishment. Furthermore, the adsorbed metal may be easily removed and the remaining biomass may be applied once again. However, this method is limited by the fact that no reactions are being continued in dried cells.

The application of living biomass enables metal to be removed during its growth which allows the processes of its reproduction, drying and storage to be avoided. Unfortunately, in this case, the metal concentration in the environment is extremely important because when it is too high, it may be toxic for the growing biomass. This problem can be avoided when applying the microorganisms of high tolerance to high concentrations of Cr(VI) [26] or getting it by adaptive processes.

The purpose of this study was to optimize the biological process of Cr(VI) removal by the application of the indigenous fungus *Trichoderma viride,* isolated from the chromium mud samples [26] and pure cultures of the microscopic fungi *Aspergillus niger* and *Penicillium citrinum,* originating from the natural environment.

MATERIAL AND METHODS

Fungal isolates

The autochtonic fungi culture *Trichoderma viride*, originating from the chromium mud and fungi cultures of *Aspergillus niger* and *Penicillium citrinum*, from the Collection of Pure Culture of Industrial Microorganism IBPRS, were selected to investigate Cr(VI) removal.

Determination of chromium(VI) contents, common for all experiments

Agents

The agents applied to determinate chromium content, such as 1,5-difenylocarbaside(I) and 2M sulfur acid(VI) were prepared according to the PN-EN 12441-10 standard [33].

Determination of chromium(VI) contents

Before starting the measurements, the pattern line was prepared. For this purpose, solutions of sulfur acid(VI), 1,5-difenylocarbaside(I) and certain volumes of pattern solution of chromium(VI) were introduced into 100 ml flasks to get the Cr^{6+} ions concentrations, such as: 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1 mg/l.

The analytical samples were prepared by mixing the solutions of sulfur acid(VI), 1,5-difenylocarbaside(I) and the sample solution in a 25 ml flask. In the case of the concentration higher than the pattern scale range, the samples were adequately diluted.

Chromium(VI) ions concentrations (c_{Cr}) were determined by measuring the absorption at 540 nm by means of spectrophotometer Cadas 200 type LPG 392 [28–32].

Investigation of the process dependence on pH

Strains of fungi were grown aerobically at 28°C in the accumulating medium prepared by mixing the Cr(VI) solution, autoclaved separately (at 120°C for at least 20 min) and the sterilized solution according to Waksman [35]. The medium pH was adjusted to the desired value by means of 0.5 M sulfuric acid(VI) solution. Cultures were performed in a 300 ml Erlenmayer flask with 100ml of the accumulation medium, containing 50 mg of ions Cr^{6+}/l. 2.5 ml samples of the medium were collected from each Erlenmayer flask daily, for 14 days, then transferred to the flasks of 25 ml volume each and chromium(VI) ions concentrations were determined.

Dependence of the accumulation process of chromium (VI) concentration in the medium

The investigation of Cr(VI) ions concentration in the medium in relation to pH allowed the optimal fungi growth to be determined. These were: *Aspergillus niger* – pH = 4.0, *Penicillium citrinum* – pH = 5.0, *Trichoderma viride* – pH = 4.5.

A certain amount of the medium and some amount of $K_2Cr_2O_7$ (of concentration 1g of ions Cr^{6+}/l) were transferred in to Erlenmayer flasks to get the required chromium concentration in a certain sample in volume of 100 ml of bed. The determination of chromium(VI) content was conducted every day at the same hour by the spectrophotometric method.

Determination of biological type of Cr(VI) ions removal

The removal of chromium(VI) from the water solution may occur because of reduction, biosorption or bioaccumulation processes. To determine which of them occurred during the investigation, Cr(III) content was determined in the samples of the medium as well chromium contents in the ooze after mycelium irrigating and in mycelium.

Determination of Cr(III) content in the medium

The Cr(III) ions content was determined on the basis of a difference between the total chromium content and chromium(VI) content in the medium after 14 days of incubation.

To determine the total chromium content, the samples of the medium of a certain volume assuring the concentration of Cr inside the pattern scale range were introduced into a beaker of 200 ml volume and were filled up to the volume of 50 ml. Next, to oxidize Cr(III) ions to Cr(VI), the solutions of sulfuric acid (VI) and ammonium persulfate were added to the sample and then it was boiled and maintained in this state for 20–25 minutes. After chilling the samples, chromium(VI) ions concentrations were determined by the spectrophotometric method.

Determination of chromium content in the ooze after mycelium irrigating

Mycelium was investigated after 14 days of incubation. To determine the presence of chromium adsorbed on the surface, mycelium was irrigated. The given ooze was then analyzed to prove the total chromium presence [28–32].

Determination of chromium content in mycelium

After 14 days of incubation the fungi dried at 105°C were burnt in the oven at 600°C. Next, the chromium compounds were transformed into dilatable nitrates by means of concentrated nitrogen acid (V). The content of Cr(VI) in the mineralized sample was determined by the spectrophotometric method. [28].

RESULTS

Investigation of the process dependence on pH

On the basis of the results given the graphs were drawn presenting the dependence of chromium(VI) ions concentration in the medium on pH. Figures 1–3 show the change of Cr(VI) concentration in the medium with different initial pH, ranging from 4.0 to 6.5. From these figures optimal pH was determined for every type of microscopic fungi. It was the one from which chromium(VI) ions were removed in the most efficient way and the growth of certain microscopic fungi was optimal.

The results suggest that the optimum initial pH value for *Aspergillus niger* was 4. This was the one from which the chromium(VI) ions were removed in the most efficient way and certain mildew fungi developed best. Similar results were obtained by Dursun et al. [10]. In the case of the biosorption process Mungasavalli et al. [13] reported that the optimum pH for the sorption of chromium(VI) by the pretreated *A. niger* was 3.0. Other authors [8, 19] showed that the complete removal of Cr(VI) by the pure colony of *A. niger* was observed only at highly acidic pH, such as 2.0, because of insufficient contact time (i.e. below 24 h) at higher pH values.

The optimum initial pH value for *Trichoderma viride* was 4.5 and for *Penicillium citrinum* it was 5.0. Morrales-Barrera et al. [22] studied Cr(VI) removal by *T. viride* in a pneumatically agitated bioreactor. In this experiment the initial pH of the culture media was 6.0±0.1 and the pH was not controlled during the experiments. In the case of biosorption by the immobilized to Ca-alginate *T. viride* biomass the optimum pH was 2.5 [34].

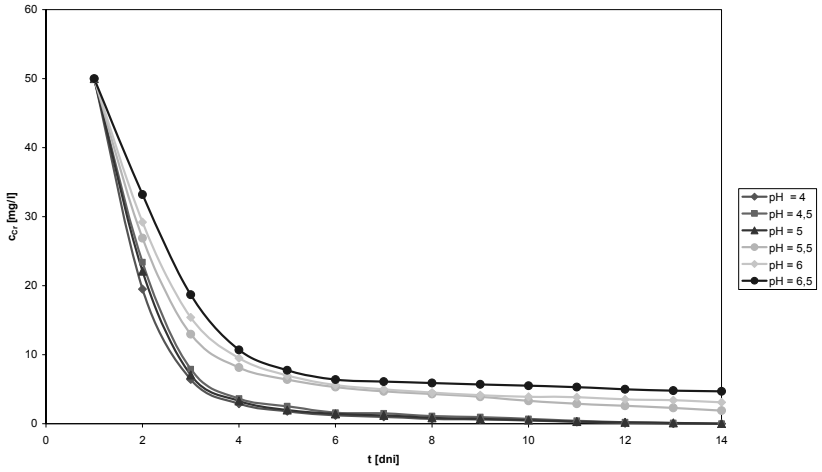

Fig. 1. Dependency c_{Cr} = f(t)on pH for Aspergillus niger (initial concentration of chromium(VI) 50 mg/l)

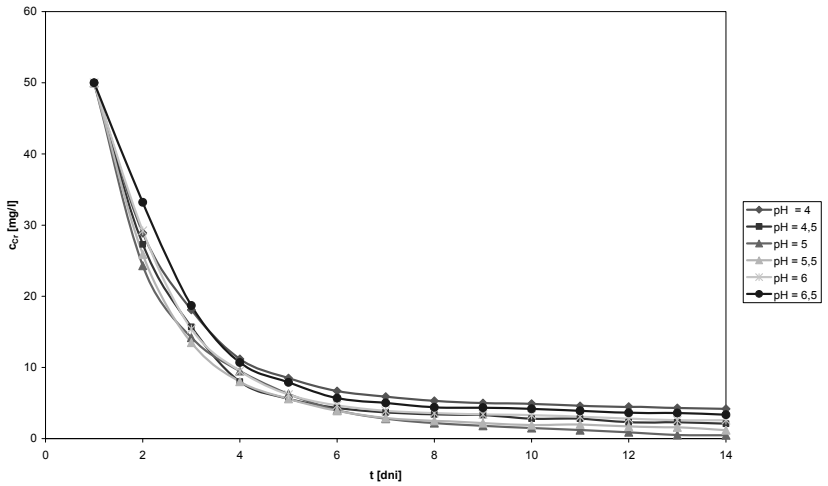

Fig. 2. Dependency $c_{Cr} = f(t)$ on pH for Penicillium citrinum (initial concentration of chromium(VI) 50 mg/l)

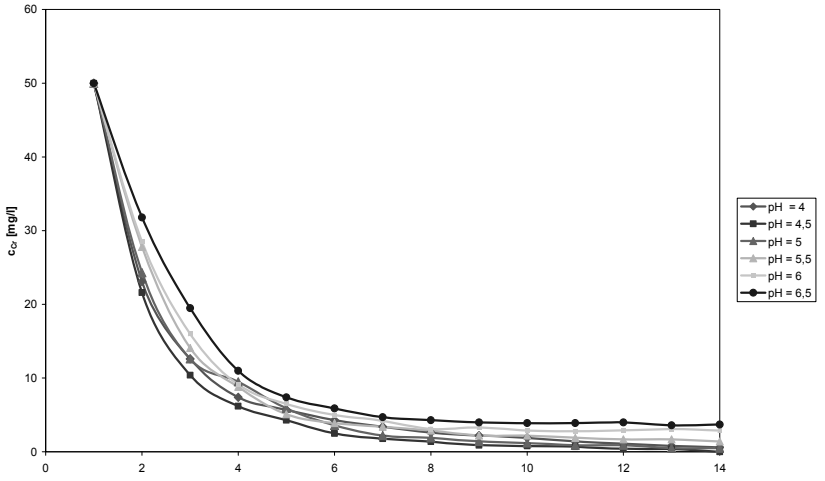

Fig. 3. Dependency $c_{Cr} = f(t)$ on pH for Trichoderma viride (initial concentration of chromium(VI) 50 mg/l)

The determined optimal pH was the basis for further research, where the relation between the chromium(VI) ions concentration and the initial concentration of chromium(VI) was determined.

Dependence of the process on chromium(VI) concentration in a sample

On the basis of measurement results the figure presenting relations of chromium(VI) ions concentration in the medium to the time of this process were prepared. The effect of initial Cr(VI) concentration was investigated in a range of about 10–125 mg/l.

Figure 4 shows that Cr(VI) removal for *Aspergillus niger* occurred even at the highest concentration of 125 mg/l, but the complete Cr(VI) removal was observed for 10, 25, 50 and 75 mg/l at 9, 11, 12 and 14 days, respectively. Similar results were obtained for *Trichoderma viride* (Fig. 6). In this case complete Cr(VI) removal was observed for 10, 25, 50 and 75 mg/l at 12, 13 and 14 days, respectively. The worst results were reported for *Penicillium citrinum* (Fig. 5). For this fungus the complete Cr(VI) removal was observed only for 10, 25, and 50 mg/l, always in 14 days. However, the change of Cr(VI) concentration indicates that in the same incubation time, more Cr(VI) was reduced at a higher initial Cr(VI) concentration.

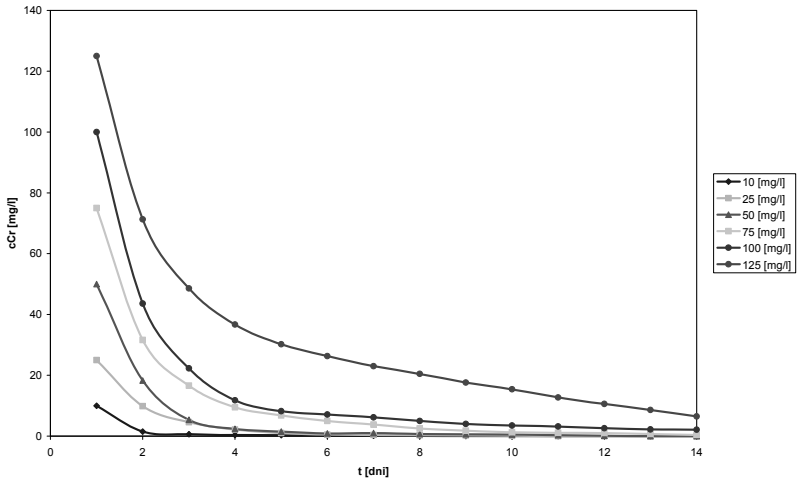

Fig. 4. Dependency $c_{Cr} = f(t)$ for various initial concentrations of chromium(VI)
Aspergillus niger, pH = 4

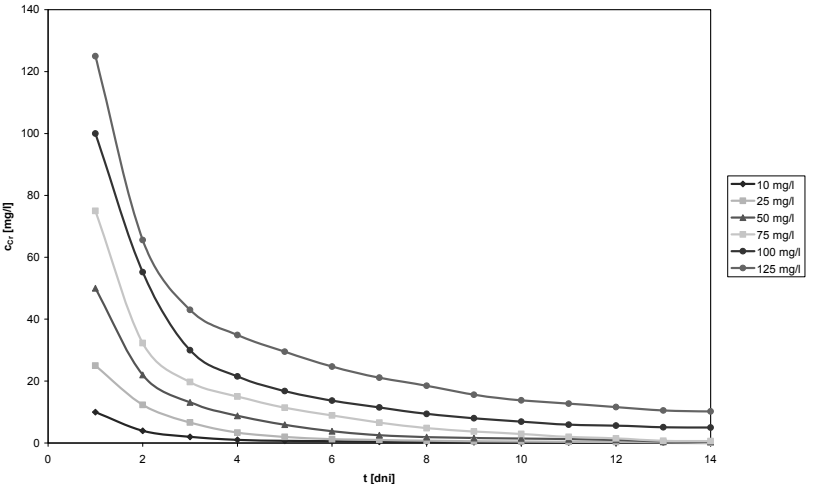

Fig. 5. Dependency $c_{Cr} = f(t)$ for various initial concentrations of chromium(VI)
Penicillium citrinum, pH = 5

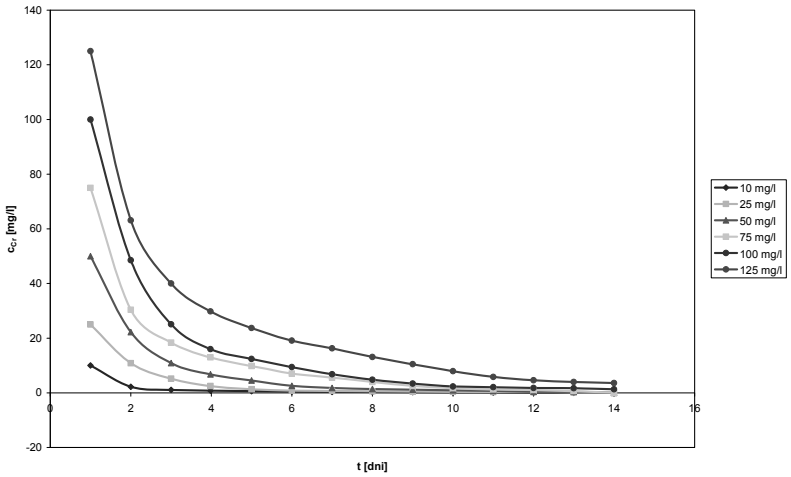

Fig. 6. Dependency $c_{Cr} = f(t)$ for various initial concentrations of chromium(VI) Trichoderma viride., pH = 4,5

For *Aspergillus niger* Dursun et al. [10] reported in the previous study that the maximum uptake capacity (defined as the ratio of bioaccumulated concentration of metal ions at the end of growth to the initial metal ions concentration) was determined at 50 mg/l initial chromium(VI) concentration and no significant microbial activity and chromium(VI) uptake were observed above this chromium(VI) concentration. The authors applied the strain of *A. niger* obtained from the U.S. Departament of Agriculture Culture Collection. The removal of hexavalent chromium by *Aspergillus niger* isolated from the soil of leather tanning effluent was reported by Srivastava et al. [15]. This strain removed more than 70% chromium in the soil contaminated by 250 and 500 ppm of chromate (*A. niger* was introduced into the soil microcosm, 40% moisture content, with different concentration of chromate). The difference in the obtained results shows how a source of colony affects its tolerance to the high concentration of toxic chromium.

In the case of biosorption, an effect of initial Cr(VI) concentration on Cr(VI) removal by the dead fungal biomass of *A. niger* was reported by D. Park et al [19]. The concentration of Cr(VI) versus time was examined at initial Cr(VI) concentrations, from 25 to 200 mg/l. According to the authors Cr(VI) was completely removed from the solution in 30 hours for Cr(VI) concentration of 25 mg/l, whereas the complete removal of 200 mg/l of Cr(VI) required about 400 h of contact time.

For *Trichoderma viride* Morrales-Barrera et al. [22] studied Cr(VI) removal by a microbial culture in a pneumatically agitated bioreactor. They have reported that in an airlift bioreactor a complete Cr(VI) removal was determined at 1.3 and 1.6 mM initial chromium(VI) concentration after 30 and 80 h of incubation, respectively. Also very high overall efficiency of Cr(VI) removal was achieved (94.3%) after more than 160 h of incubation at the initial Cr(VI) concentration of 1.94 mM. When *T. viride* was cultivated in unbaffled flasks containing the culture medium with initial Cr(VI) concentrations of 1, 1.5 and 2.0 mM, the Cr(VI) removal efficiency was 97–100%, respectively.

Determination of type of biologia Cr(VI) ions removal process

Determination of Cr(III) content in the medium

The results of the analysis of total chromium presence in the medium and initial chromium(VI) content are presented in Table 1. The Cr(III) ions content was determined on the basis of the difference between total chromium content and chromium(VI) content in the medium after 14 days of incubation.

Table 1. Results of analysis of Cr(III) presence in medium

Initial concentration Cr(VI) [mg/l]	**Total chromium concentration [mg/l]**			**Cr(III) concentration [mg/l]**		
	Aspergillus niger	*Penicillium citrinum*	*Trichoderma viride*	*Aspergillus niger*	*Penicillium citrinum*	*Trichoderma viride*
10	0	0	0	0	0	0
20	0.032	0	0.15	0.032	0	0.15
50	0.159	0.279	0.408	0.159	0.121	0.408
75	0.56	0.735	0.625	0.150	0.113	0.605
100	2.325	6.03	2.15	0.194	1.029	0.806
125	7.03	11.62	4.012	0.529	1.38	0.376

Modest amounts of Cr(III) in the medium might occur because of acidification of the environment by products of fungi metabolism at the final stage of a 14-day period of culture. Such a low chromium concentration on the III level of oxidation also proves that the reduction process is not a cause of biological removal of Cr(VI) ions by means of microscopic fungi.

Determination of overall chromium content in the ooze after mycelium irrigating

The results of the analysis of total chromium presence in the ooze depending on initial chromium(VI) content are presented in Table 2. The trace amount of total chromium in the ooze eliminates rather the ion adsorption of this element on the surface of mycelium. This process could occur in the initial phase of mycelium growth and was the first stage of intracellular accumulation.

Table 2. Results of analysis of overall chromium presence in the ooze

Initial concentration Cr(VI) [mg/l]	**Total chromium concentration in ooze [mg/l]**		
	Aspergillus niger	*Penicillium citrinum*	*Trichoderma viride*
10	0	0	0
20	0	0	0
50	0.056	0.092	0.02
75	0.11	0.33	0.092
100	0.195	0.25	0.131
125	0.23	0.411	0.3

Determination of chromium content in mycelium

Table 3 presents the results of total chromium content in mycelium depending on initial Cr(VI) ions concentration in the medium. The results indicate that the growth of Cr(VI) ions in mycelium occurred in comparison to the ions concentration in the surrounding environment. This may prove that the removal of Cr(VI) from water solutions by macroscopic fungi occurs by intracellular bioaccumulation.

Table 3. Results of analysis of overall chromium presence in mycelium

Initial concentration Cr(VI) [mg/l]	**Total chromium concentration in mycelium [mg/l]**		
	Aspergillus niger	*Penicillium citrinum*	*Trichoderma viride*
10	9.89	9.90	9.92
20	19.25	19.76	19.52
50	49.29	49.11	49.18
75	74.09	73.69	74.01
100	97.12	93.12	97.61
125	117.05	112.82	120.31

CONCLUSIONS

A wide variety of growing, living or non-living microbial biomasses has been used for chromium(VI) removal. Many species of yeasts, bacteria and fungi have been investigated to asses their suitability for Cr(VI) removal. Chromium(VI) tolerance and bioaccumulation in 51 strains of yeast was reported by Ksheminska et al. [14] while bioaccumulation in fungi was studied by Sen et al., Dursun et al., Dönmez and Koçberber and Srivastavaa et al. [7, 10–12, 15]. The use of dead fungal biomass for the detoxification of hexavalent chromium was investigated by many authors [8, 13, 16–18, 19, 23, 34]. Biosorption of Cr(VI) by biomass of different strains of bacteria isolated from various environments was also often studied [9, 20, 21, 24, 25]. In general, the authors examined the influence of pH, initial chromium concentration and biomass concentration on metal uptake.

The purpose of the investigation presented in this paper was to optimize the biological process of Cr(VI) removal by *Aspergillus niger, Trichoderma viride and Penicillium citrinum*. On the basis of the given results the following conclusions were made:

- The removal of Cr(VI) from water solutions by the application of microscopic fungi *Trichoderma viride, Aspergillus niger, Penicillium citrinum* occurs by intracellular bioaccumulation;
- The process of intracellular chromium absorption with alimentary substances is the largest during the first 5 days of mycelium growth;
- Bioaccumulation of chromium(VI) depends on environmental pH and is the most efficient by pH, respectively: 4.0 for *Aspergillus niger*, 5.0 for *Penicillium citrinum* and 4.5 for *Trichoderma sp*.;
- The higher is the chromium(VI) concentration, the lower is the accumulation of this element from the environment and the growth of mycelium is slower;

- Biological chromium(VI) removal depends on the applied fungi species and the place of its origin which occurs at higher concentrations of Cr(VI). The biggest efficiency of bioaccumulation is for autochtonic fungi from the *Trichoderma sp.* Sort, isolated from chromium mud waste.

The fungi culture investigated in this study could grow at 10–125 mg/l chromium concentration which indicated that it possessed high tolerance to various concentrations of chromium. At 125 mg/l chromium, these organisms could successfully accumulate about 90% of chromium. High tolerance of this culture makes it a potential candidate to be a heavy metal scavenger with respect to chromium.

The application of microscopic fungi to the biological removal of chromium(VI) may be a perfect alternative for expensive chemical methods. Its disadvantages are longer time of bioaccumulation and lack of possibility of metal recovery without destruction of the mycelium – this causes that the application of these microorganisms is possible only once.

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AKUMULACJA JONÓW CR(VI) PRZEZ GRZYBY MIKROSKOPOWE

W artykule przedstawiono biologiczne usuwanie jonów Cr(VI) z roztworu wodnego przy użyciu szczepu grzyba z gatunku *Trichoderma viride* pochodzącego z próbek błota pochromowego oraz czystych kultur grzybów *Aspergillus niger* i *Penicillium citrinum*, wyizolowanych z innych środowisk. Wzrost organizmu oraz usuwanie chromu(VI) przeprowadzono w roztworze wodnym o różnej zawartości chromu(VI). Badania były przeprowadzane przy optymalnym pH dla każdego rodzaju grzyba: *Aspergillus niger* 4,0, *Penicillium citrinum* 5,0, *Trichoderma viride* 4,5. W czasie 14 dni inkubacji, codziennie, pobierano 5 ml próbki roztworu w celu ozna-

czenia zawartości chromu(VI) w roztworze i na tej podstawie określono efektywność bioakumulacji tego pierwiastka. Sprawdzono również zawartość chromu w przesączu oraz grzybni w celu potwierdzenia tego rodzaju biologicznej aktywności mikroorganizmów. Czyste kultury grzybów badane w tym artykule mogły rozwijać się przy zawartościach chromu 10–125 mg/l co pokazuje ich wysoką tolerancję na różne zawartości chromu. Przy zawartości chromu równej 125 mg/l te mikroorganizmy mogły akumulować około 90% chromu zawartego w roztworze. Wysoka tolerancja tych kultur czyni je potencjalnymi kandydatami do wychwytywania chromu ze środowiska.