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Tolerance to silver of an *Aspergillus fumigatus* strain able to grow on cyanide containing wastes

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HIGHLIGHTS

- *Aspergillus fumigatus* strain able to grow on metal cyanide complexes.
- Tolerance test revealed that Ag(I) Minimum Inhibitory Concentration was 6 mM.
- The fungus reduced and sequestrated intracellularly silver forming nanoparticles.
- Best culture conditions for Ag(I) absorption were pH 8.5 at temperatures of 20–30 °C.

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ABSTRACT

We studied the strategy of an *Aspergillus fumigatus* strain able to grow on metal cyanide wastes to cope with silver. The tolerance test revealed that the Minimum Inhibitory Concentration of Ag(I) was 6 mM. In 1 mM $AgNO_3$ aqueous solution the fungus was able to reduce and sequestrate silver into the cell in the form of nanoparticles as evidenced by the change in color of the biomass and Electron Microscopy observations. Extracellular silver nanoparticle production also occurred in the filtrate solution after previous incubation of the fungus in sterile, double-distilled water for 72 h, therefore evidencing that culture conditions may influence nanoparticle formation. The nanoparticles were characterized by UV–vis spectrometry, X-ray diffraction and Energy Dispersion X-ray analysis. Atomic absorption spectrometry revealed that the optimum culture conditions for silver absorption were at pH 8.5.The research is part of a polyphasic study concerning the behavior of the fungal strain in presence of metal cyanides; the results provide better understanding for further research targeted at a rationale use of the microorganism in bioremediation plans, also in view of possible metal recovery. Studies will be performed to verify if the fungus maintains its ability to produce nanoparticles using $KAg(CN)_2$.

1. Introduction

In the jewelry industry, gold cyanide solutions are employed together with other metal ions in gold alloy electroforming. After gold recovery, these toxic solutions are often fixed with other exhaust cyanide containing solutions employed in gold surface treatments. As a consequence, the wastes are rich in cyanide which can be present as free cyanide and cyanide complexes of metals including copper, silver, nickel, zinc, aluminium, etc. Being cyanide a potent poison for humans and animals, wastewaters containing high concentrations of free cyanide and metal cyanide complexes must be released into the environment only after detoxification. To this regard, biotechnological based treatments are becoming feasible alternatives to chemical and physical methods in terms of cost and environmental impact. Actually, several authors refer the isolation of a number of bacteria and fungi able to detoxify cyanide containing wastewaters both aerobically and anaerobically [1–6]. However, when growing in media rich in cyanide and metal cyanide complexes, microorganisms must cope with the toxicity of these compounds. Metal-cyanide complexes are in general much

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less toxic than free cyanide, however their dissociation releases free cyanide and metal cations which can be toxic and represent an additional stress for microorganisms [7]. Some of these metals, such silver, cadmium, and lead, have no biological role and are potentially toxic to the microorganisms even at very low concentrations. For example, the minimal inhibitory concentration of Ag(I) in *Escherichia coli* is 0.02 mM [8]. In addition, essential heavy metals such zinc, cobalt, copper, nickel, etc., which are essential micronutrients, at high levels can also be toxic to microorganisms by forming complex compounds within the cell. Several mechanisms are involved in heavy metal toxicity including damage of the cell membrane, alteration of enzyme specificity, disruption of cellular functions, alteration in the nucleic acids and protein structure, interference with oxidative phosphorylation and osmotic balance [9,10]. In particular, the cytotoxicity of Ag(I) results from the interaction with the nucleic acids and proteins by binding to thiol, carboxyl, amino, imidazole and phosphate groups, therefore leading to the inactivation of their biological functions [8,11].

Nevertheless, it is known that the exposure of the microorganisms to harsh conditions, including potentially toxic metal concentrations, may induce stress adaptive responses that allow them to survive. Several mechanisms are involved in the resistance of fungi and bacteria to metals including enzymatic detoxification, exclusion by permeability barrier, alteration of the cellular targets, efflux pumps, accumulation by active (transport systems) and/or passive (diffusion) intracellular uptake and adsorption to the outer cellular structures (cell wall, capsule, slime), and extracellular precipitation [9,11–19]. For many metals, resistance and homeostasis involve a combination of two or three of the basic mechanisms mentioned [8].

In a previous work, the capability of an Aspergillus fumigatus strain isolated from the soil to grow on minimal medium to which a metal-cyanide containing waste had been added, was demonstrated for the first time [20]. The strain was able to utilize cyanide as source of nitrogen and concomitantly sequestrate metals. Regarding the behavior of the fungus against silver, literature data refer that resistance mechanisms differ among microbial species and may be influenced by environmental conditions [15,21,22]. Therefore, in order to better clarify the resistance mechanisms adopted by the fungus against silver, we carried out a study aimed to evaluate the behavior of the fungal strain when subjected to high concentrations of the metal. Actually, several authors report data about silver, but the studies mostly address the recovery of the precious metal and nanoparticle production [11–20,23–25] while scarce information exists in the perspective of metal-cyanide containing waste remediation [26]. This research is part of a polyphasic study aimed at an exhaustive comprehension of the survival strategies adopted by the fungal strain when growing in the above mentioned wastes. The results will provide a better understanding of the detoxification processes of toxic metals and lay the basis for further research for a rationale use of the microorganism in bioremediation plans also in view of possible metal recovery.

2. Materials and methods

The *A. fumigatus* strain, isolated and characterized as previously described [20], was maintained on Potato Dextrose Agar (PDA) (Liofilchem, Roseto degli Abruzzi, Italy) slants at 4 °C.

When the fungus was inoculated in an artificial waste containing 12,000 ppm Ag(I), 1200 ppm Cu(II), and 553 ppm Ni(II) in 40 g/L KCN in aerobic conditions it showed a decrease in the cyanide under detection limits within 24 h. The fungal growth was confirmed by the increase in the dry weight (from 0.48 mg at time 0 to 0.96 mg at day 15), and the protein content (form 1.80 μg/mg dry weight at time 0 to 25.10 μg/mg dry weight at day 15). At the same time, ICP Emission Spectrometry revealed the absorption of metals such as Ag, Cu and Ni by the fungal cells [20].

2.1. Silver tolerance

The PDA plates were supplemented with $AgNO_3$ at concentrations ranging from 2 to 10 mM of Ag(I) and inoculated with 8 mm agar plugs from 7-day-old fungal colonies. Control was performed using the medium without metal. The plates were then incubated at 28 °C for at least 7 days. The Minimal Inhibitory Concentration (MIC) was defined as the lowest metal concentration that inhibited the visible growth of the fungus.

2.2. Preparation of cultures and reaction mixtures

The fungal strain was previously grown in liquid medium containing (g/L): KH_2PO_4 7.0; K_2HPO_4 2.0; $MgSO_4{\cdot}7H_2O$ 0.1; yeast extract 0.6; glucose 10.0, with incubation at 28 °C for 72 h in a rotary shaker (150 rpm). The fungal biomass was separated by filtering through Whatman filter paper No. 1 and then washed thrice with sterile distilled water in order to remove any medium traces. After, two experiments were carried out. In the first one, 10 g of wet biomass was inoculated in 250 mL Erlenmeyer flasks containing 100 mL of an aqueous solution of 1 mM $AgNO_3$ with incubation at 28 °C for 72 h in agitation (150 rpm) in the dark (biomass containing solution). A control was performed inoculating the biomass in water without silver. In the second experiment, 10 g wet biomass was placed in 250 mL Erlenmeyer flasks containing 100 mL sterile double-distilled water and incubated at 28 °C for 72 h. Next, the fungal biomass was removed by filtration and the filtrate was mixed with 100 mL of 1 mM $AgNO_3$ solution (biomass filtrate) to evaluate extracellular silver nanoparticle production. The Ag(I) concentration was used according to the literature data [11,15,21].

2.3. Silver uptake by fungal biomass-influence of temperature and pH

The presence of silver both in the solution and within fungal biomass was detected by atomic absorption spectrometry. Solution samples were filtered using membrane filters of cellulose acetate with pore size 0.45 μm. Fungal biomass samples were dried at 105 °C until constant weight and were mineralized (MDS 2000CEM, Italy) with concentrated HNO_3 (10 mL).

All chemicals used in sample treatment were suprapure grade (HNO_3 65% Merck Suprapur, Darmstadt, Germany). Ultrapure water (Milli-Q System, Millipore Corporation, USA) was used for all solutions. All glassware was cleaned prior to use by soaking in 10% v/v HNO_3 for 24 h and rinsed with Milli-Q water. Silver concentrations were measured using PerkinElmer AAnalyst 300 atomic absorption spectrophotometer with flame atomization (FAAS). The standard solutions of metals were prepared from stock standard solutions of ultrapure grade, AA Certipur® 1000 mg/L, Merck. All samples and standard solutions were stabilized by the addition of a 5% (w/v) CH_3COOH solution (BHD AnalaR, England). Only median values of metal concentrations obtained by two replicates of the same sample with standard deviation percentage <10% were accepted.

To evaluate the influence of temperature and pH, the experiments were performed at temperatures ranging from 5 to 40 °C, each assayed at pH ranging from 4.5 to 8.5.

2.3.1. Statistical analysis

To evaluate statistically significant differences in the influence of temperature and pH on silver uptake by *A. fumigatus*, the Kruskal-

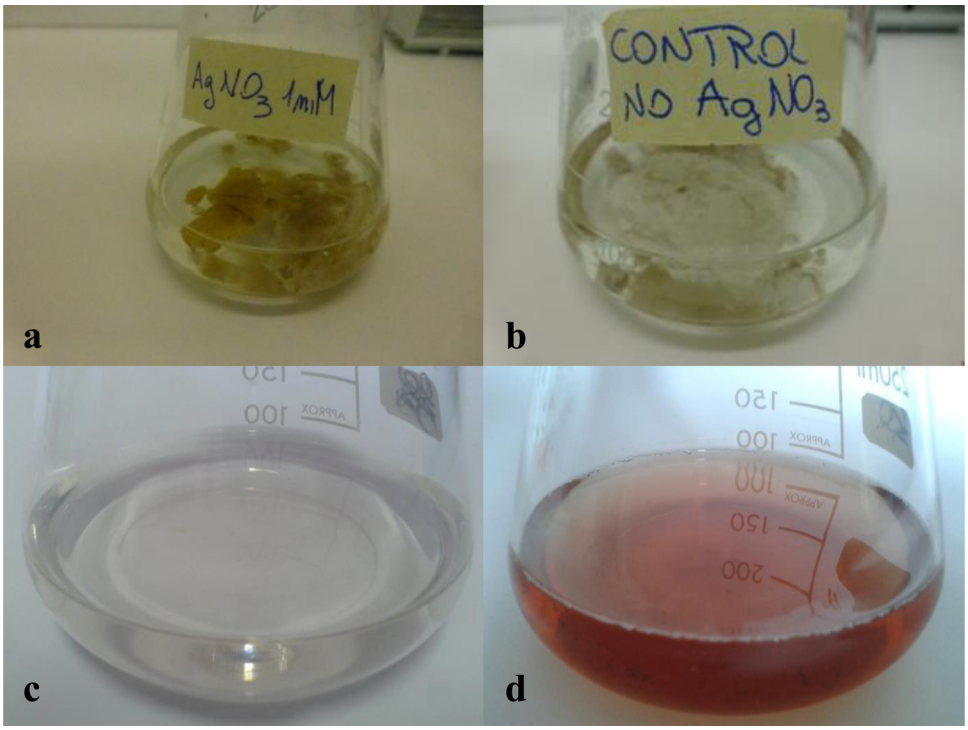

Fig. 1. Conical flasks containing: *Aspergillus fumigatus* biomass after 72 h reaction with 1mM AgNO3 (a); *A. fumigatus* biomass without silver (control) (b); biomass filtrate at the beginning of the reaction with 1 mM AgNO3 (c); biomass filtrate after 72 h reaction (d).

Wallis test (alpha = 0.05) was used. The statistical analysis was performed using the statistical software XLSTAT 2009.

2.4. UV–vis spectroscopic analysis

The silver transformation was monitored both in the biomass containing solution (100 mL) and in the biomass filtrate (100 mL) by visual inspection and measurement of the UV–vis spectra from the solutions. Sample of 1 mL were withdrawn at 2, 24, 48 and 72 h and the absorbance was measured using a Varian Cary 100 Scan UV–vis Spectrophotometer with quartz cuvettes and an optical path length of 1 cm. The absorption spectrum was registered at a resolution of 1 nm in the 200–800 nm wavelength range. An absorption band in the range of about 380–440 nm is attributed to the presence of silver nanoparticles [25].

2.5. X-ray diffraction measurements (XRD)

To determine the crystal shape of silver nanoparticles, samples of the biomass filtrate were previously subjected to separation and concentration to remove the protein fraction. Then the samples, dried at 45 °C in a vacuum drying oven, were coated as a thin film on glass slides and tested using Philips diffractometer operating at a voltage of 35 kV and current of 30 mA with CuKα radiation scanning from 30° to 80° 2 theta. The peaks match with JCPDF card no. 087-0719.

2.6. Sampling for electron microscopy observation

To evaluate the localization of silver in the fungal cell at time intervals of 2, 24 and 72 h incubation, specimens of fungal biomass, inoculated in the $AgNO_3$ solution, were processed for Transmission Electron Microscopy (TEM) and Scansion Electron Microscopy (SEM). Controls, at the beginning and at the end of the trials (72 h), were also processed. Briefly, control and treated samples were washed, and immediately fixed with 2.5% glutaraldehyde in 0.15 M phosphate buffer (pH 7.5) for 1 h at room temperature [27].

2.6.1. TEM

After gentle wash in phosphate buffer, a post-fixation was performed for 1 h in 1% OsO_4 in the same buffer and then fragmented into pieces of a few millimeters. Alcohol dehydration and araldite embedding were performed, and thin sections, collected on 400 mesh nickel grids, were stained with uranyl acetate and lead citrate. The observations were carried out with a Philips CM 10 electron microscope at 80 kV [27].

TEM was also used to visualize the silver nanoparticles formed in the aqueous solution. To this purpose, a drop of the sample was placed on the carbon coated grid of the microscope.

2.6.2. SEM

Samples, after fixation in glutaraldehyde, were dehydrated in ethanol gradients and subjected to critical point drying with CO_2. The samples were then attached to aluminium stubs and coated with a carbon film (40 nm) Emitech K250 Sputter Coater. A Zeiss SUPRA40, field emission scanning electron microscope equipped with a Bruker Quantax Z200 Energy Dispersive X-Ray (EDX) microanalysis, was used to analyze both the surface microstructure and the mean composition of the crystals. SEM observations were carried out by using both secondary electrons (SE) to evidence surface topography, and backscattered electrons (BSE) to observe compositional variations eventually present in the sample.

All the experiments were carried out in triplicate for each culture and control and the results were expressed as the mean of the values.

3. Results

The silver tolerance test revealed that at 2 mM Ag(I) the fungal strain was very resistant and exhibited a growth similar to the control. Even though at a reduced rate, growth was still observed in

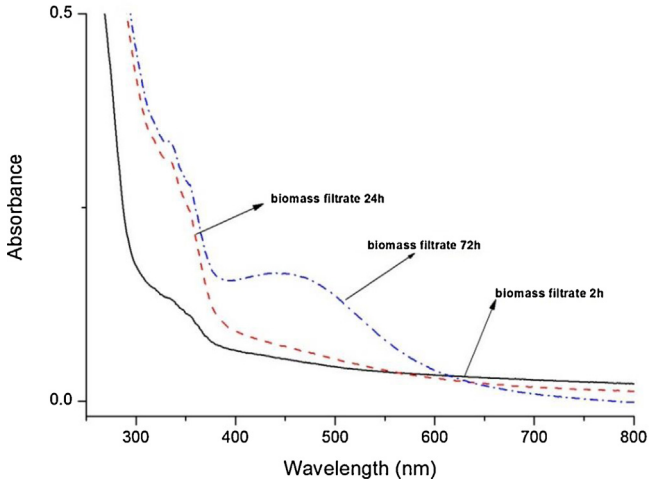

Fig. 2. UV–vis spectrarecordedfrom biomassfiltrate with 1 mM AgNO3 at different incubation times.

presence of up to 5 mM; instead, the concentration of 6 mM was completely inhibitory.

The flasks containing the fungal biomass and the filtrate were initially subjected to visual inspection. In the case of the samples containing biomass, the mycelium turned to brown within 72 h after 1 mM $AgNO_3$ addition, while the solution did not show any change in color (Fig. 1a). On the contrary, in absence of silver, the fungal biomass maintained the pale yellow color during the incubation time (Fig. 1b). In the second experiment, the biomass filtrate showed change in color to brown after exposure to 1 mM $AgNO_3$ for 72 h (Fig. 1c,d). The brown color indicates the formation of colloidal silver in solution and results from the excitation of surface plasmon vibrations in the nanoparticles. These results were confirmed by UV–vis spectroscopy; in fact, as reported in Fig. 2 the biomass filtrate showed an absorption curve at around 430 nm after 72 h of contact with Ag(I); on the contrary in the fungal biomass containing solution, the UV–vis spectra did not reveal the presence of silver nanoparticles (data not shown).

The silver sorption ability of the cell mass was determined by estimating residual metal concentration in the fungal biomass containing solution. Table 1 shows silver concentrations detected in *A. fumigatus* (μg/g d.w.) and in the solution (μg/mL) at different pH and temperatures, and percentage of silver absorption [(silver initial concentration-silver culture medium) × 100]/silver initial concentration]. At 5 °C, silver in fungal biomass ranged between 17195.7 and 51271.6 μg/g d.w. with a maximum percentage of absorption (87%) at pH 8.5. At 20 °C, silver concentrations ranged between 14921.0 and 59237.2 μg/g d.w. and the percentage of metal removal (97%) was the same at alkaline pH. At 30 °C and 40 °C, the concentrations were 15866.5–51811.9 and 18327.3–39357.6 μg/g d.w., respectively. As at 5 °C, also in these cases, the highest removal percentages were found at pH 8.5. Fig. 3 shows that the uptake by the fungus corresponded to a decrease in the silver concentration in the solution and that above pH 5.5, metal uptake increased rapidly at all temperatures.

The transmission electron micrographs of fungal samples collected at different time intervals evidenced a different localization of silver nanoparticles during the incubation. As shown in Fig. 4a, a representative TEM image recorded from the mycelium without Ag(I) revealed the absence of electron dense particles on the fungal cells; conversely, after two hours of contact with the metal the presence of nanoparticles was observed on the cellular surface (Fig. 4b), while in the following period (72 h) particles were found both on the cellular surface and within the cytoplasm (Fig. 4c). TEM analysis of the biomass filtrate revealed the presence of electrondense particles in solution (Fig. 4d) which were confirmed as elemental Ag(0) using XRD (Fig. 5). The results regarding the silver uptake by fungal cells were confirmed by SEM. In fact, as shown in Fig. 6a, nanoparticles were densely distributed on the hyphal surface after 2 h of exposure to Ag(I) and apparently decreased in the subsequent period (Fig. 6c). The presence of silver atoms in the fungal biomass was confirmed by EDX; in fact Fig. 6b, d shows spectra recorded in the spot-profile mode from regions of the mycelia at different time contacts; signals from silver atoms in the nanoparticles increased according to the contact time of the mycelium with the Ag(I) aqueous solution an these results were confirmed by the semiquantitative analysis (data not shown). Conversely, the control SEM micrograph and EDX spectrum did not reveal any presence of silver on the fungal biomass (Fig. 6e,f).

TEM micrographs of the fungal cells and the biomass filtrate both revealed that most of the silver nanoparticles were almost spherical and in the range of about 3–80 nm in size. Several agglomerated nanoparticles were also observed both intracellularly and in solution.

4. Discussion

The release of high concentrations of metal cyanide complexes by industrial activities causes environmental pollution coupled with serious public health problems. Therefore, it is necessary to develop innovative processes able to remove these hazardous substances from industrial wastes without causing further ecological damage. Biotechnological based treatments are becoming feasible alternatives to chemical and physical methods in terms of costs and environmental impact. Microorganisms such as bacteria, fungi, yeasts and algae are known to degrade cyanides and tolerate relatively high concentrations of heavy metals. Fungi are good candidates for these purposes because of their metabolic versatility and their superior capacity to adapt to severe environmental constrains.

In this study, we investigated the ability of a strain of *A. fumigatus* to cope with silver at concentrations that may be found in metal cyanide containing wastes. Initially, the silver tolerance test revealed fungal growth in presence of metal concentrations from 2 to 5 mM, although with a gradual decrease concomitantly with the concentration increase. Our results are comparable to those of Salunke et al. [22] who referred growth of a strain of *Cochliobolus lunatus* in presence of silver concentrations up to 635 ppm, but our results are very high if compared with the MICs observed by other authors for fungal strains of medical concern. Thus, Xu et al. [28] reported values of MIC_{90}s for silver nitrate of 2 μg/mL and 1 μg/mL for ocular *Fusarium* spp. and *Aspergillus* spp., respectively; *Aspergillus* strains included ten isolates of *A. fumigatus*.

Taking into account that the process is not yet completely understood, detoxification mainly relies on the reduction of silver ions to non-toxic metallic solid nanoparticles, which can take place both intra and extracellularly in bacteria and fungi according to microbial species and culture conditions being dependent upon enzymes and other cellular components [29] Several bacteria including *Pseudomonas antarctica*, *Pseudomonas proteolytica*, *Pseudomonas meridiana*, *Arthrobacter kerguelensis*, *Arthrobacter gangotriensis*, *Bacillus indicus*, *Bacillus cecembensis* [30], *Bacillus megaterium* [31], and *Bacillus strain* CS 11 [32] have been proven to produce silver nanoparticles extracellularly; on the other hand, intracellular production has been referred in species such as *Pseudomonas stutzeri* AG259 [33], *Idiomarina* sp. PR58-8 [34], *Bacillus licheniformis* [35], and *Lactobacillus* sp. [36].

Regarding fungi, most reports in literature refer the reduction of silver ions as an extracellular process with formation of nanoparticles in solution using *Alternaria alternata* [37], *Aspergillus niger*

Table 1
Silver absorption by fungal biomass at different values of pH and temperature.

Silver concentrations in *A. fumigatus* (μg/g d.w.) and in solution (μg/mL) (mean ± SD)			Silver absorption by fungal biomass from the solution
Sample	*Aspergillus* pellets	Culture medium	%
5 °C pH 4.5	17195.7 ± 86.4	70.68 ± 0.45	28
5 °C pH 5.5	18600.3 ± 14.7	68.84 ± 0.74	30
5 °C pH 6.5	32318.5 ± 190.2	42.23 ± 0.33	57
5 °C pH 7.5[a]	50579.4 ± 347.8	13.71 ± 1.06	86
5 °C pH 8.5[a]	51271.6 ± 408.5	12.73 ± 0.04	87
20 °C pH 4.5	14921.0 ± 142.6	71.09 ± 0.13	28
20 °C pH 5.5	19646.5 ± 127.1	42.78 ± 3.59	57
20 °C pH 6.5	39943.6 ± 92.1	16.95 ± 1.40	83
20 °C pH 7.5[a]	45542.8 ± 23.3	2.95 ± 0.03	97
20 °C pH 8.5[a]	59237.2 ± 37.7	3.02 ± 0.03	97
30 °C pH 4.5	15866.5 ± 72.4	61.51 ± 3.13	38
30 °C pH 5.5	15132.1 ± 66.1	58.75 ± 1.99	40
30 °C pH 6.5	22556.0 ± 15.8	52.80 ± 1.44	46
30 °C pH 7.5[a]	38428.7 ± 26.3	12.96 ± 0.10	87
30 °C pH 8.5[a]	51811.9 ± 32.4	4.79 ± 0.01	95
40 °C pH 4.5	18327.3 ± 99.1	51.77 ± 3.10	47
40 °C pH 5.5	18453.9 ± 125.2	50.18 ± 0.03	49
40 °C pH 6.5	24865.7 ± 97.7	46.70 ± 0.06	53
40 °C pH 7.5[a]	39888.9 ± 216.2	26.72 ± 0.14	73
40 °C pH 8.5[a]	39357.6 ± 141.0	23.06 ± 0.03	77

[a] The differences of mean concentrations at pH 7.5 and 8.5 with mean at pH4.5 and 5.5 is significant at level 0.05.

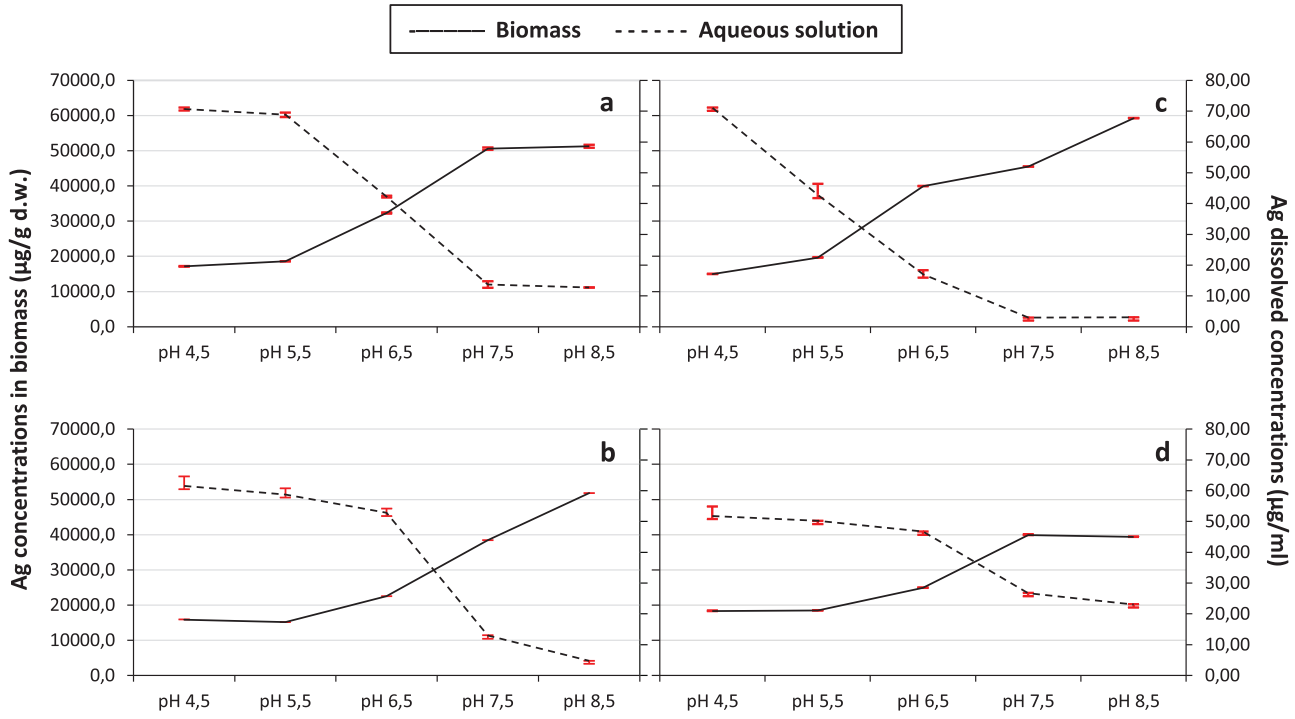

Fig. 3. Silver concentrations in fungal biomass and in aqueous solution at 5 °C (a), 20 °C (b), 30 °C (c), 40 °C (d).

[38], *Cladosporium cladosporioides* [13], *Fusarium oxysporum* [15], *Fusarium semitectum* [39], *Hormoconis resinae* [25], *Trichoderma viridae* [40], *Fusarium acuminatum* [41], *Fusarium solani* [42], and *Trichoderma reesei* [43]. Studies performed on strains of *Fusarium oxysporum* [15] showed that the reduction of silver ions occurs through a nitrate dependent reductase and a shuttle quinone extracellular process.

Biosorption of silver nanoparticles to the mycelial cell wall has been referred by Chen et al. [44] for the strain *Phoma* sp. 3.2883, by Salunkhe et al. [22] for *C. lunatus,* and by Vigneshwaran et al. [45] for *Aspergillus flavus,* therefore indicating that the reduction process takes place on the cell surface. In these cases, the resistance to silver relies on wall-binding capacity resulting from interactions of the ions in solution with oxygen functional groups such as carboxylate anion, the carboxyl peptide bond of proteins, and hydroxyl groups of saccharides in the fungal cell wall followed by reduction by enzymes located on the cell surface to form nanoparticles [46,47].

Mukherjee et al. [17] report surface/intracellular reduction of silver ions by the fungus *Verticillium* sp. The authors speculate that besides the surface trapped nanoparticles, some silver ions, after electrostatic interactions with negatively charged groups present on the cell wall, may diffuse through the wall to be reduced by enzymes present on the cytoplasmic membrane and within the cytoplasm. There is also the possibility that some silver nanopar-

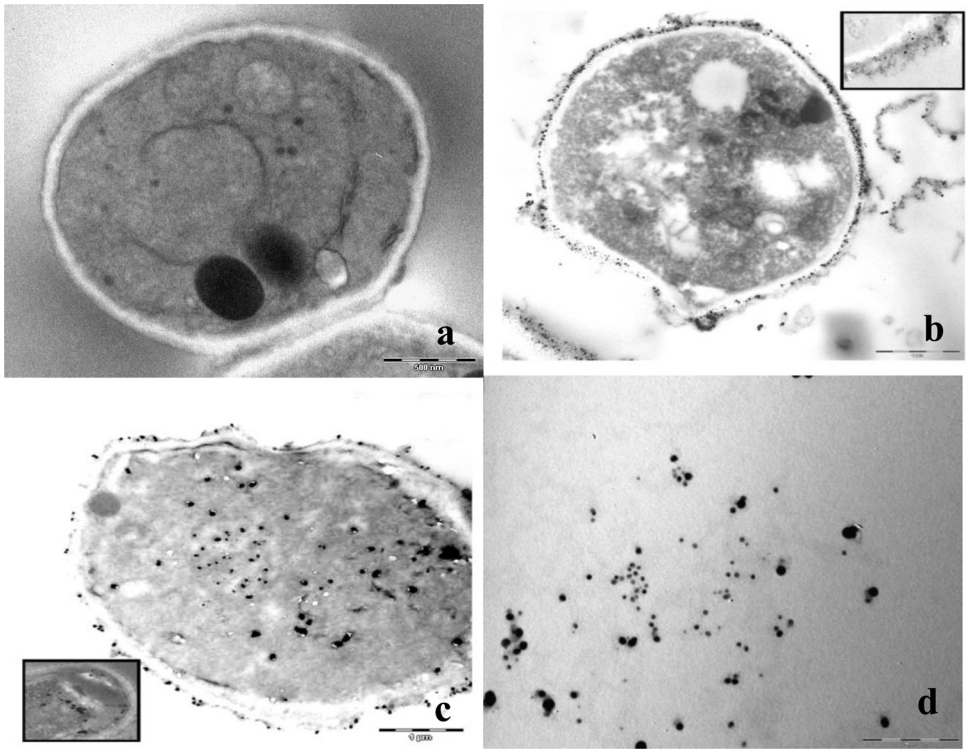

Fig. 4. TEM images of *Aspergillus fumigatus* cells without silver (a), after immersion in 1 mMaqueous AgNO3 solution for 2 h (b) -inset shows the magnificence view- and 72 h (c)-inset shows the magnificence view-; micrograph of silver nanoparticles in the biomass filtrate after 72 h of reaction with 1 mM AgNO3 (d).

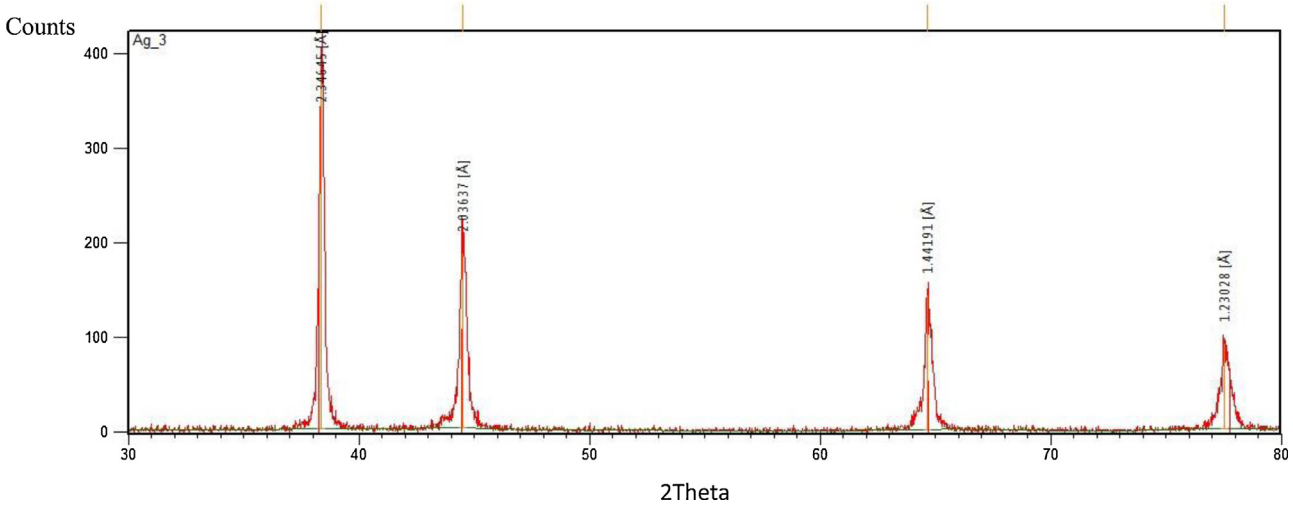

Fig. 5. XRD pattern of silver nanoparticles.

ticles of smaller dimensions can cross the cell wall and be trapped within the cytoplasm.

Our results demonstrated the ability of the *A. fumigatus* strain to sequestrate silver into the cell in the form of nanoparticles as evidenced by the change in color of the biomass after contact with silver aqueous solution and TEM and SEM observations. These findings were confirmed by the absence of change in color of the biomass aqueous solution and the absence of the surface plasmon absorption band at 420 nm that are indicative of the absence or very low concentration of nanoparticles in solution. Conversely, we observed extracellular nanoparticle formation in the biomass filtrate added with Ag(I) when the biomass was previously incubated in deionized water for 72 h, evidencing that extracellular biosynthesis may also occur. Therefore, our results showed that the silver nanoparticle synthesis by *A. fumigatus* is primarily an intracellular process and the extracellular production only occurs when the culture conditions allow the fungal cells to release sufficient amounts of components with metal reducing properties into the medium [21,48]. Our assertion is also strengthened by the results obtained with atomic absorption spectrometry, which evidenced silver intracellular accumulation and concomitant decrease in the metal concentration in solution.

Regarding the cellular localization, our study showed that after an initial adsorption to the cell wall, most particles were subsequently internalized within the cytoplasm as observed by TEM and SEM, therefore evidencing a greater tendency of the fungus to accumulate, over time, silver nanoparticles in the cytoplasm rather than on the wall surface. Sastry et al. [49] referred that in the fungus *Ver-*

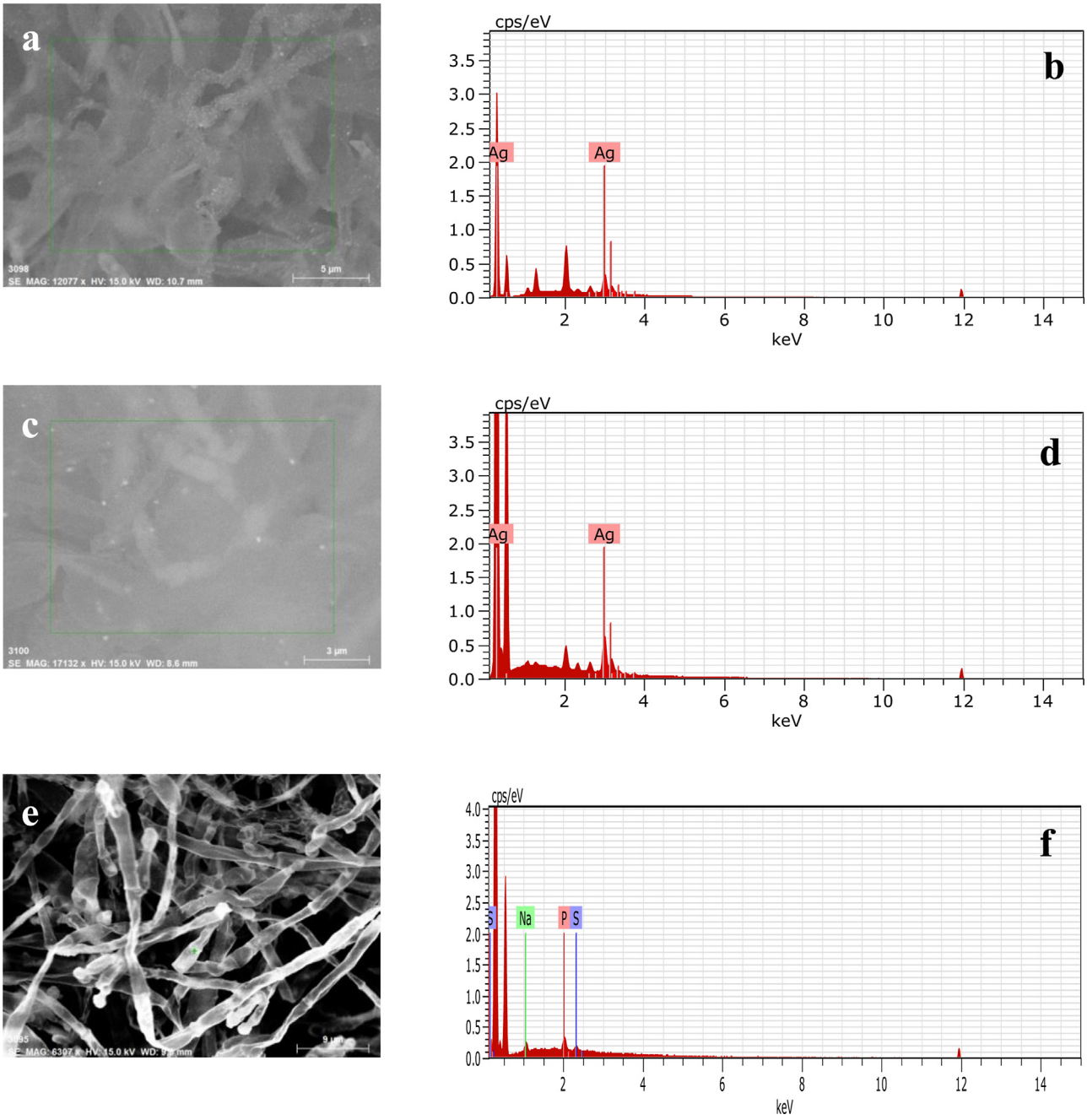

Fig. 6. SEM image (a) and corresponding EDX spectrum (b) of *Aspergillus fumigatus* cells after immersion in 1 mM Ag(I) solution for 2 h; SEM image (c) and corresponding EDX spectrum (d) of *A. fumigatus* cells after immersion in 1 mM Ag(I) solution for 72 h; SEM image (e) and corresponding EDX spectrum (f) of *A. fumigatus* cells after 72 h of incubation without Ag(I) (control).

ticillium sp., after 72 h of contact of the biomass with silver aqueous solution, particles were prevalently distributed on the cell wall and only few were located on the cytoplasmic membrane and within the cytoplasm, therefore indicating that the process was mainly localized on the cell wall. These findings highlight how individual fungal species may adopt different mechanisms to cope with silver. In this regard, while there is a great knowledge of the extracellular detoxification process, little information is still available about intracellular silver detoxification by fungi. According to our results, we suppose that, after an initial adsorption on the cell wall, a number of silver nanoparticles and ions as well are channeled through transporters involved in the uptake of essential metals and other nutrients into the cytoplasm where they are sequestrated by induced metal binding molecules including metallothioneins and other metal-binding proteins [50]. On the other hand, in bacteria such as *Salmonella*, *E. coli,* and *Pseudomonas diminuta,* silver resistance associated proteins have been shown to specifically bind silver [51–53].

It is well known that pH and temperature are critical parameters in the biosorption of heavy metal ions. Regarding pH, literature reports different data. Thus, Kathiresan et al. [54] and Salunkhe et al. [22] observed that the highest accumulation of silver occurred at pH 6.5 ± 0.2 for *C. lunatus* and pH 6.2 ± 0.2 for *Penicillium fellutanum*. Instead Navazi et al. [21] found that pH 4.5 represented the best culture condition for silver nanoparticle biosynthesis by *A. fumigatus*. In our case, statistical analysis (p-value = 0.003) evidenced

that the silver uptake was significantly higher at pH 7.5 and 8.5 than at lower pH; in particular we found the maximum absorption of silver from culture medium at pH 8.5. On the contrary, the Kruskal–Wallis test did not show a significant influence of temperature (p-value = 0.832) on silver uptake, therefore confirming that pH was the most relevant parameter for silver absorption.

Our results agree with those of Sanghi and Verma [48] who found an increase in silver reduction under alkaline conditions. The low bioaccumulation capacity at pH values <5 is attributed to the competition of hydrogen ion with metal ion on the sorption site [55]. Thus, at lower pH, due to the protonation of binding site resulting from a high concentration of protons, negative charge intensity on the site is reduced, resulting in the reduction or inhibition for the binding of metal ion. In general, microbial surfaces are negatively charged due to the ionization of functional groups, thereby contributing to metal binding. However, at low pH, some of the functional groups will be positively charged and would not be able to interact with metal ions [56].

The alkaline conditions in which the maximum absorption of silver was observed are important considering the possible use of the fungus in the bioremediation of metal cyanide complexes. In fact, cyanide dissociation and stability are influenced by pH; alkaline pH reduces the risk of hydrogen cyanide volatilization, making the use of alkaline tolerant organisms more attractive for the degradation of cyanide-containing effluents [57]. In this regard, several authors refer the degradation of cyanides at pH ranging from 6.5 to 10.5 by fungi and bacteria [4,20,58–61]. For example Patil and Paknikar [61] demonstrated that the degradation of silver-cyanide by a bacterial consortium occurred optimally at pH 6.5.

5. Conclusion

Developing reliable protocols for bioremediation plans for polluted industrial wastes requires a number of preliminary tests to clarify the dynamics by which microorganisms cope with the pollutants. In view of the microbial use for the remediation of metal-cyanide containing wastes from electroplating industries, the presence of high concentrations of heavy metals has to be taken in account due to their toxicity.

The results of the present study together with the proved ability of the *A. fumigatus* strain to utilize cyanide for growth (20 nostro aspergillus) make the fungus a good candidate for biotechnological use for the detoxification of polluted industrial effluents and the possible recovery of the precious metal as well.

In any case, further research is needed to better clarify the mechanisms of resistance to silver by analyzing the enzymatic pathways involved and verifying the ability of the fungus to produce nanoparticles using $KAg(CN)_2$ in consideration of the presence of metal-cyanide complexes in wastes. The resistance mechanisms against the other metals which can be found in metal cyanide wastes will also be investigated.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2015.12.014.

References

[1] C.-F. Wu, Q. Xu, L. Deng, J. Feng, J.-P. Yuan, J.-H. Wang, An effective method for the detoxification of cyanide-rich wastewater by *Bacillus* sp. CN-22, Appl. Microbiol. Biotechnol. 98 (2014) 3801–3807, http://dx.doi.org/10.1007/s00253-013-5433-5.
[2] S. Mirizadeh, S. Yaghmaei, Z. Ghobadi Nejad, Biodegradation of cyanide by a new isolated strain under alkaline conditions and optimization by response surface methodology (RSM), J. Environ. Health Sci. Eng. 12 (2014) 1–9, http://dx.doi.org/10.1186/2052-336X-12-85.
[3] R.R. Dash, C. Balomajumder, A. Kumar, Removal of metal cyanides from aqueous solutions by suspended and immobilized cells of *Rhyzopus oryzae* (MTCC 2541), Eng. Life Sci. 9 (2007) 53–59, http://dx.doi.org/10.1002/elsc.200700024.
[4] Y.K. Özel, S. Gedikli, P. Aytar, A. Ünal, M. Yamaç, A. Çabuk, N. Kolankaya, New fungal biomasses for cyanide biodegradation, J. Biosci. Bioeng. 110 (2010) 431–435.
[5] D. Naveen, C.B. Majumder, P. Mondal, D. Shubha, Biological treatment of cyanide containing Wastewater, Res. J. Rhem. Sci. 1 (2011) 15–21, ISSN 2231-606X.
[6] D. Novak, I.H. Franke-Whittle, E.T. Pirc, V. Jerman, H. Insam, R.M. Logar, B. Stres, Biotic and abiotic processes contribute to successful anaerobic degradation of cyanide by UASB reactor biomass treating brewery waste water, Water Res. 47 (2013) 3644–36453.
[7] J.D.C. Desai Ramakrishna, Microbial degradation of cyanides and its commercial application, J. Sci. Ind. Res. 57 (1998) 441–453.
[8] D.H. Nies, Microbial heavy-metal resistance, Appl. Microbiol. Biotechnol. 51 (1999) 730–750, http://dx.doi.org/10.1007/s002530051457.
[9] M.R. Bruins, S. Kapil, F.W. Oehme, Microbial resistance to metal in the environment, Ecotox. Environ. Safe. (2000) 198–207, http://dx.doi.org/10.1006/eesa.1999.1860.
[10] I.V.N. Rathnayake, M. Megharaj, N. Bolan, R. Naidu, Tolerance of heavy metals by gram positive soil bacteria, Int. J. Civil. Environ. Eng. 2 (2010) 191–195.
[11] M.L. Merroun, N. Ben Omar, E. Alonso, J.M. Arias, M.T. GonzÁlez-MuÑoz, Silver sorption to Myxococcus xanthus biomass, Geomicrobiol. J. 18 (2001) 183–192, http://dx.doi.org/10.2174/1573413711309990092.
[12] A. Ahmad, P. Mukherjee, D. Mandal, S. Senapati, M. Islam Khan, R. Kumar, M. Sastry, Enzyme mediated extracellular synthesis of CdS nanoparticles by the fungus *Fusarium* oxysporum, J. Am. Chem. Soc. 124 (2002) 12108–12109, http://dx.doi.org/10.1021/ja027296o.
[13] D.S. Balaji, S. Basavaraja, R. Deshpande, D. Bedre Mahesh, B.K. Prabhakar, A. Venkataraman, Extracellular biosynthesis of functionalized silver nanoparticles by strains of *Cladosporium cladosporioides* fungus, Colloids Surf. B 68 (2008) 88–92, http://dx.doi.org/10.1016/j.colsurfb.2008.09.022.
[14] M. Deschatre, F. Ghillebaert, J. Guezennec, C. Simon Colin, Sorption of copper(II) and silver(I) by four bacterial exopolysaccharides, Appl. Biochem. Biotechnol. 173 (2010) 1313–1327, http://dx.doi.org/10.1007/s12010-013-0343-7.
[15] P.D. Durán, O.L. Marcato, G.I.H. De Souza, E. Esposito, Mechanistic aspects of biosynthesis of silver nanoparticles by several *Fusarium oxysporum* strains, J. Nanobiotechnol. 13 (2005) 1–7, http://dx.doi.org/10.1186/1477-3155-3-8.
[16] X.-Z. Li, H. Nikaido, K.E. Williams, Silver-resistant mutants of *E. coli* display active efflux Ag^+ and are deficient in porins, J. Bacteriol. 179 (1997) 6127–6132.
[17] P. Mukherjee, A. Ahmad, D. Mandal, S. Senapati, S.R. Sainkar, M.I. Khan, R. Parishcha, P.V. Ajaykumar, M. Alam, R. Kumar, M. Sastry, Fungus-mediated synthesis of silver nanoparticles and their immobilization in the mycelial matrix: a novel biological approach to nanoparticle synthesis, Nano Lett. 1 (2001) 515–519, http://dx.doi.org/10.1021/nl0155274.
[18] M. Solioz, A. Odermatt, Copper and silver transport by CopA-ATPase in membrane vesicles of *Enterococcus hirae*, J. Biol. Chem. 270 (1995) 9217–9221, http://dx.doi.org/10.1074/jbc.270.16.9217.
[19] H. Zhang, Q. Li, Y. Lu, D. Sun, X. Lin, X. Deng, N. He, S. Zheng, Biosorption and bioreduction of diamine silver complex by *Corynebacterium*, J. Chem. Technol. Biotechnol. 80 (2005) 285–290, http://dx.doi.org/10.1002/jctb.1191.
[20] L. Sabatini, C. Ferrini, M. Micheloni, A. Pianetti, B. Citterio, C. Parlani, F. Bruscolini, Isolation of a strain of *Aspergillus fumigatus* able to grow in minimal medium added with an industrial cyanide waste World, J. Microbiol. Biotechnol. 28 (2012) 165–173, http://dx.doi.org/10.1007/s11274-011-0805-4.
[21] R.Z. Navazi, M. Pazouki, F.R. Halek, Investigation of culture conditions for biosynthesis of silver nanoparticles using *Aspergillus fumigatus*, Iran J. Biotechnol. 8 (2010) 56–61.
[22] R.B. Salunkhe, S.V. Patil, B.K. Salunke, C.D. Patil, A.M. Sonawane, Studies on silver accumulation and nanoparticle synthesis by *Cochliobolus lunatus*, Appl. Biochem. Biotechnol. 165 (2011) 221–234, http://dx.doi.org/10.1007/s12010-011-9245-8.
[23] N. Krumov, I. Perner-Nochta, S. Oder, V. Gotcheva, A. Angelov, C. Posten, Production of inorganic nanoparticles by microorganisms, Chem. Eng. Technol. 32 (2009) 1026–1035, http://dx.doi.org/10.1002/ceat.200900046.
[24] S. Saha, J. Sarkar, D. Chattopadhyay, S. Patra, A. Chakraborty, K. Achary, Production of silver nanoparticles by a phytopathogenic fungus *Bipolaris nodulosa* and its antimicrobial activity, Dig. J. Nanomater. Bios. 5 (2010) 887–895.
[25] R. Varshney, A.N. Mishra, S. Bhadauria, M.S. Gaur, A novel microbial route to synthetize silver nanoparticles using fungus *Hormoconis resinae*, Dig. J. Nanomater. Bios. 4 (2009) 349–355.
[26] N.C.M. Gomes, M.M. Figueira, E.R.S. Camargos, L.C.S. Mendonça-Hagler, J.C.T. Dias, V.R. Linardi, Cyano-metal complexes uptake by *Aspergillus niger*, Biotechnol. Lett. 21 (1999) 487–490.
[27] M. Battistelli, S. Salucci, E. Olivotto, A. Facchini, M. Minguzzi, S. Guidotti, S. Pagani, F. Flamigni, R.M. Borzì, A. Facchini, E. Falcieri, Cell death in human articular chondrocyte: a morpho-functional study in micromass model,

Apoptosis 19 (2014) 1471–1483, http://dx.doi.org/10.1007/s10495-014-1017-9.
[28] Y. Xu, G. Pang, C. Gao, D. Zhao, L. Zhou, S. Sun, B. Wang, In vitro comparison of the efficacies of natamycin and silver nitrate against ocular fungi, Antimicrob. Agents Chemoter. 53 (2009) 1636–1638, http://dx.doi.org/10.1128/aac.00697-08.
[29] V. Deepak, K. Kalishwaralal, S. Ram Kumar Pandian, S.S. Gurunathan, An Insight into the bacterial biogenesis of silver nanoparticles, industrial production and Scale-up, in: M. Rai, N. Duran (Eds.), Metal Nanoparticles in Microbiology, Springer, VerlagBerlin Heidelberg, 2011, pp. 17–35, http://dx.doi.org/10.1007/978-3-642-18312-6_2.
[30] S. Shivaji, S. Madhu, S. Singh, Extracellular synthesis of antibacterial silver nanoparticles using psychrophilic bacteria, Process Biochem. 46 (2011) 1800–1807, http://dx.doi.org/10.1016/j.procbio.2011.06.008.
[31] M. Saravanan, A.K. Vemu, S.K. Barik, Rapid biosynthesis of silver nanoparticles from Bacillus megaterium (NCIM 2326) and their antibacterial activity on multi drug resistant clinical pathogen, Coll. Surf. B 88 (2011) 325–331, http://dx.doi.org/10.1016/j.colsurfb.2011.07.009.
[32] V.L. Das, R. Thomas, R.T. Varghese, E.V. Soniya, J. Mathew, E.K. Radhakrishnan, Extracellular synthesis of silver nanoparticles by the *Bacillus* strain CS 11 isolated from industrialized area, Biotech 4 (2014) 121–126, http://dx.doi.org/10.1007/s13205-013-0130-8.
[33] T. Klaus, R. Joerger, E. Olsson, C.-G. Granqvist, Silver-based crystalline nanoparticles, microbially fabricated, Proc. Nati. Acad. Sci. U S A 96 (1999) 13611–13614, http://dx.doi.org/10.1073/pnas.96.24.13611.
[34] S. Seshadri, A. Prakash, M. Klowshik, Biosynthesis of silver nanoparticles by marine bacterium, *Idiomarina* sp. PR58-8, Bull. Mater. Sci. 35 (2012) 1201–1205.
[35] K. Kalimuthu, R.S. Babu, D. Venkataraman, M. Bilal, S. Gurunathan, Biosynthesis of silver nanocrystals by *Bacillus licheniformis*, Coll. Surf. B 65 (2008) 150–153, http://dx.doi.org/10.1016/j.colsurfb.2008.02.018.
[36] B. Nair, T. Pradeep, Coalescence of nanoclusters and formation of submicron crystallites assisted by *Lactobacillus* strains, Cryst. Growth Des. 2 (2002) 293–298, http://dx.doi.org/10.1021/cg0255164.
[37] M. Gajbhiye, J. Kesharwani, A. Ingle, A. Gade, M. Rai, Fungus-mediated synthesis of silver nanoparticles and their activity against pathogenic fungi in combination with fluconazole, Nanomed. Nanotechnol. 5 (2009) 382–386, http://dx.doi.org/10.1016/j.nano.2009.06.005.
[38] A.K. Gade, P. Bonde, A.P. Ingle, P.D. Marcato, N. Durán, M.K. Rai, Exploitation of *Aspergillus niger* for synthesis of silver nanoparticles, J. Biobased Mater. Bio. 2 (2008) 1–5, http://dx.doi.org/10.1166/jbmb.2008.401.
[39] G.B. Shelar, A.M. Chavan, Fusarium semitectum mediated extracellular synthesis of silver nanoparticles and their antibacterial activity, Int. J. Biomed. Adv. Res. 5 (2014) 348–351, http://dx.doi.org/10.7439/ijbar.
[40] M. Fayaz, C.S. Tiwary, P.T. Kalaichelvan, R. Venkatesan, Blue orange light emission from biogenic synthesized silver nanoparticles using *Trichoderma viride*, Colloids Surf. B 75 (2010) 175–178, http://dx.doi.org/10.1016/j.colsurfb.2009.08.028.
[41] A. Ingle, A. Gade, S. Pierrat, C. Sönnichsen, M. Rai, Mycosynthesis of silver nanoparticles using the fungus Fusarium acuminatum and its activity against some human pathogenic bacteria, Curr. Nanosci. 4 (2008) 141–144, http://dx.doi.org/10.2174/157341308784340804.
[42] A. Ingle, M. Rai, A. Gade, M. Bawaskar, Fusarium solani : a novel biological agent for the extracellular synthesis of silver nanoparticles, J. Nanopart. Res. 11 (2009) 2079–2085, http://dx.doi.org/10.1007/s11051-008-9573-y.
[43] K. Vahabi, G.A. Mansoori, S. Karimi, Biosynthesis of silver nanoparticles by fungus *Trichoderma reesei* (a route for large-scale production of AgNPs), Insciences J. 1 (2011) 65–79, http://dx.doi.org/10.5640/insc.010165.
[44] J.C. Chen, Z.H. Lin, X.X. Ma, Evidence of the production of silver nanoparticles via pretreatment of *Phoma* p.3.2883 with silver nitrate, Lett. Appl. Microbiol. 37 (2003) 105–108, http://dx.doi.org/10.1046/j.1472-765X.2003.01348.x.
[45] N. Vigneshwaran, A.A. Kathe, P.V. Varadarajan, R.P. Nachane, R.H. Balasubramanya, Biomimetics of silver nanoparticles by white rot fungus, *Phaenerochaete chrysosporium*, Colloids Surf. B 53 (2006) 55–59, http://dx.doi.org/10.1016/j.colsurfb.2006.07.014.
[46] Z. Lin, J. Wu, R. Xue, Y. Yang, Spectroscopic characterization of Au^{3+} biosorption by waste biomass of *Saccharomyces cerevisiae*, Spectrochim. Acta A Mol. Biomol. Spectrosc. 61 (2005) 761–765, http://dx.doi.org/10.1016/j.saa.2004.03.029.
[47] N. Vigneshwaran, N.M. Ashtaputre, P.V. Varadarajan, R.P. Nachane, K.M. Paralikar, R.H. Balasubramanya, Biological synthesis of silver nanoparticles using the fungus Aspergillus flavus, Mater. Lett. 61 (2007) 1413–1418, http://dx.doi.org/10.1016/j.matlet.2006.07.042.
[48] R. Sanghi, P. Verma, Biomimetic synthesis and characterisation of protein capped silver nanoparticles, Bioresour. Technol. 100 (2009) 501–504, http://dx.doi.org/10.1016/j.biortech.2008.05.048.
[49] M. Sastry, A. Ahmad, M.I. Khan, R. Kumar, Biosynthesis of metal nanoparticles using fungi and actinomycete, Curr. Sci. 8 (2003) 162–170.
[50] I. Pócsi, Toxic metal/metalloid tolerance in fungi—a biotechnology-oriented approach, in: G. Bánfalvi (Ed.), Cellular Effects of Heavy Metals, Springer, 2011, pp. 31–58, http://dx.doi.org/10.1007/978-94-007-0428-2_2.
[51] A. Gupta, K. Matsui, J.F. Lo, S. Silver, Molecular basis for resistance to silver cations in Salmonella, Nat. Med. 5 (1999) 183–188.
[52] R.H. Sedlak, M. Hnilova, C. Grosh, H. Fong, F. Baneyx, D. Schwartz, M. Sarikaya, C. Tamerler, B. Traxler, Engineered *E. coli* silver-binding periplasmic protein that promotes silver tolerance, Appl. Environ. Microbiol. 78 (2012) 2289–2296, http://dx.doi.org/10.1128/AEM.06823-11.
[53] Z. Ibrahim, W.A. Ahmad, A.B. Baba, Bioaccumulation of silver and the isolation of metal-binding protein from *P. diminuta*, Braz. Arch. Biol. Technol. 44 (2001) 223–225, ISSN 1516-8913.
[54] K. Kathiresan, S. Manivannan, M.A. Nabeel, B. Dhivya, Studies on silver nanoparticles synthesized by a marine fungus, *Penicillium fellutanum* isolated from coastal mangrove sediment, Colloids Surf. B 71 (2009) 133–137, http://dx.doi.org/10.1016/j.colsurfb.2009.01.016.
[55] S. Congeevaram, S. Dhanarani, J. Park, M. Dexilin, K. Thamaraiselvi, Biosorption of chromium and nickel by heavy metal resistant fungal and bacterial isolates, J. Hazard Mater. 146 (2007) 270–277, http://dx.doi.org/10.1016/j.jhazmat.2006.12.017.
[56] G. Yan, T. Viraraghavan, Heavy-metal removal from aqueous solution by fungus Mucor rouxii, Water Res. 37 (2003) 4486–4496, http://dx.doi.org/10.1016/j.jhazmat.2008.08.105.
[57] A. Dumestre, T. Chone, J.-M. Portal, M. Gerard, J. Berthelin, Cyanide degradation under alkaline conditions by a strain of *Fusarium solani* isolated from contaminated soils, Appl. Environ. Microbiol. 63 (1997) 2729–2734.
[58] M.D. Adjei, Y. Ohta, Factors affecting the biodegradation of cyanide by *Burkholderia cepacia* strain C-3, J. Biosci. Bioeng. 89 (2000) 274–277, http://dx.doi.org/10.1016/s1389-1723(00) 88833-7.
[59] L.J. Basile, R.C. Willson, B. Trevor Sewell, J. Michael, M.J. Benedik, Genome mining of cyanide-degrading nitrilases from filamentous fungi, Appl. Microbiol. Biotechnol. 80 (2008) 427–435, http://dx.doi.org/10.1007/s00253-008-1559-2.
[60] M.J. Huertas, L.P. Sáez, M.D. Roldán, V.M. Luque-Almagro, M. Martínez-Luque, R. Blasco, F. Castillo, C. Moreno-Vivián, I. García-García, Alkaline cyanide degradation by *Pseudomonas pseudoalcaligenes* CECT5344 in a batch reactor. Influence of pH, J. Hazard Mater. 179 (2010) 72–78, http://dx.doi.org/10.1016/j.jhazmat.2010.02.059.
[61] Y.B. Patil, K.M. Paknikar, Biodetoxification of silver-cyanide from electroplating industry wastewater, Lett. Appl. Microbiol. 30 (2000) 33–37.