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ORIGINAL ARTICLE

Bioaccumulation and biosorption of inorganic nanoparticles: factors affecting the efficiency of nanoparticle mycoextraction by liquid-grown mycelia of *Pleurotus eryngii* and *Trametes versicolor*

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Abstract Nanoparticles (NPs) could reach the food chain from diverse wastes containing these potentially toxic substances. We studied the mycoextraction of alumina (Al_2O_3) NPs by mycelia of edible fungi: *Pleurotus eryngii* and *Trametes versicolor*. Mycelia were cultivated in liquid medium supplemented with alumina nanoparticles (concentrations 0.001–0.1 mol L^{-1}) to investigate accumulation of metal in the mycelium. The accumulation of Al in the mycelium depended on the duration of exposure, biomass of the mycelium and concentration of NPs. The efficiency of alumina-NP removal from the medium depended only on the duration of exposure and the fungal biomass, but not on NP concentration. Live hyphae of *P. eryngii* were more efficient in the removal of the NPs (~86 % of total amount of NPs removed from medium) than *T. versicolor* (61 %). Dead mycelium of *P. eryngii* was less efficient (51 %), but also useful in the mycoextraction. These results were confirmed by scanning and transmission electron microscopy and laser ablation inductively coupled plasma mass spectrometry. Additionally, it was found that the mycoextraction efficiency by *P. eryngii* depended on NP type and was lower for NPs other than alumina: platinum – 58 % and cobalt – 13 %.

Keywords Alumina nanoparticles · Platinum nanoparticles · Cobalt nanoparticles · Bioaccumulation · Biosorption · Mycoextraction · Bioremediation · *Pleurotus eryngii* · *Trametes versicolor*

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Introduction

Mycoextraction is a process in which fungi are used for the removal of contaminants from soil or water. The main mechanisms of accumulation of toxic substances by organisms are biosorption and bioaccumulation. Biosorption is a metabolically passive process, in which pollutants are bound to the surface of cell wall. Bioaccumulation is a metabolically active process, based on incorporation of compounds or ions inside living biomass (Chojnacka 2010; Velásquez and Dussan 2009).

A high accumulation level is crucial for efficient bioextraction of pollutants from the environment and its bioremediation. Bioremediation is the application of biological systems to the cleanup of organic and inorganic pollution, with bacteria and fungi being the most important organisms for reclamation, immobilization or detoxification of metallic and radioactive pollutants (Gadd 2010). Certain fungi, in particular "white fungi", can inactivate insecticides, herbicides, heavy fuels, and diverse toxic elements (Pointing 2001; Christian et al. 2005). It was documented that macrofungi are effective accumulators of silver (Borovicka et al. 2010). Among others, fungi have been shown to biomineralize uranium oxides, suggesting that they may have application in the bioremediation of radioactively polluted sites (Fomina et al. 2007, 2008). One of such fungi is *Pleurotus eryngii*, analyzed in our laboratory, which accumulates cesium in the fruitbody (Bystrzejewska-Piotrowska et al. 2008; Bazała et al. 2005, 2008) and in biotechnologically cultivated mycelium. The ubiquity and importance of fungi in environmental processes make mycoextraction one of the most promising strategies of bioremediation. On the other hand, the ability of fungi to accumulate toxins to high concentration makes them potentially dangerous for consumers.

Recently, new potentially toxic products in the form of manufactured nanoparticles have appeared on the market. Nanotechnology is one of the most rapidly developing areas of technology, dealing with nanoparticles (NPs)—particles in which at least one of the dimensions does not exceed 100 nm. Such particles exhibit novel physical, chemical and biological properties, different from their larger counterparts and resulting from their high ratio of surface area to volume or mass (Aitken et al. 2004). NPs are more reactive than conventional micron-sized particles (Rai et al. 2006). Owing to rapidly developing nanotechnologies, the worldwide market for nanomaterials is estimated to reach 100 billion dollars per annum for 2011–2014 (Podila and Brown 2013), and the potential impact of these new materials on the environment requires careful consideration. The increasing implementation of nanotechnologies brings with it the risk of creating a new generation of waste (nanowaste) and new potential threats to the environment (as reviewed by Bystrzejewska-Piotrowska et al. 2009). Production, use, and waste-disposal of NPs may lead to their release into the environment, so there is a growing need for the investigation of the accumulation and toxicity of NPs and means of their efficient removal or deactivation.

Aluminum oxide (Al_2O_3) nanoparticles (alumina NPs) are among the most important nanomaterials and are widely used in diverse areas. Alumina is estimated to account for approximately 20 % of the 2005 world market of nanoparticles (Rittner 2002). Alumina was listed as a high-priority group by the Organization for Economic Cooperation and Development (OECD) Steering Group for Test Guidelines (Park et al. 2010). Alumina NPs are widely used in catalysts, fine ceramics, paint, waterproofing, polishing, and fluorescent materials, and as a cosmetic UV filter; therefore, they can be found in many commercial products (Stenger et al. 2005; Huang et al. 2007; Schmid and Riediker 2008). Consequently, an increasing accumulation of Al_2O_3 NPs in the environment can be expected.

Few studies have been conducted to investigate the toxicity of Al_2O_3-NPs. Aluminum is relatively stable in the form of alumina (aluminum oxide) and can enter the body through drinking water, food intake, inhalation and skin contact (Yang et al. 2012). Manufactured alumina NPs have been found to decrease the expression of tight junction proteins in brain vasculature (Chen et al. 2008). The effect of alumina nanoparticles (the growth inhibition and decrease in chlorophyll content) on microalgae *Scenedesmus* sp. and *Chlorella* sp. was demonstrated (Sadiq et al. 2011). Additionally, the temporal changes in physico-chemical behavior of Al_2O_3 NPs in the environment were found (Pakrashi et al. 2012).

Efficient removal of NPs from a contaminated environment should prevent their ecotoxicity (as reviewed by Oberdorster et al. 2005). There is a lack of information about the accumulation of Al_2O_3-NPs in fungi. Recently, Bakircioglu et al. (2010) demonstrated that filamentous fungal biomass-loaded TiO_2 NPs could be used for the biosorption of lead (II) ions. Based on their results, establishing conditions for optimal recovery of nNPs from the environment seems worthwhile.

The aim of this study was the examination of:

i. applicability of biotechnological method of culturing of *Pleurotus eryngii* and *Trametes versicolor* mycelium;
ii. factors affecting the efficiency of Al_2O_3 NP mycoextraction by live and dead fungal biomass;
iii. comparison of mycoextraction efficiency of alumina and other NPs (platinum [Pt], cobalt [Co]).

Materials and methods

Cultivation of *Pleurotus eryngii* and *Trametes versicolor*

The king oyster mushroom *P. eryngii* (DC.) Quel 1872 was isolated from a fruitbody growing on the roots of *Eryngium campestre* L., a plant found in the locality of Camarma de Esteruelas (3°22′45″W, 40°33′15″N, 30T 4684489 UTM; Madrid Province, Spain) (Bystrzejewska-Piotrowska et al. 2008). The mushroom *T. versicolor* (L.) Lloyd 1920 (cat. no. CCBAS612) was obtained from the Institute of Microbiology, Academy of Sciences of the Czech Republic. Procedures for cultivation of *Pleurotus eryngii* under laboratory conditions have been described (Manjon et al. 2004; Bystrzejewska-Piotrowska et al. 2008; Bazała et al. 2008; Bystrzejewska-Piotrowska and Bazała 2008), but are limited to fungi with fruitbodies growing on solid medium, usually barley seeds. In this work, a method of cultivation of mycelia of *P. eryngii* and *T. versicolor* in liquid growth medium is proposed. For this purpose, PDY medium consisting of 4 g L^{-1} potato extract (Fluka, Poznan, Poland), 20 g L^{-1} dextrose (Sigma, Poznan, Poland) and 2 g L^{-1} yeast extract (Fluka, Poznan, Poland) was used. To the PDY medium, NPs of Al_2O_3 (Al_2O_3 NPs particle size < 50 nm, Sigma-Aldrich) were added, to obtain concentrations of 0.001, 0.01, 0.05 or 0.1 mol L^{-1}. In each experiment, control cultivation without NPs was performed. All growth media were autoclaved at 121 °C for 15 min and inoculated with three pieces of mycelium under sterile conditions. Each variant of the experiment was performed in at least three replicates. Mycelium was grown for 2 or 7 days on an orbital shaker (120 rpm) at 25 °C. The same method was applied for cultivation of *P. eryngii* on medium contaminated with nanoparticles of Pt and Co (Pt-NPs, particle size < 50 nm, Co-NPs, particle size < 50 nm, both from Sigma-Aldrich).

After finishing the cultivation, the mycelium was separated from the medium by filtration through a double layer of sterile gauze.

For investigation of alumina-NPs accumulation by dead mycelium, control mycelium of *P. eryngii* was frozen at −22 °C, lyophilized and again introduced into NP-contaminated growth

medium. After 7 days, mycelium was separated from the medium as above.

For total analysis of the metal content, mycelia samples were dried at 60 °C for 48 h, weighed and homogenized in a mortar.

Analysis of metal content

For determination of Al, Pt and Co content in the mycelia, about 250 mg of dried material was digested with a mixture of 2.5 mL HNO_3 and 0.5 mL $HClO_4$ using a microwave laboratory system ETHOS 1 with ATC-400-CE automatic temperature control (Milestone, Italy). After digestion samples were quantitatively transferred into volumetric flasks (25 mL) and analyzed by inductively coupled plasma mass spectrometry ICP MS (ELAN 6000 ICP mass spectrometer, PE-SCIEX, Concord, Canada). Accumulation efficiency was calculated according to the equation:

$$\text{total metal accumulated in mycelium [mg]/metal content in solution [mg]} \times 100\%$$

A thin slice of a fresh *P. eryngii* mycelium sphere (Al_2O_3 NPs concentration 0.1 mol L^{-1}) was cut and Al was determined in the cross section of the mycelium using laser ablation inductively coupled plasma mass spectrometry (LA ICP MS, ELAN 6000 ICP mass spectrometer).

Electron microscopy analysis

Mycelia cultivated with Al_2O_3 NPs at 0.1 mol L^{-1} were chosen for electron microscopy analysis. Medium–sized fresh mycelium spheres were fixed with 3 % glutaraldehyde in 0.1 mol L^{-1} cacodylate buffer (pH 7.2) for 24 h at 4 °C. Samples were rinsed five times with 0.1 mol L^{-1} cacodylate buffer, then dehydrated stepwise in an ethanol solution series of 30, 50, 70, 90, 96 and 100 %, 15 min per step. Finally, samples were dehydrated twice for 5 min in acetone. For scanning electron microscopy (SEM), samples were dried and sputter-coated with gold. Before examination in a LEO 912AB transmission electron microscope (TEM), samples dehydrated in acetone were embedded in epoxy resin, polymerized and hardened at 60 °C. Ultrathin

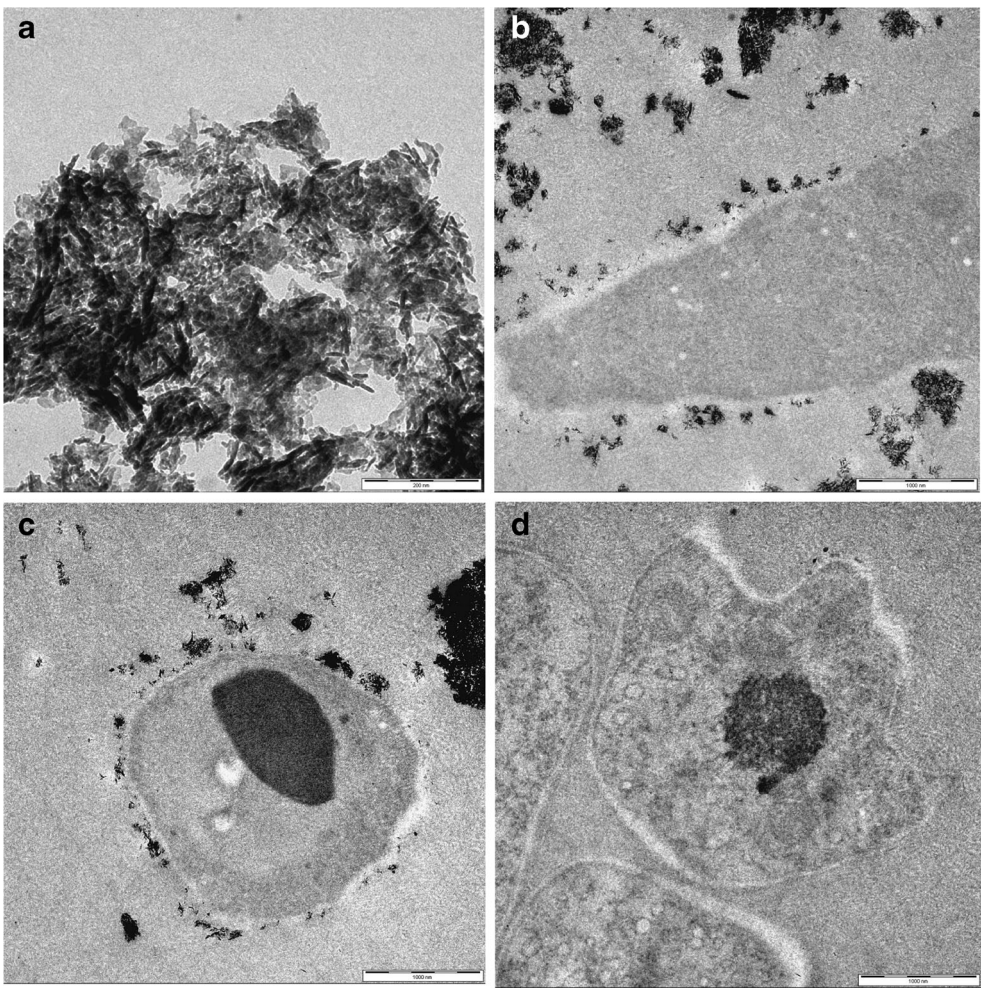

Fig. 1 TEM images of Al_2O_3-NPs in medium before autoclaving (**a**), longitudinal section of hyphae of *Pleurotus eryngii* exposed to Al_2O_3-NPs (**b**), cross section of hyphae exposed to Al_2O_3-NPs (**c**) and control (**d**). Scale bar 200 nm (**a**) and 1,000 nm (**b**–**d**)

sections (70 nm) were cut with an MTX ultramicrotome (RMC, Japan), placed on a copper grid and analyzed. Micrographs were taken with the Proscan High Speed Slow Scan CCD-camera.

For microscopic (TEM) characteristic of particles, one drop of a 0.1 mol L^{-1} solution of Al_2O_3 NPs was placed on a formvar-coated grid and dried. Different variants of Al_2O_3 solution were investigated: solution in water, in PDY medium before autoclaving and in PDY after autoclaving. In each solution, similar aggregates of NPs were observed.

Results and discussion

Microscopic analysis of alumina-NPs accumulation by *P. eryngii* mycelium

Both investigated species, *T. versicolor* and *P. eryngii*, efficiently accumulated Al_2O_3 nanoparticles. TEM images present Al_2O_3 NPs aggregates in the medium before autoclaving (Fig. 1a) and on a cross section of hyphae with NPs adsorbed on the surface (Fig. 1c) in comparison with control hyphae (Fig. 1d). Longitudinal section of the mycelium (Fig. 1b)

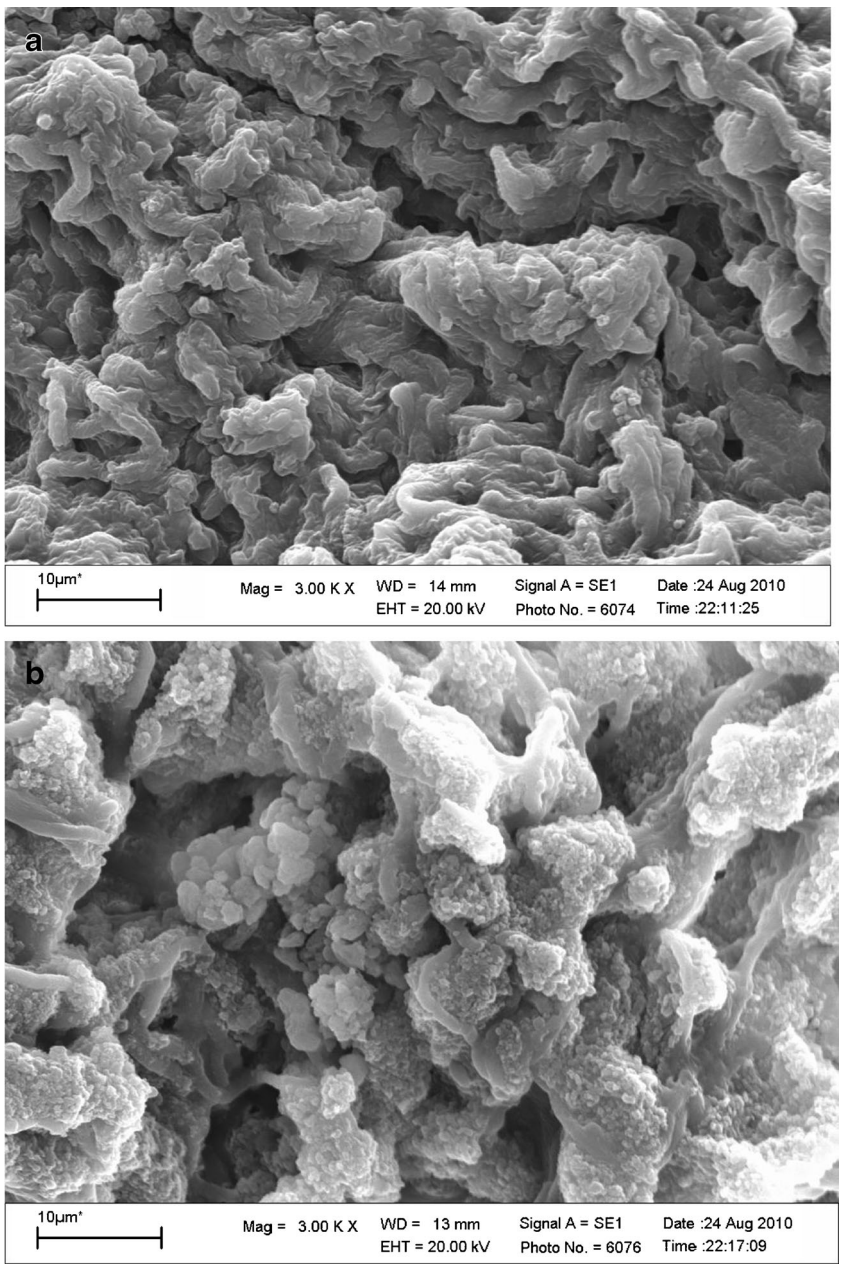

Fig. 2 SEM images of *Pleurotus eryngii* mycelium: **a** – control; **b** – exposed to Al_2O_3 NPs

Table 1 Aluminum content in live mycelium of *Pleurotus eryngii* and *Trametes versicolor* exposed for 2 or 7 days to Al_2O_3 NPs and accumulation efficiencies

Al_2O_3 NPs concentration in medium [mol L^{-1}]	*Pleurotus eryngii*				*Trametes versicolor*			
	2 days		7 days		2 days		7 days	
	Al content [mg g^{-1} d.w]	Accumulation efficiency [%]	Al content [mg g^{-1} d.w]	Accumulation efficiency [%]	Al content [mg g^{-1} d.w]	Accumulation efficiency [%]	Al content [mg g^{-1} d.w]	Accumulation efficiency [%]
0.001	35±3	39±9	16±0	75±3	16±1	17±2	9±1	83±7
0.01	134±8	33±2	121±11	71±5	89±5	11±2	61±5	71±8

Results are presented as means ± standard deviations, $n \geq 3$

confirmed the binding of NPs with the surface of cell wall. Scanning electron micrographs (SEM) show alumina NPs aggregates on *P. eryngii* hyphae walls after exposition (Fig. 2b) compared with control (Fig. 2a).

Effect of exposure time on Al_2O_3 NPs accumulation

The intensity of NP accumulation depended on the exposure time (Table 1). The concentration of Al bound by the hyphae

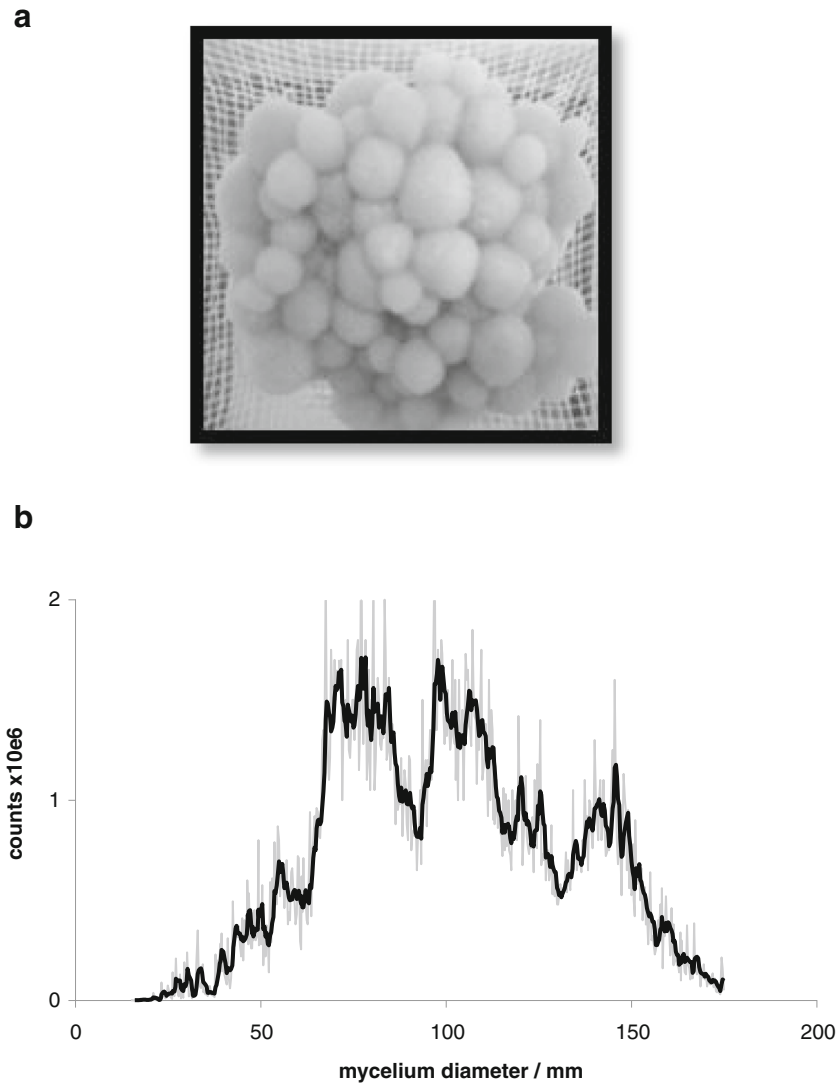

Fig. 3 Macroscopic image *Pleurotus eryngii* mycelium spheres (**a**) and LA ICP MS analysis of aluminum content in the cross section of a mycelium sphere (**b**)

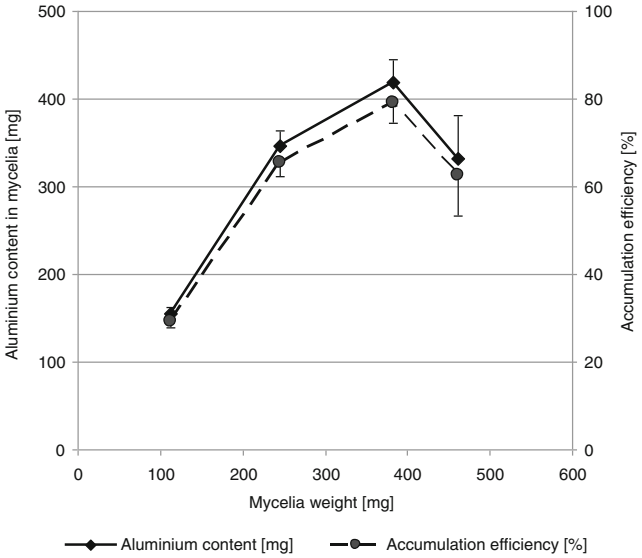

Fig. 4 Content of accumulated Al in the mycelium after exposure to alumina-nanoparticles and accumulation efficiency as a function of mycelia weight

after 2 days of cultivation was significantly higher than that determined after 7 days, both for *P. eryngii* and *T. versicolor*. An opposite tendency was noticed for accumulation efficiency. After 2 days, the efficiency was about 36 % and 14 % for *P. eryngii* and *T. versicolor*; and after 7 days, effeicieny was 73 % and 77 % respectively.

We used LA ICP MS to study the distribution of Al_2O_3 NPs in spherical colony (Fig. 3a, b). The highest concentration of Al was found in the center of the sphere formed at the beginning of mycelium growth. The Al content decreased with the increasing distance from the center, i.e., the later stages of growth. These results confirmed previously obtained data (Table 1) that the intensity of NPs accumulation is highest at the beginning of mycelium growth.

Relationship between mycelium mass and Al accumulation

The relationship between mycelium mass and Al accumulation was investigated for *P. eryngii* grown in the medium supplemented with 0.001 mol L^{-1} Al_2O_3-NPs (Fig. 4). It was found that the Al content in mycelia increased with the dry mycelia weight. The same tendency was observed for Al accumulation efficiency.

Effect of Al_2O_3-NPs concentration on Al accumulation by *P. eryngii* and *T. versicolor*

The investigations of the effect of alumina nanoparticles concentration on their accumulation by live *P.eryngii* and *T. versicolor* mycelia showed a good correlation between the NPs concentration (0.001–0.1 mol L^{-1}, exposition time 7 days) and the Al content of the mycelium (Table 2). It was found that Al content in mycelia increased with the NP concentration, while accumulation efficiencies were at the same level. Differences were observed between the two mushroom species. *P. eryngii* showed a higher Al accumulation efficiency (79–92 %) in comparison with *T. versicolor* (60–64 %). The obtained efficiencies of Al_2O_3 NPs accumulation were similar to those of Simonescu and Ferdes 2012, who evaluated fungal strains (*Aspergillus oryzae, Aspergillus niger, Fusarium oxysporum, Polyporus squamosus)* for their ability to remove copper (in the form of CuS nanoparticles) from aqueous systems. They found that

Table 2 Aluminum content in mycelium of *Pleurotus eryngii* (live or dead) and *Trametes versicolor* (live) exposed to varying concentrations of Al_2O_3 NPs and accumulation efficiencies

Al_2O_3 NPs concentration in medium [mol L^{-1}]	*Pleurotus eryngii*				*Trametes versicolor*	
	Live		Dead		Live	
	Al content [mg g^{-1} d.w.]	Accumulation efficiency [%]	Al content [mg g^{-1} d.w.]	Accumulation efficiency [%]	Al content [mg g^{-1} d.w.]	Accumulation efficiency [%]
0	0	–	–	–	0	–
0.001	15±4	84±16	–	–	10±1	64±1
0.01	89±14	92±7	–	–	73±4	61±6
0.05	243±50	90±8	235±13	57±49	261±12	60±4
0.1	266±39	79±11	307±8	45±27	350±10	61±5

Results are presented as means ± standard deviations, $n \geq 3$

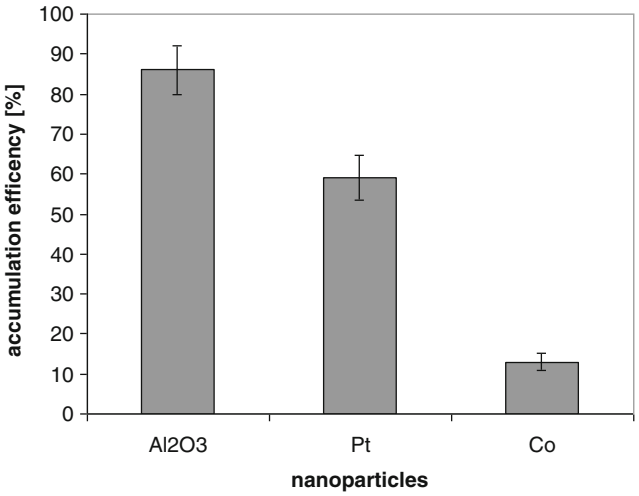

Fig. 5 Accumulation efficiency for different NPs

strains of *Aspergillus oryzae* are capable of removing up to 88 % of copper.

Accumulation of Al_2O_3 NPs by live and dead *P. eryngii* mycelium

The accumulation efficiency of Al by dead mycelium of *P. eryngii* was within the range of 45–57 %, so somewhat lower than by the live one (on average 85 %). This indicates a more effective interaction of NPs with live mycelia compared to biologically nonactive hyphae. An ability of dead fungal biomass to adsorb zinc was shown by Faryal et al. (2006). Alkali-extracted *A. niger* mycelium biomass (biosorbant) was effective in sequestration of metal ions, especially Zn and Cd, present at very low concentrations in lake waters (Akhtar and Mohan 1995). The lack of an effect of the NPs concentration on their accumulation efficiency seems to be due to a dynamic equilibrium between the NPs dispersed in medium and those adsorbed by the mycelium. Accumulation of particles by the mycelium can involve two modes—surface binding of NPs to the cell wall and uptake into the cell across the cell membrane (Bishnoi and Garima 2005). The first mode takes place in the case of both live and dead cells, while the second, which is energy-dependent, occurs only in live cells. As shown by our results, biosorption is undoubtedly the dominant mechanism of alumina NP accumulation by *P. eryngii*.

Ability of *P. eryngii* mycelia to mycoextract different nanoparticles

The ability of *P. eryngii* mycelia to sequester Pt and Co NPs was investigated. The mycoextraction efficiency depended on the NP type (Fig. 5), and was the highest for Al_2O_3 NPs (86 %), lower for Pt (58 %) and the lowest for Co (13 %).

Summary

Our results have shown that the efficiency of NP accumulation depends on the duration of exposure, biomass of the mycelium, NP type and species of fungi. It was higher for live mycelia in comparison with dead ones. The accumulation efficiency was not related to NP concentration.

Fungi are a promising alternative for bioremediation of heavy metal-contaminated aqueous environments and, according to the above results, can also be successfully applied for mycoextraction of nanoparticles. The obtained results document an excellent ability of biotechnological cultures of mycelium of *P. eryngii* and *T. versicolor* to efficiently remove alumina NPs from a contaminated environment which may prevent their toxicity. Additionally, the NP-loaded biomass can be separated and the biosorbed particles can potentially be reclaimed. Recovery of biosorbed particles should be investigated in future studies. Results will answer for the question of nanoparticles stability. Another possibility is that the biomass of the fungus can be used as an alumina-loaded biosorbant for absorption of the other pollutants. This should be a subject of further studies. To the best of our knowledge, this is the first paper on the use of fungi in bioremediation of metallic nanoparticle wastes, presenting a novel convenient cultivation method addressing the yield and mechanisms of Al_2O_3 NPs sequestration. These data can be built upon when optimizing conditions for large scale bioremediation or when dealing with other nanoparticle contaminants.

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