Bioaccumulation of toxic metals by fungi of the genus *Aspergillus* isolated from the contaminated area of Ostramo Lagoons

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**Abstract.** The study compares the ability to bioaccumulate toxic metal ions using microscopic filamentous fungi of the genus *Aspergillus* isolated from the anthropogenically contaminated site of the Ostramo Lagoons (Ostrava, Czech Republic). The experiment comprised six species of indigenous fungal isolates: *A. niger, A. candidus, A. iizukae, A. westerdijkiae, A. ochraceus* and *A. clavatus*. Nutrient liquid media enriched with Cu(II), Zn(II), Ni(II) and Cr(III) were individually inoculated with spores of these fungi. After thirty days of incubation, the content of metal ions in the dried fungal biomass and medium was measured by the AAS. It was found that the average bioaccumulation capacity of selected toxic metal within the tested strains decreases in the following order: *A. ochraceus* > *A. candidus* > *A. clavatus* > *A. westerdijkiae* > *A. iizukae* > *A. niger*. The highest bioaccumulation efficiency was achieved by the *A. ochraceus* strain which accumulated Cu(II) with an efficiency of 57.42 %, Zn(II) with 56.88 %, Cr(III) with 37.73 %. When comparing the ability of bioaccumulation of the toxic metals, the following was found: Zn(II) > Cu(II) > Cr(III) > Ni(II). Understanding of bioaccumulation processes that take place in fungal cells at the molecular level may lead to better strategies for the application of these interesting microorganisms in bioremediation processes.

1. Introduction

Microorganisms, including microscopic filamentous fungi, have adapted to the presence of toxic metals through various resistance mechanisms. Tolerance/resistance of microscopic filamentous fungi to toxic metals is conditioned by their environment [1, 2]. Microorganisms that show a high level of tolerance to toxic metal ions were isolated from environments with a high incidence of these metals [3, 4, 5], respectively toxic metals in combination with other toxic chemicals, both organic and inorganic [6, 7]. It has previously been confirmed that toxic metals can accumulate in the living biomass of resistant fungi strains growing on organic waste material [8, 9]. The Ostrava lagoons Ostramo represented an old ecological burden of extraordinary scale with high concentrations of hydrocarbons and toxic metals [10] and thus represent a unique environment suitable for the adaptation of microorganisms resistant to these substances. This study compares the ability of six different *Aspergillus* species isolated from the Ostramo Lagoons to bioaccumulate Cu(II), Zn(II), Ni(II), Cr(III) toxic metal ions.

Contamination of the environment with toxic metals poses a serious environmental risk, especially due to the bioaccumulation of metals in living organisms and their subsequent distribution through the food chain, which leads to serious ecological and health risks [11]. The ability of living organisms to resist...
the toxic effects of metals and subsequently accumulate these metals in living cells can also be used in bioremediation processes. The method using the properties of cells of living organisms, including their metabolism, is called bioaccumulation. Bioaccumulation of toxic metals is a complex process involving the active transport of metals through cell membranes into the cell and using physical, chemical, and biological mechanisms [12]. It is a combination of surface reactions, intra- and extracellular clotting, and complexation reactions [13]. Bioaccumulation depends on the internal structural and biochemical properties of cells, on genetic and physiological adaptation of the organism, environmental modification of the metal, its availability, and toxicity which is mainly influenced by the oxidation state of the metal [14]. Currently, the application of bioaccumulation processes is one of the adaptation mechanisms for the removal of pollutants from contaminated environments, including wastewater and semi-liquid sludge [15].

1.1. Interactions of microscopic fungi with toxic metals
Fungal metabolism can very significantly affect the mobility and toxicity of metals and metalloids. There are various ways in which metals can be transformed into a form that is suitable for interactions with cell mass [5]. One of these methods is solubilization, which involves, for example, the formation of complex with organic acids, other metabolites or siderophores [16]. In contrast, metal immobilization results from sorption to cellular exopolymers (cell walls), cell transport, and intra- and extracellular sequestration or precipitation [17]. Dissolution of metal compounds and immobilization of metals are key elements of the biogeochemical cycles of toxic metals. Metals are directly and indirectly involved in all stages of microbial growth, physiology, and morphogenesis [18].

Resistance/tolerance of organisms to toxic metals is defined as the ability to resist metal toxicity using one or more resistance mechanisms that are activated in direct interaction with a particular metal [19, 20]. Some metals, such as cobalt, copper and nickel, serve as micronutrients and are used during redox processes to stabilize the molecule through electrostatic interactions or as components of certain enzymes, such as to regulate osmotic pressure in a cell. However, many metals do not play a significant role, have no nutritional value and are potentially toxic to microorganisms. All metals interact with cellular structures through covalent and ionic bonds. Essential and non-essential metal ions interact with fungal cells and are accumulated by physico-chemical mechanisms and transport systems of varying specificity [21]. In high concentrations, essential and non-essential metals can damage cell membranes, alter the specificity of enzymes, disrupt cellular functions and damage DNA. In these cases, different mechanisms of resistance of microorganisms to toxic metals may apply, which may vary depending on the microorganism, the metal, and the environment (pH, metal ion concentration, etc.) [22]. The key to understanding these mechanisms is, among other things, their genetic nature. These complex processes taking place at the molecular level in the cells of microorganisms may lead to better strategies for their detoxification from the environment.

1.2. Origin of microscopic filamentous fungi of the genus Aspergillus
The landfill, also known as OSTRAMO lagoons (Ostrava, Czech Republic), was established at the end of the 19th century by depositing waste from refinery production. Since 1965, waste from the regeneration of used lubricating oils has also been deposited here, later also other toxic waste, which was often not specified. The landfill consisted of several lagoons originally designated R1, R2 and R3, which were separated by dikes with earth mounds 5 m above the surrounding terrain; lagoon R0 was located in the earth pit of the former brickyard, the existence of which was confirmed only in 1999 [23]. The Ostrava lagoons are classified as very serious and old ecological burdens of extraordinary extent. The actual area of the lagoons was burdened by pollution of the rock environment mainly by organic compounds. The main organic contaminants were polycyclic aromatic hydrocarbons, polychlorinated
biphenyls, and phenols. In addition to organic pollution, there were several toxic metals - As, Cd, Cu, Hg, Ni, and Pb, the pH of the lagoons was acidic to neutral [23]. Microscopic fungi were isolated from lagoons during their microbiological survey in the period 2012–2015. Isolates were identified at the workplace of the Slovak Academy of Sciences in Bratislava and their sequences were stored in the GenBank database: MK243706 - MK243708, MG639905 - MG639907.

2. Material and methods

2.1. Sampling
Samples of semi-liquid sludge were taken from the R2 lagoon area at a depth of 0.1 m, then individual strains of microscopic filamentous fungi were isolated and cultured according to a standard procedure using Sabouraud Dextrose Agar (HiMedia Laboratories, India). Identification and taxonomic classification of individual species was performed by sequence analysis of the Internal Transcribed Spacer of Ribosomal DNA.

2.2. Microorganisms
The following Aspergillus isolates from the contaminated site of the Ostramo lagoons were used in the study: A. niger, A. candidus, A. iizukae, A. westerdijkiae, A. ochraceus, A. clavatus. For a comparative study of the bioaccumulation potential of these species, 2 ml of a spore suspension was used. This suspension was aseptically transferred to a mixture of nutrient medium and metal solution.

2.3. Chemicals and media
The spore suspension was transferred to a nutrient medium containing the appropriate metal ions. First, the amount of the respective salt was determined so that the final concentration of metal ions in the medium was 1000 mg L\(^{-1}\). The following chemicals were used as a source of metal cations (Table 1).

| Metal ion | Chemical compound | Quantity [g] |
|-----------|------------------|-------------|
| Cu(II)    | Cu(NO\(_3\))_2 \cdot 3H_2O | 3.8023      |
| Zn(II)    | Zn(NO\(_3\))_2 \cdot 6H_2O | 4.5475      |
| Ni(II)    | NiCl\(_2 \cdot 6H_2O\) | 4.0502      |
| Cr(III)   | Cr(NO\(_3\))_3 \cdot 3H_2O | 7.6982      |

Sabouraud Dextrose Broth (Sabouraud liquid medium, HiMedia Laboratories, India) was prepared according to the manufacturer's instructions and used to cultivate the microscopic filamentous fungi. Demineralized water was used to prepare the medium and the resulting pH of the nutrient medium was 5.6 ± 0.2. The medium was autoclaved (15-lbs, 121°C). The appropriate amount of metal salt was quantitatively transferred to 1 L of sterile medium. This was followed by further dilution of the solution with sterile metal-free nutrient medium in a ratio of 1:10 so that the final metal concentration in the solution was 100 mg L\(^{-1}\). The experiments were then performed in 1 L Erlenmeyer flasks.

2.4 Method
Bioaccumulation experiments took place for 30 days in the form of static culture. After 30 days of growth, the microfungal biomass was filtered (Gridded MCE sterile filter, pore size 0.45 μm, Membrane Solutions, USA), dried and weighed. The amount of bioaccumulated metal in the biomass of microscopic fungi was analyzed by atomic absorption spectrophotometry (AAS, VARIAN AA 280FS, Agilent, USA), as well as the metal balance values in the nutrient medium. Bioaccumulation efficiency
was calculated by the ratio of the amount of metal accumulated by the microfungal biomass after 30 days to the original amount of metal in the nutrient medium before culturing the microscopic fungi.

3. Results
There were $6 \times 0.5$ L of nutrient media for each metal ion – Cu(II), Zn(II), Ni(II) and Cr(III) at a mass concentration of 100 mg·L$^{-1}$. After 30 days of incubation, the average weight of the harvested and dried biomass of Aspergillus species depending on the type of toxic metal cation in the medium as follows: Cr(III) > Cu(II) > Zn(II) > Ni(II) (Table 2).

|                | Cu(II) | Zn(II) | Ni(II) | Cr(III) |
|----------------|--------|--------|--------|---------|
| A. niger       | 3.5892 | 1.7978 | 0.5984 | 4.8059  |
| A. candidus    | 0.5028 | 1.1479 | 0.3470 | 2.5924  |
| A. iizukae     | 2.5447 | 2.8851 | 1.0160 | 3.0829  |
| A. westerdijkiae | 2.0000 | 2.5902 | 1.0096 | 2.2676  |
| A. ochraceus   | 2.6071 | 3.1493 | 0.6388 | 3.3511  |
| A. clavatus    | 2.3089 | 2.6401 | 1.2456 | 3.2324  |

These results show that Ni(II) ions had the highest inhibitory effect on the growth of microscopic filamentous fungi of the genus Aspergillus and, conversely, Cr(III) ions showed the least growth inhibition. Of the examined species of the genus Aspergillus, A. niger recorded the highest increase in biomass, in the presence of Cr(III) ions, a high increase was also recorded in the presence of Cu(II) ions. Interestingly, A. niger did not grow very well in the presence of Ni(II) ions, only 0.5984 g of microfungal biomass was found, which was the second lowest result among Ni(II) ions in the medium. The average amount of biomass detected in the genus Aspergillus was in the following order: A. niger > A. ochraceus > A. iizukae > A. clavatus > A. westerdijkiae > A. candidus. Thus, A. niger can be considered the most tolerant strain, A. candidus was the least tolerant of the mentioned metals.

Another results analyzed was the amount of accumulated ions in 1 g of dry microfungal biomass [mg g$^{-1}$]. This result showed the ability of the biomass of microscopic fungi of the genus Aspergillus to accumulate toxic metal ions (Table 3).

|                | Cu(II) | Zn(II) | Ni(II) | Cr(III) |
|----------------|--------|--------|--------|---------|
| A. niger       | 2.83   | 2.20   | 5.36   | 0.71    |
| A. candidus    | 9.54   | 40.30  | 6.25   | 1.48    |
| A. iizukae     | 1.18   | 5.12   | 7.65   | 3.43    |
| A. westerdijkiae | 5.31   | 9.20   | 3.85   | 1.69    |
| A. ochraceus   | 11.10  | 9.03   | 5.70   | 5.63    |
| A. clavatus    | 7.28   | 5.84   | 6.01   | 1.19    |

Within the studied microscopic filamentous fungi, the best results was shown by A. candidus, which accumulated 40.30 mg of Zn(II) in 1 g of dry biomass. This amount was accumulated simultaneously
with the smallest amount of grown biomass after 30 days of cultivation, thus accumulating up to 92.52% of the initial amount of Zn(II) in the medium. The remaining strains accumulated on average per 1 g of dry microfungal biomass toxic metal ions in the following order: Zn(II) > Cu(II) > Ni(II) > Cr(III). The lowest amount of toxic metal accumulated in 1 g of dry microfungal biomass was found in *A. niger*, the best result was obtained in *A. candidus* for Zn(II), Cu(II) and Ni(II). The bioaccumulation capacity of this fungi for the indicated toxic metal ions in 1 g of dry microfungal biomass is as follows: *A. candidus* > *A. ochraceus* > *A. clavatus* > *A. westerdijkiae* > *A. iizukae* > *A. niger*.

We have also calculated the bioaccumulation efficiency of the data obtained. The total amount of bioaccumulated toxic metal in the dried biomass, expressed as a percentage, is shown in Table 4.

**Table 4. Bioaccumulation efficiency (percentage of metal accumulated in dry microfungal biomass after 30 days of incubation [%]).**

|       | Cu(II) | Zn(II) | Ni(II) | Cr(III) |
|-------|--------|--------|--------|---------|
| *A. niger* | 20.15  | 7.91   | 5.89   | 6.82    |
| *A. candidus* | 9.52   | 92.52  | 3.98   | 7.67    |
| *A. iizukae* | 5.96   | 29.54  | 14.26  | 21.15   |
| *A. westerdijkiae* | 21.07  | 47.66  | 7.13   | 7.66    |
| *A. ochraceus* | 57.42  | 56.88  | 6.68   | 37.73   |
| *A. clavatus* | 33.35  | 30.84  | 13.74  | 7.69    |

Bioaccumulation efficiency was calculated for each strain examined and for each toxic metal applied. The value was calculated according to the following formula:

$$\eta = \frac{m_{acc}}{m_{in}} \cdot 100$$  (1)

where \( \eta \) is the bioaccumulation efficiency [%], \( m_{acc} \) is the amount of metal accumulated in the total amount of dried microfungal biomass and \( m_{in} \) is the amount of metal in the medium at the beginning of the incubation, in this case the metal concentration is 100 mg L\(^{-1}\) in 0.5 L of medium (\( m_{in} = 50 \) mg).

The results of bioaccumulation efficiency show that *A. ochraceus* best accumulated ions of the toxic metals, which achieved an efficiency of 57.42 % during Cu(II) bioaccumulation, 56.88 % during Zn(II) bioaccumulation and 37.73 % during Cr(III) bioaccumulation. This strain is characterized by a high ability to resist the toxic effects of these metals, which are classified as transition metals. At the same time, it is able to bioaccumulate these metals into mycelial cells with relatively high efficiency and thus use the potential of its metabolism. The maximum of bioaccumulation potential was found in *A. candidus* for Zn(II), the efficiency of bioaccumulation in this case was up to 92.52 %. Thus, this strain showed a high degree of adaptation to the presence of Zn(II) ions in the environment, although a significant inhibitory effect of these ions on mycelial growth was demonstrated. Interesting results were also obtained for the *A. clavatus* strain, where the efficiency of copper bioaccumulation was 33.35 % and the efficiency of zinc bioaccumulation was 30.84 %. *A. westerdijkiae* accumulated Zn(II) the best with an efficiency of 47.66 %. Ni(II) the best accumulated the *A. iizukae*, which, however, was not very successful in the bioaccumulation of other metals. It remains interesting that Ni(II) accumulated the best, which was accumulated overall by all *Aspergillus* species with the lowest efficiency. The average bioaccumulation capacity of selected toxic metals within the tested strains decreases in the following order: *A. ochraceus* > *A. candidus* > *A. clavatus* > *A. westerdijkiae* > *A. iizukae* > *A. niger*. *A. ochraceus* accumulated metals with the highest efficiency, *A. candidus*, which showed the highest content of metal ions in 1 g of dry biomass, was placed behind it.
4. Discussion

Non-essential toxic metals can pass through the cell wall using transporters primarily responsible for obtaining cellular nutrients. As a micronutrient, Zn is bioaccumulated in higher concentrations compared to toxic metals such as As and Cd, but its high concentrations initiate the blockade of its absorption into the cell [24]. These findings are consistent with our experiments, where Zn(II) ions were accumulated with the highest efficiency. Many studies have been performed showing that Ni in the form of Ni(II) is able to interact with cellular components such as nucleotides, organic acids, phospholipids and amino acids, thereby disrupting the biochemical and physiological cycles in living cells [25]. However, it is known that microfungal species of the genus Aspergillus isolated from areas contaminated with toxic metals are able not only to survive in the presence of Ni(II) but also to accumulate Ni in their cells in the form of nickel oxalate crystals which can be incorporated into the fibril matrix along cell walls and to a lesser extent also in the cell wall and on its inner wall. Active cellular metabolism, in this case oxalate formation, plays a major role in the removal of Ni(II) from liquid media, with minimal adsorption to non-growing microfungal biomass [26]. The formation of oxalate complexes also plays a role during the detoxification of Zn(II) and Cu(II) [27]. The enzyme oxaloacetate hydrolase, which is also produced by A. niger, is responsible for the formation of oxalates. The gene oah, which encodes the production of this enzyme, is not expressed in an environment with pH < 4.0. That fact proves how important role the pH of the environment plays in bioaccumulation processes [28]. In the study [29], the microscopic filamentous fungi A. niger and A. flavus also showed the lowest ability to accumulate Ni (25.20 %), which is more than in our case the best Ni accumulating A. iizuka (14.26 %), A. niger accumulated Ni(II) with a success rate of only 5.89 %. These differences are due to the fact that indigenous species of microscopic filamentous fungi tend to be isolated from areas that are not contaminated with only one, but more species of toxic metals. Uneven contamination in these localities causes resistance mechanisms to be influenced either by the predominant metal or by the synergistic action of different metals. For this reason, fungi of the same species isolated from different habitats do not show the same tolerance to toxic metal. The study [15] suggests the existence of different types of tolerance strategies or resistance mechanisms to toxic metals in three isolates of the genus Aspergillus. In this case, all three isolates grew better in the presence of Cu(II) than Ni(II), as did the strains we studied. In the case of Cu(II), intracellular detoxification dependent on the sequestration of metal ions in the cytosol by metal-binding molecules predominates. These molecules include metallothioneins, which are low molecular weight cysteine-rich proteins that are able to bind, for example, Zn, Cu and Cd ions [22, 30].

5. Conclusions

The aim of this study was to compare indigenous species of microscopic filamentous fungi of the genus Aspergillus isolated from the contaminated site of the Ostramo Lagoons depending on their ability to bioaccumulate toxic metal ions: Cu(II), Zn(II), Ni(II) and Cr(III). It was confirmed that the ability to bioaccumulate toxic metals depends on the type of organism and the type of metal. The data obtained certainly deserves closer examination. The highest bioaccumulation potential was achieved by A. candidus bioaccumulating Zn(II), but on average the best was A. ochraceus. Interesting results were obtained by A. niger, which grew the best in the presence of toxic metals, also but showed the lowest bioaccumulation potential.

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