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Copper-induced adaptation, oxidative stress and its tolerance in *Aspergillus niger* UCP1261

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ABSTRACT

Background: The effects of exposure to copper, during growth, on the production of biomass, total protein, catalase, glutathione-S transferase, glutathione peroxidase, peroxidase, polyphosphate, acid and alkaline phosphatases, ultrastructure and the ability to remove this metal from *Aspergillus niger*, obtained from caatinga soil, were evaluated.
Results: All parameters tested were influenced by the concentration of metal in the culture medium. The presence of metal induced high levels of antioxidant enzymes, including lipid peroxidation, thereby revealing the appearance of an oxidative stress response. The variation in polyphosphate levels indicates the participation of the polymer in response to stress induced by copper. The activities of the phosphatases were positively influenced by growing them in the presence of copper. Ultrastructure changes in the cell surface, electron density, thickness, and septation were visualized by exposing cells to increasingly larger concentrations of metal. The isolate was able to remove the agent from the growth medium, while maintaining its physiological functions. The metal removed from the cultures exposed to 0.5 mM, 1 mM and 2 mM copper exhibited percentages of removal equivalent to 75.78%, 66.04% and 33.51%.
Conclusions: The results indicate that the isolate was able to grow in high concentrations of copper, activates mechanisms for adaptation and tolerance in the presence of metal, and is highly efficient at removing the agent. Such data are fundamental if a better understanding is to be reached of the cellular and molecular abilities of native isolates, which can be used to develop bioprocesses in environmental and industrial areas.

1. Introduction

Heavy metals are among the pollutants of greatest importance and concern in the world today [1,2]. They are not degraded, which results in the increase of their concentration in different natural environments, and consequently in food chains.

In general the level of metal concentration in the environment is associated with the geographical location, there being an increase in the toxicity level in areas near industrial and mining activities. In these places, plants and animals nearby can absorb and accumulate such concentrations in their fibers and bodies (bioaccumulation), causing severe poisoning along the food chain [3,4,5,6].

Although Cu is an essential microelement, and is also necessary for a wide range of metabolic processes in living things, an accumulation of an over-dosage of Cu can be toxic for an animal depending on the species [7,8]. It is emphasized that the concentrations of metal normally encountered in industrial effluents is considered toxic to a wide variety of organisms, especially microbial populations, including nitrifying and denitrifying bacteria, algae, fungi and many aquatic invertebrates [7,8,9].

The presence of these elements in high concentrations poses a serious ecological risk, since they cause the deterioration of the quality of air, the environment, and groundwater and surface water, and thus provoke health problems. The toxicity of metals depends on several factors: the nature of the metal, its existing toxic form, bioavailability, solubility, mobility, the rate of accumulation in living organisms, local accumulation in the body, position in the food chain, and so on. Bioavailability is related both to the association of toxic metals with other particles and the rate of uptake and elimination. Their toxicity results from their direct effect on cellular and molecular levels, and the capacity of the organism to adapt to this pollutant [2].

One of the effects of heavy metal toxicity is that reactive oxygen species (ROS) are formed and accumulate [10]. ROS, singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radicals interact

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with molecules, and alter their structure or metabolic activity, resulting in the oxidation of proteins and nucleic acids and lipid peroxidation. An interesting fact is that such processes also result from normal cellular activities and as a consequence, cells and living organisms have developed enzymatic and non-enzymatic, antioxidant defense systems. Changes related to attack by ROS may be caused by excessively training them and/or the failure of antioxidant defenses to intercept them, thus generating oxidative stress [11,12,13].

The consequences of oxidative stress can be varied according to the cell type and the intensity of cell proliferation. The effects are: some cells may respond to oxidative stress by increasing the adaptation rate of cell division: thus increasing cellular defenses, such as catalase, superoxide dismutase and glutathione (GSH), thereby giving the cells total or partial protection. Furthermore, the targets can be redirected. Oxidative damage or even basal ROS production can be reduced. Cell injury may involve one or more types of biomolecule such as lipids, proteins, DNA, carbohydrates, etc. In cases of minor damage, the cell can survive with persistent oxidative damage and irreparable damage, or repair itself; senescence: cell survival, but with the system is compromised; cell division and cell death: after damage, the cell can trigger the process of cell death. Oxidative damage to DNA, mitochondria, or other cellular targets, may cause cell death by apoptosis or necrosis [11].

Microorganisms as efficient tools and biogeochemical cycling exhibit several mechanisms to deal with heavy metals. Among these are: external mechanisms -which act so as to restrict the absorption and transport of metal, there being substances and molecules present on the cell surface or which are produced and secreted and act to sequester and exclude metals by selective absorption and retention, thus preventing their entry into the cell, and there are internal tolerance mechanisms such as immobilization strategies which include complexing and compartmentalizing within the cell. Depending on the type of metal, ligands, which act in the chelation of metal, can be produced: namely, organic acids (such as citric and malic acid), amino acids (such as proline and histidine) and peptides, and polyphosphates [1,10,14].

Aspergillus niger was used considering the biotechnological potential of enzyme production and its applicability to produce organic acids, especially citric acid, in industry since 1919, and is extensively applied to food and pharmaceutical industries. The genus *Aspergillus* is used in the bioremediation process removing heavy metals due to its high resistance and tolerance to different metal ions [15,16,17,18,19]. Particularly, *A. niger* was used as a model system, considering its defense strategies against cell-surface acting compounds such as caspofungin, fenpropimorph, inhibitor of chitin synthesis by antifungal protein (AFP), and inhibitor of chitin microfibril by calcofluor white [20,21,22].

The experimental approach of the present study was to evaluate the effects of copper on growth of *A. niger* related to the antioxidant enzyme system (acid and alkaline phosphatases), in polyphosphate production, ultrastructure behavior, and their potential for removal the metal.

2. Materials and methods

2.1. Microorganism and culture conditions

The microorganism *A. niger* UCP/WFCC 1261, was isolated from caatinga soil of Serra Talhada, Pernambuco, Brazil, and belongs to the Culture Collection of the Catholic University of Pernambuco (UCP)/ World Federation for Culture Collection (WFCC), and was used to study growth at various concentrations of copper. The strain was maintained on Potato Dextrose Agar (PDA) at 5°C. For the production of the pre-inoculum, the strain *A. niger* was grown on Sabouraud Dextrose Agar (SDA) medium and incubated at 28°C for 7 d.

2.2. Metallic solution

To prepare the stock solution of 100 mM, Cu^{2+} ions were prepared by dissolving 24.968 g of $CuSO_4 \cdot 5H_2O$ in 1 L of distilled and deionized water. From the stock, a standard solution was prepared of working solutions of 0.5 mM, 1 mM and 2 mM of copper used in these experiments.

2.3. Determination of growth curve

Pre-inoculum corresponding to 5% of a spore suspension of 10^7 cells/mL, obtained from cultures of *A. niger* were inoculated in 250 mL Erlenmeyer flasks containing 100 mL of Sabouraud (broth) medium added copper at levels of 0.5 mM, 1 mM and 2 mM at pH 5, and were incubated in a shaker at 150 rpm, at 28°C, for 15 d. Control samples were grown in the absence of metal. The mycelia collected during the cultivation ranges reported were subjected to freeze drying, and subsequently kept in a vacuum desiccator until constant weight. The average dry weight in triplicate was used to establish the graph corresponding to the growth curve.

2.4. Biochemical determinations

2.4.1. Glucose consumption

Treated and control samples corresponding to the supernatants from cultures grown in Sabouraud broth medium, served to determine the consumption of glucose in the medium. The glucose was measured by the enzymatic colorimetric method (Test-Lab) [23]. A standard curve was prepared using glucose solution (0.5–5.0 g/mL), and the concentration of glucose was determined by reading the absorbance at 510 nm, using a digital spectrophotometer (Spectronic Genesys 2 Mod).

2.4.2. Determination of pH

The pH of the growth in the culture media was monitored by the mean of three measurements. Subsequently, a pH curve was established with these values.

2.4.3. Extraction and determination of total protein

Samples of *A. niger* mycelium, collected at intervals of 3, 6, 9, 12 and 15 d of cultivation were collected and washed in phosphate buffered saline, pH 7.2 three times to remove waste. Samples of 10 mg of mycelium were lyophilized, and subjected to the extraction process using urea buffer/trichloroacetic acid. Initially, the samples were incubated in 0.5 mL of 10% trichloroacetic acid for 5 min at room temperature, washed three times with 90% acetone and allowed to air dry. Subsequently, the samples were incubated in 0.2 mL buffer containing 1% of SDS, 9 M of urea, 25 mM of Tris–HCl, pH 6.8, 1 mM of EDTA and 0.7 M of mercaptoethanol. The sample was stirred, boiled for 2 min, and stirred and boiled again for another minute. The samples were submitted to quantification by the Biuret method [24], which is based on the principle that the copper ions in an alkaline medium (the Biuret reaction) will interact with the peptide bonds of the protein, forming a purple color, which has absorbance to 545 nm and is proportional to the protein concentration in the sample.

2.5. Preparation of samples for determination of oxidative enzymes

Samples of 1 g of mycelium, collected at intervals of 3, 6, 9, 12 and 15 d were collected by filtration, washed with deionized water and mixed with a solution containing potassium chloride 1.15% phenyl methyl sulfonyl fluoride (protease inhibitor) at a concentration of 100 mM in isopropanol. The homogenized samples were centrifuged for 10 min at 3000 rpm in a refrigerated centrifuge. The supernatants were collected and used to determine the enzymatic activities antioxidants and lipid peroxidation.

2.5.1. Catalase (Cat)

The high speed of reaction of this enzyme associated with lower "affinity" enables its activity with high concentrations of H_2O_2 (10 mM) to be determined. This was done using the rate of consumption of H_2O_2 in the first minute of the reaction, 240 nm ($\varepsilon = 40\ M^{-1}\ cm^{-1}$) [25]. The disappearance of hydrogen peroxide without the presence of the sample is discounted. The enzyme assay was performed in 50 mM of 40-second potassium phosphate buffer (KPi); 0.5 mM of EDTA, pH 7, containing 0.012% of Triton X-100. 10 mM of H_2O_2 is used as the substrate initiator. The absorbance baseline is deduced from the readings of the reaction in the absence of the test sample. The data are expressed as IU/mg protein. Proteins were measured by the Biuret method [24].

2.5.2. Glutathione-S-transferase (GST)

Conjugation of GSH with the substrate chlorodinitrobenzene (CDNB) catalyzed by GST produces a compound which can be detected at 340 nm ($\varepsilon = 9600\ M^{-1}\ cm^{-1}$). Enzyme activity is proportional to the speed of production of the conjugate compound [26]. This activity is a discounted reaction baseline reading obtained from the reaction between GSH and CDNB assay without the presence of the sample. The 5 min enzyme assay was performed in 100 mM of KPi, 1 mM of EDTA, pH 7, containing 1 mM of GSH. The substrate, 1 mM of CDNB, was used as the initiator. The baseline absorbance was reduced from the readings of the reaction in the absence of the test sample. The data is expressed as IU/mg protein. Proteins were measured by the Biuret method [24].

2.5.3. Glutathione peroxidase (GPx)

The enzyme, using GSH to degrade an organic peroxide such as t-butyl peroxide (t-BOOH), or cumene, generates oxidized glutathione (GSSG), which in turn is reduced by glutathione reductase, was added to the medium reaction with consumption of NADPH ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$). The consumption of NADPH is monitored spectrophotometrically at 340 nm as glutathione reductase [27] is determined. This is a discounted rate of consumption of NADPH. The consumption of basal is obtained by reading the enzymatic assay without the presence of the substrate (peroxide). The enzyme assay is performed for 5 min in KPi, 50 mM of ethylenediamine tetraacetic acid, 0.5 mM of EDTA, pH 7, containing 0.2 mM of NADPH, and 1 mM of GSH, 0.2 U/mL of GR purified from yeast. 5–10 min of incubation is needed with the reagents (except initiator substrate) to activate the enzyme. 1 mM of CuOOH (cumene hydroperoxide) is used as substrate initiator. The data are expressed as IU/mg protein. Proteins were measured by the Biuret method [24].

2.5.4. Peroxidase

The presence of peroxidase activity was determined using pyrogallol and hydrogen peroxide as substrates [28]. Samples (0.1 mL for each activity measured) were subjected to reaction with 0.5 ml of H_2O_2 (5 mmoL L^{-1}), and 1.0 mL of pyrogallol (12.69 mmoL L^{-1}), in 1.4 mL of sodium phosphate buffer 0.1 moL L^{-1}, pH 6. The reading activity was conducted at 420 nm after 1 min of reaction. The enzymatic activity was measured in enzyme units mg^{-1} protein. The proteins were measured by the Biuret method [24].

2.6. Lipid peroxidation

Lipid peroxidation was estimated by the TBARS [29], method with some modifications. The method forms a pinkish-red pigment comprising two molecules of thiobarbituric acid (TBA) and malondialdehyde (MDA). An aliquot (100 μL) of sample was added to 1 mL of solution containing 400 μL of acetic acid buffer, 1.3 M, 400 μL of TBA at 0.8% and 200 μL of sodium dodecyl sulfate (SDS) at 8.1%. This was mixed and incubated to 95°C for 60 min. The reaction of TBA with MDA produces a chromophore which can be measured photometrically at 532 nm. Data were expressed as nmol/mg protein. Proteins were measured by the Biuret method [24].

2.7. Extraction and polyphosphate determination

The polyphosphate was extracted and assayed according to the method described by McGrath and Quinn [30]. Samples of 10 mg of mycelium (dry weight) obtained from the incubation medium during the cultivation of the fungus at intervals of 3, 6, 9 12 and 15 d were collected and washed twice in 1.5 M NaCl solution containing 0.01 M EDTA and 1 mM NaF. They were then placed in wash solution and sonicated on ice for 2 min 12 times with intervals of 1 min at 16 KHz. The resulting extract was centrifuged at 12000 × g for 10 min at 4°C to remove the cell debris. To determine the total cell content of the polyphosphate, 100 μL of concentrated HCl was added to 0.5 mL of cell extract and heated at 100°C for 45 min. The phosphate released was determined using the colorimetric method Subbarow [31], which is based on the reaction of inorganic phosphorus with ammonium molybdate in acidic media, resulting in a complex phosphomolybdate blue color, quantified spectrophotometrically at 600 nm, and the intensity of which is directly proportional to the concentration of phosphorus. A standard curve was prepared, for which a solution of phosphorus (0.5–5.0 mg/dL) was used. Data were collected in a digital spectrophotometer, Spectronic, Genesys model 2. The concentration of polyphosphate was expressed in milligrams per deciliter (mg/dL) of phosphorous and given as means of triplicates. A non-hydrolyzed sample was used as a control to determine the level of free phosphate in the cell. The overall polyphosphate is expressed in milligrams per gram of biomass.

2.8. Activity of acid and alkaline phosphatase

To determine the enzymatic activity of acid and alkaline phosphatase in culture medium, aliquots of the supernatant fluid and metabolic biomass were collected at intervals of 3, 6, 9, 12, and 15 d during cultivation. Samples of 36 mg of mycelium (wet weight) were collected at intervals of 3, 6, 9, 12, and 15 d of cultivation, washed with deionized water and incubated in 3 mL of extraction solution, consisting of 0.02 M of sodium acetate buffer, pH 4.5 for acid phosphatase, and 50 mM of alkaline phosphatase Tris–HCl, pH 7.5, containing 5% of glycerol. The samples were macerated by mortar and pestle for 5 min, and then homogenized for 2 min on ice. The resulting extract was centrifuged at 12000 × g for 10 min at 4°C to remove cellular debris. To determine enzyme activity, the Lab-Test Kit was used and cell extracts were collected and subjected to spectrophotometric determination using Lab-Test Kits. These are based on the enzymatic hydrolysis of monophosphate thymolphthalein releasing thymolphthalein released in its blue at different pH values. The resulting color of the reaction is directly proportional to the enzyme concentration and is measured at 590 nm. The values of the concentrations were given in International Units (IU), which represent the amount of enzyme that catalyzes the splitting of 1 μmol substrate/minute/liter of sample, according to Joh et al. [32].

2.9. Scanning electron microscopy

A scanning electron microscope was used to analyze the mycelium surface, of control and exposed cells, to different concentrations of copper, after 3 and 15 d of contact. The mycelium was collected after centrifugation and filtration, washed twice with phosphate buffered saline, pH 7.2, and fixed with 0.1 M of cacodylate buffer containing 2.5% of glutaraldehyde, pH 7.2. After 3 h, the mycelium was washed twice with 0.1 M of cacodylate buffer, pH 7.2. Samples were post-fixed with 0.1 M of cacodylate buffer containing 0.05% of malachite green for two hours in the dark. This was followed by washing in 0.1 M of cacodylate buffer, pH 7.2, 2.5% of glutaraldehyde, pH 7.2. The

mycelium was dehydrated in ethanol (v/v): 50%, 70%, 90% and 100%. The mycelia were then mounted on metal supports, observed and photographed in a LV5.600 JEOL scanning microscope operating at 20 keV.

2.10. Determination of copper removal

To determine copper removal from the culture medium, supernatant samples withdrawn at intervals of 3, 6, 9, 12 and 15 d were subjected to an atomic absorption spectrophotometer, model spectrophotometer (GBC 932 AA). All experiments were performed in triplicate. The biomass biosorption and the removal efficiency were evaluated. The assay was performed in triplicate. The sorption efficiency (%) was calculated according to the expressions:

$$\text{Sorption efficiency}\,\% = \frac{C_o - C_e}{C_o} \times 100 \qquad \text{[Equation 1]}$$

where C_o and C_e are the initial and equilibrium concentrations (mg L^{-1}).

2.11. Statistical analysis

To evaluate the influence of copper on growth, antioxidant enzyme activity, lipid peroxidation, polyphosphate, acid and alkaline phosphatase, and removal of copper, the data were submitted to variance analysis using Statistica 7.0 software. The means between treatments were compared by Tukey's t-test at 5% probability.

3. Results and discussion

3.1. Effects of copper on the growth profile

Initially, the behavior of the strain was evaluated on the characteristics of growth by increasing the concentrations of copper in the medium. The growth profile of *A. niger* at time intervals in cultures in the absence and presence of copper at concentrations of 0.5 mM, 1 mM and 2 mM, was associated with a corresponding glucose consumption. Fig. 1a and Fig. 1b, present the data obtained for isolated growth in the presence of copper and glucose consumption.

Approximately 58.45% of glucose was consumed in the indicated time intervals of the culture control during the first 3 d of cultivation and the cultures were treated with 0.5 mM, 1 mM and 2 mM copper respectively and 63.1%, 50.4% and 23.6% were consumed. Based on the growth curve, it appears that after 15 d of cultivation, although glucose consumption had occurred, all cultures kept growing even after the carbon source in the presence of the metal was exhausted (Fig. 1a). This represents the tolerance of *A. niger* to the presence of copper.

It was found that the length of time and concentration had an influence on the biomass of *A. niger*. Treatments with 0.5 mM and 1 mM copper did not differ statistically from the control culture and after 15 d of culture, an increase in the biomass concentration of 1 mM with 1.7 g/L was observed as was a control sample with 1.3 g/L. However, a significant reduction was observed in the biomass concentration of 2 mM copper, with 1.0 g/L being obtained (Fig. 1b).

Assessing the potential of growth of *A. niger* in the presence of copper, it was found that the fungus is able to grow at concentrations of up to 300 mg L^{-1} in the culture medium [33]. In this study, the growth of *A. niger*, accompanied by the production of biomass revealed that copper at concentrations of 0.5 mM, 1 mM and 2 mM has a positive effect on this variable. However, higher copper concentrations increased the lag phase and decreased the carbon source consumption. These data confirmed the information regarding the effects of copper on the growth of *A. niger*.

3.2. Effect of copper on total protein and lipid peroxidation (TBARS)

The data obtained from analyzing the TBARS in response to the presence and absence of copper in the cultivation time intervals are shown in Fig. 2a and Fig. 2b.

Treatment with copper in diverse concentrations induced the appearance of a distinct protein profile for all the samples tested (Fig. 2a).

The results showed that despite the changes in total protein content in the samples treated with copper, no significant differences were observed between treatments ($P > 0.05$). However, the content of total protein indicates that a significant effect on protein content has been found between cultivation times. There was an increase over the control which might demonstrate the inductive effect of copper content on protein isolated from *A. niger*.

Many microorganisms synthesize proteins, and are able to sequester metal ions, especially Cu, Pb and Cd, found at high concentrations in polluted soils. Metal transport proteins may be involved in metal tolerance either by extruding toxic metal ions from the cytosol out of the cell or by allowing metal sequestration into intracellular compartments [10,34].

In this study, increasing total protein content in response to copper (0.5, 1 and 2 mM) concentration and the protein levels can support the synthesis of key proteins involved in detoxification system *A. niger* against excess copper indicating that it is able to neutralize the toxic effect of heavy metals.

Throughout the experimental period, it was found that the control culture exhibited lower levels of lipid peroxidation compared to treated cultures (Fig. 2b). Treatments with 0.5 mM, 1 mM and 2 mM of copper induced an increase in the peak levels for TBARS according to time intervals. Treatment with 2 mM copper induced the highest

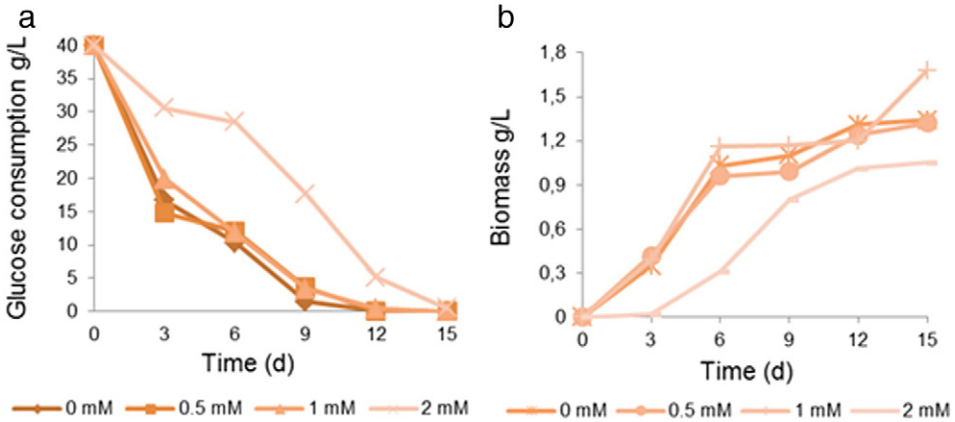

Fig. 1. (a) Glucose consumption and (b) biomass production of *A. niger* cultures within 15 d at 28°C and 150 rpm.

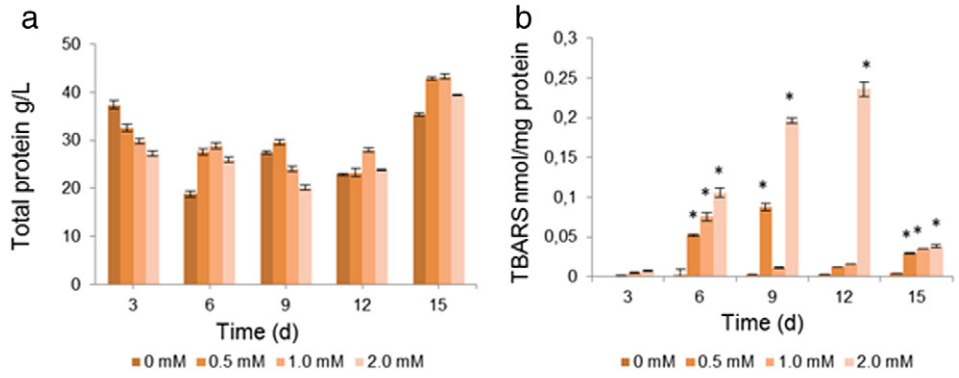

Fig. 2. (a) Total protein profile and (b) Lipid peroxidation of *A. niger* grown in the presence and absence of copper. Results are means (μ) ± standard deviations (σ) for thee replicates. * discriminates significant differences ($P < 0,5$) from control using Tukey test.

TBARS difference from the average, relative to treatment with 0.5 mM and 1 mM copper. Note that the TBARS levels among the treated samples varied according to the culture and differed significantly 5% from the control sample over time.

These data reveal that oxidative stress resulting, among others, in membrane lipid peroxidation is involved in the mechanisms of the toxicity of copper toxicity and suggests that this fungus exhibits (an ability to copper), to some extent, with the increased level of lipid peroxides. *Curvularia lunata* in the mycelia exposed to Ni^2, the levels of TBARS (lipid peroxidation products) increased and ranged between 156 and 823% over the control [35].

3.3. Activity of antioxidant enzymes

The results presented in Fig. 3a, Fig. 3b, Fig. 3c, and Fig. 3d reveal the activity of antioxidant enzymes — catalase, glutathione S transferase, glutathione peroxidase, and peroxidase, in response to the concentration of copper (0.5, 1 and 2 mM).

These results showed that the time and concentration of copper in catalase activity had a significant effect ($P < 0.05$). At all-time intervals, the catalase activity remains higher in samples treated with copper (Fig. 3a).

It appears that enzyme activity increases with the length of cultivation and that within 3 d of cultivation, catalase activity increased 50% in the samples treated with 0.5 mM and 1 mM copper respectively 52.6 UI/mg and 61.8 UI/mg compared to control with 26 UI/mg. Furthermore, the presence of copper at concentrations of 0.5 mM and 1 mM led to a progressive increase in enzymatic activity compared to the concentration of 2 mM with 40.46 UI/mg. The highest level of CAT was obtained at the end of the experiment after 15 d in culture with 110.26 UI/mg, 119.26 UI/mg and 90.68 UI/mg treatments respectively 0.5 mM, 1 mM and 2 mM compared to control with 64.4 UI/mg.

Results on the effect of exposure concentrations of copper and the interaction of time and copper on peroxidase activity were similar to those for catalase activity (Fig. 3b). It is observed that the presence of

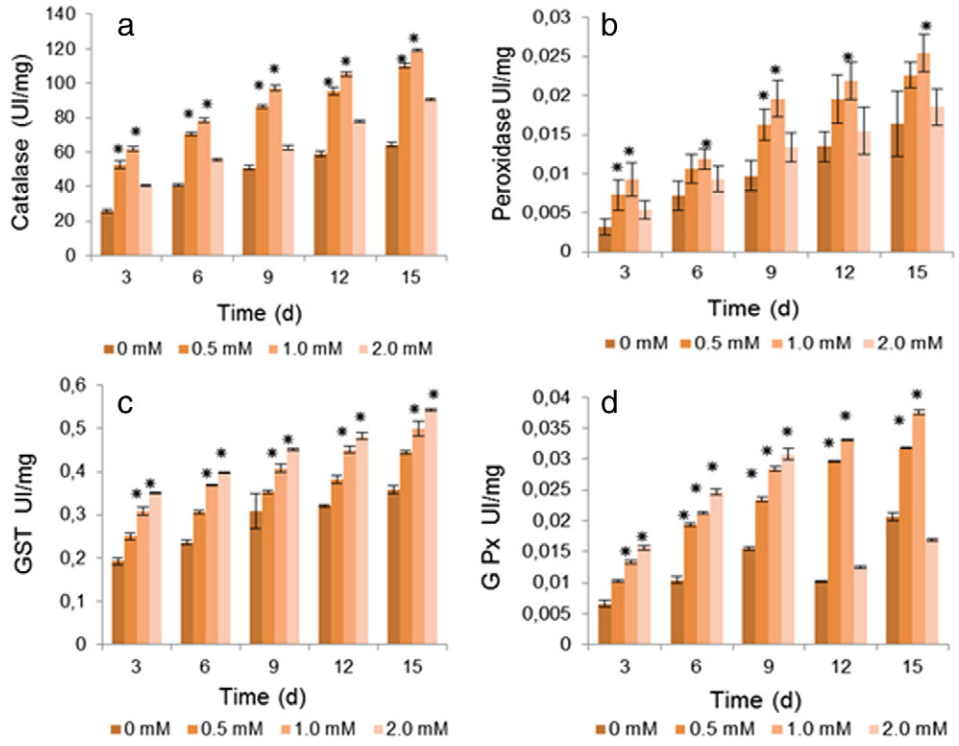

Fig. 3. Biological responses of *A. niger* apices exposed to Cu (a, b, c, and d) for the activity of the enzymes catalase, peroxidase, glutathione S-transferase (GST) and glutathione peroxidase (GPx) respectively, in the culture medium within 15 d. Results are means (μ) ± standard deviations (σ) for thee replicates. * discriminate significant differences ($P < 0.5$) from control using Tukey test.

metal in the culture medium resulted in increased activity compared to control culture ($P < 0.05$). Peroxidase activity in time intervals of cultivation among treated and control samples shows that a progressive increase in the activity of the enzyme was obtained at the end of the experiment after 15 d in culture. This increased 60% and demonstrated that time was a significant factor in the enzyme expression. The treatment with 0.5 and 1 mM copper with respectively 0.02262 UI/mg and 0.02546 UI/mg compared to control with 0.0164 UI/mg, and 2 mM 0.01852 UI/mg had the highest level of peroxidase activity.

On determining the activity of glutathione S transferase (GST), the positive effect of exposure to copper compared to control culture and the culture time was observed. The enzymatic response was dependent on the concentration of copper used (Fig. 3c). The data on the levels of glutathione S-transferase activity were increasing the time intervals between a 5% of significance. The results differing among the concentrations used achieved after 15 d of cultivation levels of 0.358 IU/mg 0.445 IU/mg, 0.5 IU/mg and 0.5434 IU/mg respectively stop control, 0.5 mM, 1.0 mM and 2 mM, thus verifying the inductive effect of copper on the activity of the enzyme.

The activity of glutathione peroxidase (GPx) in response to the presence and absence of copper in the time intervals in treated and control samples is shown in Fig. 3d. The glutathione peroxidase activity shows a progressive increase in the activity of the enzyme for treatment and control samples in the time intervals. It is observed that the presence of metal in the culture medium resulted in increased activity compared to control culture ($P < 0.05$). The treatment with 1 mM of copper had the highest level of GPx activity with 0.03758 UI/mg compared to control with 0.02066 UI/mg.

Cellular responses related to the adaptation process when the occurrence of oxidative stress includes increased activity of antioxidant enzymes and/or increasing the concentration of non-enzymatic antioxidant components [36].

Enzymes like CAT, which detoxifies hydrogen peroxide and GPx, which catalyzes the reduction of hydrogen peroxide and other peroxides, are essential to maintain cellular redox balance, and are activated during stress induced by heavy metals [11,37]. Additionally, glutathione S-transferase is the enzyme responsible for the conjugation of glutathione to electrophilic xenobiotics, thereby reducing its toxicity. This makes them more hydrophilic, thus enabling the conveyor system to eliminate these conjugates into the extracellular environment. They are metabolized via the mercapturic acid pathway [38].

In the present study it was demonstrated that the isolate of *A. niger*, obtained from the soil of caatinga, exhibited catalase, glutathione S-transferase, glutathione peroxidase, and peroxidase activities. These enzyme activities increased during exposure to copper. This result possibly indicates an induction mechanism of H_2O_2 formation. The increase in enzyme activities is a mechanism for metal detoxification via degradation of hydrogen peroxide, thus confirming the data in the literature.

A. niger has an activity of superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase and glutathione reductase in response to oxidative stress induced by the presence of hydrogen peroxide. The existence and activity of antioxidant enzyme catalase and superoxide dismutase have also been reported in the protection of the *A. niger* conidiophores subjected to thermal stress [39,40]. Several species of fungi under stress induced by copper have been shown to have activation of enzymes, such as catalase. An increase in peroxidase activity and a reduction in glutathione reductase have also been reported [41,42].

3.4. Effects of copper on polyphosphate behavior

The behavior of polyphosphate isolated from *A. niger* over time for the control samples and treated with copper sulfate is shown in Fig. 4.

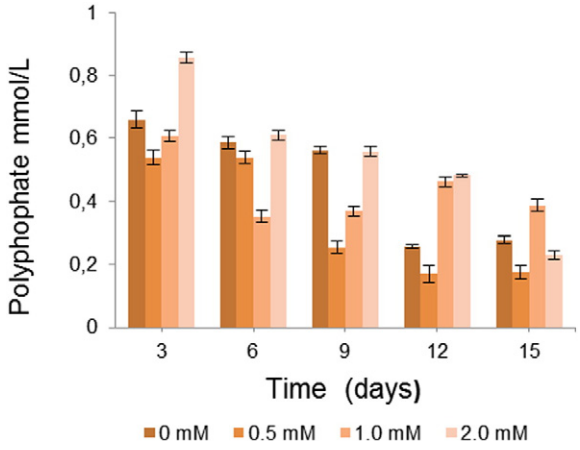

Fig. 4. Polyphosphate profile of *A. niger* cultivated in the absence and presence of copper.

The highest total polyphosphate was obtained at 3 d of cultivation for control cultures and those treated with 2 mM copper, with values of 0.66 mmoL L^{-1} and 0.86 mmoL L^{-1}, respectively. For treatment with 0.5 mM and 1 mM of copper, polyphosphate content of 0.54 mmoL L^{-1}, and 0.61 mmoL L^{-1} was obtained, respectively. The content of the polymer decreased over cultivation time. For the treatment with 2 mM, a decrease of 73% in the polymer was found after 15 d. The results revealed, for the treated and control cultures, that the cultivation time and metal concentration were important factors for the polymer behavior in *A. niger*, and showed significant variations at the level of 5%.

The data indicate the potential for polyphosphate accumulation by the fungus *A. niger*, as well as its degradation related to tolerance/survival in the presence of coper ions.

The detoxification of heavy metals, including copper, is also reported as dependent on the metabolism of polyphosphate. The polymer is associated with the potential of tolerance to heavy metals, as well as resistance to stress induced by heavy metals in various bacteria, yeasts and filamentous fungi [43,44,45,46,47,48].

The metabolism of polyphosphate was investigated in *Trichoderma harzianum* showed that the presence of cadmium induced a reduction in polyphosphate content related to the concentration used [49].

The presence of heavy metals, cells can activate two mechanisms: the increase in or accumulation of degradation of the polymer and the start of the synthesis cycle for the release of phosphorus sequestering and chelating of metal ions by reducing intracellular flow and consequently their toxicity [50,51,52].

In this study, we observed variations in the content of polyphosphate between control cultures and those exposed to copper. This variation was related to the length of cultivation, the growth phase, and the concentration of metal. Thus, reducing the polymer content in the cultures of *A. niger* exposed to metal revealed the reduction of the polymer, indicating its use to maintain cell viability, which supports the functions of the metal in the maintenance of cell metabolism. Additionally, the data obtained for control cultures also demonstrate the use of metal at different stages of growth. These data are the first reports of the behavior of polymer *A. niger*.

3.5. Acid and alkaline phosphatase behavior

The results obtained for the intracellular acid and alkaline phosphatases detected in treated and control samples are presented in Fig. 5a, Fig. 5b.

High levels of acid phosphatase activity of control and treated samples were evaluated in relation to the culture period. Variance analysis showed significant variations for the cultivation time.

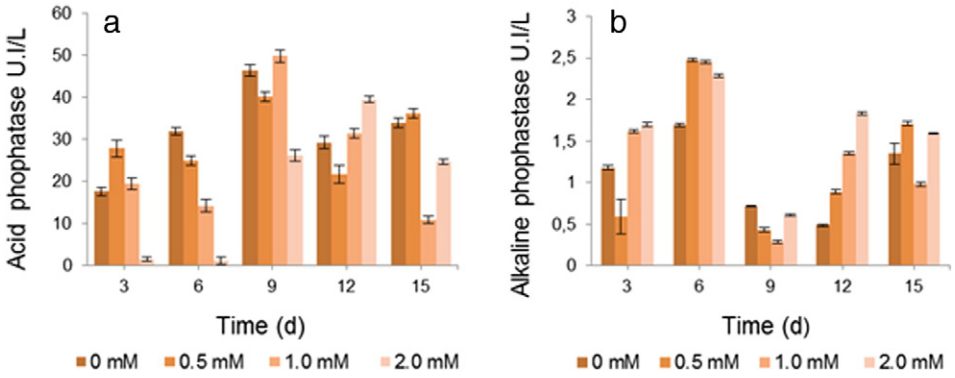

Fig. 5. Intracellular phosphatases of *A. niger* grown in the presence and absence of copper. (a) Acid phosphatase; (b) alkaline phosphatase.

The level of acid phosphatase at 3 d of culture for culture control was 17.56 IU/L. Samples treated with 0.5 mM, 1 mM and 2 mM of copper exhibited 27.8 IU/L, 19.39 IU/L. and 1.45 IU/L, respectively. The highest enzymatic activities were obtained after 9 d of cultivation. The control culture exhibited a level of 46.24 IU/L and treatment with 0.5 mM and 1 mM of copper revealed activities of 40.24 IU/L and 49.83 IU/L. The sample exposed to 2 mM of copper exhibited the highest activity, 39.51 IU/L, after 12 d of cultivation.

The results obtained for alkaline phosphatase activity also showed different values among samples during the time intervals. After 6 d of cultivation, increased levels of the enzyme were detected in the cultures treated with 0.5 mM, 1 mM and 2 mM of copper, respectively, which corresponded to 2.48 IU/L, 2.45 IU/L and 2.29 IU/L, an increase of 25% compared to the control with 1.69 IU/L. At intervals of 9 and 12 d phosphatase activity varied for samples treated with 0.5 mM, 1 mM and 2 mM copper, and maintained a behavior similar to the control culture. At 15 d of cultivation, values were very similar between the treated and control sample. As demonstrated by the variance analysis at the 5% level, there was no significant effect on the enzyme activity related to copper concentrations. However, it was identified that cultures treated with copper had a slight increase in phosphatase activity, which was proportional to the concentration, thus showing an inductive effect of exposure to copper.

The results obtained for alkaline phosphatase activity also demonstrated a significant effect of culture time on the expression of enzymatic activity.

The activity of the phosphatase also induced copper in *A. niger*, was described by Tsekova and Todorova [33]. Where observed the increased activity of the phosphatase with exposure to copper. Acid phosphatase activity in *Yarrowia lipolytica* increased with increasing concentrations of Cu^{2+} in the medium. In addition, the content of phosphate involving cellular polyphosphate was reduced by the addition of Cu^{2+} [53].

The microorganisms to reduce the concentration of free heavy metal, may sequester the form of phosphate salts. The hydrolysis of organic phosphorus acid phosphatase and by a concurrent precipitation of heavy metals on the surface of the cell help to protect and enables these support microorganisms to tolerate higher doses of heavy metals [2].

3.6. Scanning electron microscopy

Ultrastructural analysis showed changes in the density of mycelia, the electron density pattern, the texture of the cell surface and the thickness of hyphae in treated samples compared to control culture. The intensity variations are related to the concentration of the metal to which cells were exposed (Fig. 6).

Heavy metals are reported to induce structural changes and the effects associated with the type of metal and its concentration. Basically, exposure to metals and their subsequent connection modify the properties of the structure, such as the texture of electron density [41,45,47]. The data obtained in this study confirm the effects of copper in ultrastructure *A. niger*, isolated from soil of the caatinga.

The reports about the tolerance of microorganisms to copper display data indicated that within the same genus, species and strains react differently to different heavy metals and their concentrations.

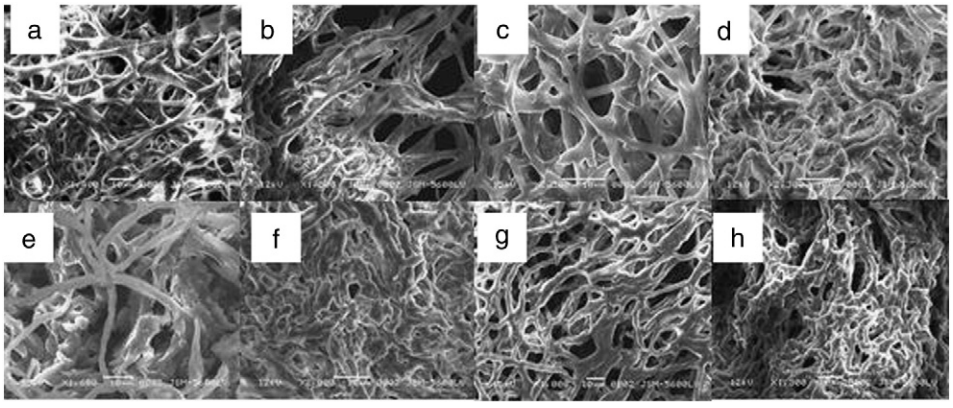

Fig. 6. Electron micrographs of *A. niger*. (a–b) Control samples, 3 and 15 d; (c–h) samples exposed to copper sulfate; (c–d) 0.5 mM, after 3 and 15 d; (e–f) 1 mM, with 3 and 15 d; (g–y) 2 mM, after 3 and 15 d.

Additionally, the sensitivity is also related to the stage of cell growth. Thus, these studies do not enable a concentration limit to be determined. For example, while other microorganisms can only tolerate up to 6000 mg/kg of metal, showing metal resistance mechanisms, adsorption and intracellular compartmentalization [54].

In this study, data on the ability of removing copper revealed that the strain was able to remove a high percentage of the metal, when exposed to copper during growth and has metabolically active cells, suggesting the involvement of cumulative processes and binding to the cell surface, related to the presence of chitin and chitosan in its walls.

3.7. Copper removal

The results revealed that *A. niger* sample was able to remove the metal from culture supernatant. Additionally, the removal depended on the initial concentration used. Fig. 7 shows the residual copper in the medium.

Cells exposed to 0.5 mM and 1 mM of copper showed higher removal percentages compared to cultures treated with 2 mM. At 3 d of cultivation, the corresponding percentages of removal were 31.76%, 21.39% and 12.72% for cultures exposed to 0.5 mM, 1 mM and 2 mM of copper, respectively. At the end of 15 d of cultivation, the cultures exposed to 0.5 mM, 1 mM and 2 mM of copper exhibited percentages of removal equivalent to 75.78%, 66.04% and 33.51%. The percentage removal was used to perform the variance analysis relating the time of cultivation and the concentration of copper. The data express a significance level of 5%.

In this study, the data show that the strain was able to show growth even with the reduction of biomass, in concentrations of up to 2 mM of copper. This finding was confirmed by the expression of adaptive responses and tolerance that allowed cell survival.

The genus Aspergillus fungi is considered to be a fungus which has one of the greatest potential biotechnological and industrial uses nowadays due to its potential for producing numerous metabolites [15,55,56].

Microbial cells are used in bioremediation and biotransformation processes of textile dyes, oil and oil products, pesticides and heavy metals [2,56,57]. Thus, the biomass of fungi is an excellent sorbent material for metal ions [2,48].

Data in the literature show that microorganisms exhibit high potential for the removal and mobilization of copper. This removal can be performed efficiently by bacteria such as Pseudomonas, Bacillus, Staphylococcus, and yeasts such as Candida, Saccharomyces, Kluyveromyces, Schizosaccharomyces and filamentous fungi, such as Aspergillus, Mucor, Rhizopus, Trichoderma and Cunninghamella [2].

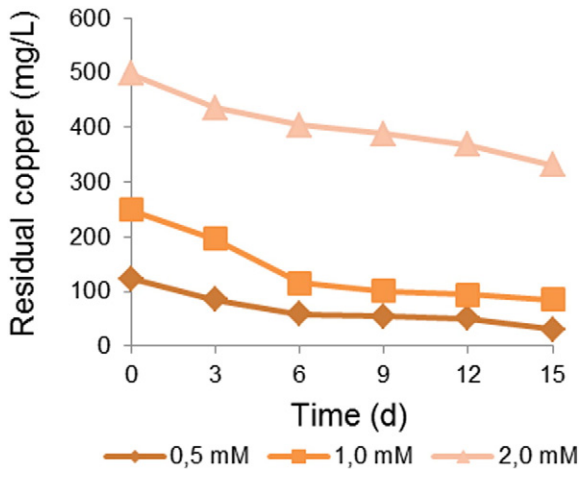

Fig. 7. Removal of copper ions by *A. niger* UCP/WFCC 1261 in the culture medium within 15 d at 28°C and 15 rpm.

Therefore, we evaluated the potential for copper removal in *A. niger* isolate from soil of the caatinga. The data presented show that the isolate removed copper in stages during growth, such that by the end of the experiment, the percentage removed was 75.78%, 66.04% and 33.51% at concentrations of 0.5 mM, 1 mM and 2 mM, thus indicating there were greater efficiencies at lower metal content.

The species *A. niger* has been shown to be effective in the biotransformation of pesticides [57]. Additionally, many strains of *A. niger* have been extensively tested for their ability to leach and remediate heavy metals, lead, cadmium, nickel, zinc, copper, based on their capacity for physiological adaptation and tolerance resistance [58,59,60,61,62,63].

The cell walls of fungi act as a first barrier restricting the internalization of solutes. Chitin, chitosan, glycoproteins and melanin, the main components of cell walls of fungi, confer protection against metal ions [2]. The metals bound to the walls act like other materials used in ion exchange, adsorption, complexation, precipitation and crystallization [2]. Thus, it has been reported that yeast tolerant metals exhibit a higher binding potential of metals in relation to a wall of lesser tolerance. Some studies have shown that the walls of fungi can retain from 37% to 77% of copper [64].

4. Conclusions

It was found that there was influence of copper on the growth of the *A. niger* isolate UCP/WFCC 126, used in this study and a significant decrease in biomass concentration of 2 mM was observed. The isolate showed changes in the protein profiles directly related to the concentration of copper, and the microorganism was able to keep the content of proteins in the presence of copper in the medium which may suggest a possible detoxification engine due to exposure to this metal. The lipid peroxidation was observed as a result of exposure to the copper. The isolate exhibited activity of catalase, glutathione S-transferase, glutathione peroxidase, and peroxidase. These activities in cells exposed to copper were increased, revealing the activation of antioxidant response against stress induced by metal. The behavior of the polyphosphate had changed, indicating the role of polymer molecular responses of tolerance to stress induced by copper. Modifications of the fine structure, such as increased electron density, septation and shortening of the hyphae were observed. The intensity of the changes was proportional to the concentration of the copper. The isolate was able to remove the metal from the cultivation medium and has the potential for higher remediation. These data indicate the physiological and biotechnological potentials of the isolate, which should be evaluated more specifically for the development of bioprocesses.

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References

[1] Dixit RW, Deepti Malaviya D, Kuppusamy Pandiyan K, Singh UB, Asha Sahu A, Shukla R, et al. Bioremediation of heavy metals from soil and aquatic environment: An

overview of principles and criteria of fundamental processes. Sustainability 2015;7: 2189–212. http://dx.doi.org/10.3390/su7022189.
[2] Gadd GM. Metals, minerals and microbes: Geomicrobiology and bioremediation. Microbiology 2010;156:609–43. http://dx.doi.org/10.1099/mic.0.037143-0.
[3] Callender E. Heavy metals in the environment — Historical trends. Treatise Geochem 2003;9:67–105. http://dx.doi.org/10.1016/B0-08-043751-6/09161-1.
[4] Järup L, Akesson A. Current status of cadmium as an environmental health problem. Toxicol Appl Pharmacol 2009;238:201–8. http://dx.doi.org/10.1016/j.taap.2009.04.020.
[5] Elekwachi CO, Andresen J, Hodgman TC. Global use of bioremediation technologies for decontamination of ecosystems. J Biorem Biodegrad 2014;5:1–9. http://dx.doi.org/10.4172/2155-6199.1000225.
[6] Simeonov LI, Kochubovski MV, Simeonova BG, editors. Environmental heavy metal pollution and effects on child mental development: Risk assessment and prevention strategies. Springer; 2010.
[7] Ali H, Khan E, Sajad MA. Phytoremediation of heavy metals-concepts and applications. Chemosphere 2013;91:869–81. http://dx.doi.org/10.1016/j.chemosphere.2013.01.075.
[8] Buss W, Kammann C, Koyro HW. Biochar reduces copper toxicity in *Chenopodium quinoa* willd. In a sandy soil. J Environ Qual 2012;41:1157–65.
[9] Achal V, Pan X, Zhang D. Remediation of copper-contaminated soil by *Kocuria flava* CR1, based on microbially-induced calcite precipitation. Ecol Eng 2011;37:1601–5. http://dx.doi.org/10.1016/j.ecoleng.2011.06.008.
[10] Bellion M, Courbot M, Jacob C, Blaudez D, Chalot M. Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. FEMS Microbiol Lett 2006;254:173–81. http://dx.doi.org/10.1111/j.1574-6968.2005.00044.x.
[11] Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. J Free Radic Biol Med 1985;1:331–2. http://dx.doi.org/10.1016/0748-5514(85)90140-0.
[12] Valko M, Morris H, Cronin MTD. Metals, toxicity and oxidative stress. Curr Med Chem 2005;12:1161–208. http://dx.doi.org/10.2174/0929867053764635.
[13] Jomova AM, Valko M. Advances in metal-induced oxidative stress and human disease. Toxicology 2011;283:65–87. http://dx.doi.org/10.1016/j.tox.2011.03.001.
[14] Rao MA, Scelza R, Scotti R, Gianfreda L. Role of enzymes in the remediation of polluted environments. J Soil Sci Plant Nutr 2010;10:333–53. http://dx.doi.org/10.4067/S0718-95162010000100008.
[15] Dijksterhuis J, Wösten HAB. Development of *Aspergillus niger*. CBS-KNAW Fungal Biodiversity Centre. Colloids Surf A 2013;74.
[16] Amini M, Younesi M, Bahramifar N. Statistical modeling and optimization of the cadmium biosorption process in an aqueous solution using *Aspergillus niger*. Colloids Surf A 2009;337:67–73. http://dx.doi.org/10.1016/j.colsurfa.2008.11.053.
[17] Andersen MR, Salazar MP, Schaap PJ, de Vondervoort PJI Van, Culley D, Thykaer J, et al. Comparative genomics of citric-acid-producing *Aspergillus niger* ATCC 1015 versus enzyme-producing CBS 513.88. Genome Res 2011;21:885–97. http://dx.doi.org/10.1101/gr.112169.110.
[18] Abdullah-Al-Mahin, Sharifuzzaman ABM, Faruk MO, Kader MA, Alam J, Begum R, et al. Improved citric acid production by radiation mutant *Aspergillus niger* using sugarcane bagasse extract. Biotechnology 2012;11:44–9. http://dx.doi.org/10.3923/biotech.2012.44.49.
[19] Mudhoo A, Garg VK, Wang S. Removal of heavy metals by biosorption. Environ Chem Lett 2012;10:109–11. http://dx.doi.org/10.1007/s10311-011-0342-2.
[20] Ouedraogo JP, Hagen S, Spielvogel LA, Engelhardt S, Meyer V. Survival strategies of yeast and filamentous fungi against the antifungal protein AFP. J Biol Chem 2011; 286:13859–68. http://dx.doi.org/10.1074/jbc.M110.203588.
[21] Damveld RA, Arentshorst M, Franken A, Van Kuyk PA, Klis FM, den Hondel CA Van, et al. The *Aspergillus niger* MADS-box transcription factor RlmA is required for cell wall reinforcement in response to cell wall stress. Mol Microbiol 2005;58:305–19. http://dx.doi.org/10.1111/j.1365-2958.2005.04827.x.
[22] Meyer V, Damveld RA, Arentshorst M, Stahl U, Van Den Hondel CAMJJ, Ram AFJ. Survival in the presence of antifungals: Genome-wide expression profiling of *Aspergillus niger* in response to sublethal concentrations of caspofungin and fenpropimorph. J Biol Chem 2007;282:32935–48. http://dx.doi.org/10.1074/jbc.M705856200.
[23] Henry RJ, Cannon DC, Winkelman JW. Principles and techniques. Clinical chemistry. 2nd ed. Hagerstown, MD: Harper and Row; 1974.
[24] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[25] Aebi H. Catalase in vitro. Methods Enzymol 1984;105:121–6. http://dx.doi.org/10.1016/S0076-6879(84)05016-3.
[26] Habig WH, William BJ. Assays for differentiation of glutathione S-transferases. Methods Enzymol 1981;77:398–405. http://dx.doi.org/10.1016/S0076-6879(81)77053-8.
[27] Wendel A, Feuerstein S. Drug-induced lipid peroxidation in mice — I Modulation by monooxegenase activity, glutathione and selenium status. Biochem Pharmacol 1981;30:2513–20. http://dx.doi.org/10.1016/0006-2952(81)90576-1.
[28] Halpin B, Pressey R, Jen J, Mondy N. Purification and characterization of peroxidase isoenzymes from green peas (*Pisum sativum*). J Food Sci 1989;54:644–9. http://dx.doi.org/10.1111/j.1365-2621.1989.tb04672.x.
[29] Draper HH, Hadley M. Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol 1990;186:421–31. http://dx.doi.org/10.1016/0076-6879(90)86135-I.
[30] McGrath JW, Quinn JP. Intracellular accumulation of polyphosphate by the yeast *Candida humicola* G-1 in response to acid pH. Appl Environ Microbiol 2000;66: 4068–73. http://dx.doi.org/10.1128/AEM.66.9.4068-4073.2000.
[31] Fiske CH, Subbarow Y. The colorimetric determination of phosphorus. J Biol Chem 1925;66:375–400.
[32] Joh T, Malick DH, Yazaki J, Hayakawa T. Purification and characterization of secreted acid phosphatase under phosphate-deficient condition in *Pholiota nameko*. Mycoscience 1996;37:65–70. http://dx.doi.org/10.1007/BF02461459.
[33] Tsekova K, Todorova D. Copper (II) accumulation and superoxide dismutase activity during growth of Aspergillus niger B-77. Z Naturforsch 2002;57:319–22. http://dx.doi.org/10.1515/znc-2002-3-421.
[34] Jarosławiecka A, Piotrowska-Seget Z. Lead resistance in microorganisms. Microbiology 2014;160:12–25. http://dx.doi.org/10.1099/mic.0.070284-0.
[35] Paraszkiewicz K, Bernat P, Naliwajski M, Długoński J. Lipid peroxidation in the fungus *Curvularia lunata* exposed to nickel. Arch Microbiol 2010;192:135–41. http://dx.doi.org/10.1007/s00203-009-0542-3.
[36] Gaetke LM, Chow CK. Copper toxicity, oxidative stress, and antioxidant nutrients. Toxicology 2003;189:147–63. http://dx.doi.org/10.1016/S0300-483X(03)00159-8.
[37] Pócsi I, Prade RA, Penninckx MJ. Glutathione, altruistic metabolite in fungi. Adv Microb Physiol 2004;49:1–76. http://dx.doi.org/10.1016/S0065-2911(04)49001-8.
[38] Yang J, Wei X, Xu J, Yang D, Liu X, Yang J, et al. A sigma-class glutathione S-transferase from *Solen grandis* that responded to microorganism glycan and organic contaminants. Fish Shellfish Immunol 2012;32:1198–204. http://dx.doi.org/10.1016/j.fsi.2012.03.010.
[39] Abrashev R, Dolashka P, Christova R, Stefanova L, Angelova M. Role of antioxidant enzymes in survival of conidiospores of *Aspergillus niger* 26 under conditions of temperature stress. J Appl Microbiol 2005;99:902–9. http://dx.doi.org/10.1111/j.1365-2672.2005.02669.x.
[40] Abrashev R, Pashova SB, Stefanova LN, Vassilev SV, Dolashka PAA, Angelova MB. Heat-shock-induced oxidative stress and antioxidant response in *Aspergillus niger* 26. Can J Microbiol 2008;54:977–83. http://dx.doi.org/10.1139/W08-091.
[41] Kim IS, Kim YS, Yoon HS. Glutathione reductase from *Oryza sativa* increases acquired tolerance to abiotic stresses in a genetically modified *Saccharomyces cerevisiae* strain. J Microbiol Biotechnol 2012;22:1557–67. http://dx.doi.org/10.4014/jmb.1202.02028.
[42] Braha B, Tintemann H, Krauss G, Ehrman J, Bärlocher F, Krauss GJ. Stress response in two strains of the aquatic hyphomycete *Heliscus lugdunensis* after exposure to cadmium and copper ions. Biometals 2007;20:93–105. http://dx.doi.org/10.1007/s10534-006-9018-y.
[43] Keasling JD. Regulation of intracellular toxic metals and other cations by hydrolysis of polyphosphate. Ann N Y Acad Sci 1997;829:242–9. http://dx.doi.org/10.1111/j.1749-6632.1997.tb48579.x.
[44] Keasling JD, Van Dien SJ, Renninger N, Mcmahon K. Application of polyphosphate metabolism to environmental and biotechnological problems. Biochemistry 2000; 65:324–31.
[45] Lima MABD, Nascimento AED, Souza WD, Fukushima K, Campos-Takaki GMD. Effects of phosphorus on polyphosphate accumulation by *Cunninghamella elegans*. Braz J Microbiol 2003;34:363–72. http://dx.doi.org/10.1590/S1517-83822003000400016.
[46] Alvarez S, Jerez CA. Copper ions stimulate polyphosphate degradation and phosphate efflux in *Acidithiobacillus ferrooxidans*. Appl Environ Microbiol 2004;70: 5177–82. http://dx.doi.org/10.1128/AEM.70.9.5177-5182.2004.
[47] Souza PM, Marinho PH, Lima MAB, Nascimento AE, Campos GMT. Copper influence on polyphosphate metabolism of *Cunninghamella elegans*. Braz J Microbiol 2005;36: 315–20. http://dx.doi.org/10.1590/S1517-83822005000400002.
[48] Remonsellez F, Orell A, Jerez CA. Copper tolerance of the thermoacidophilic archaeon *Sulfolobus metallicus*: Possible role of polyphosphate metabolism. Microbiology 2006;152:59–66. http://dx.doi.org/10.1099/mic.0.28241-0.
[49] Freitas de Lima A, Ferreira de Moura G, de Lima MA Barbosa, Mendes de Souza P, da Silva CA Alves, Campos Takaki GM, et al. Role of the morphology and polyphosphate in *Trichoderma harzianum* related to cadmium removal. Molecules 2011;16: 2486–500. http://dx.doi.org/10.3390/molecules16032486.
[50] Lima MAB, Franco LDO, de Souza PM, do Nascimento AE, Silva CA, Maia RDC, et al. Cadmium tolerance and removal from *Cunninghamella elegans* related to the polyphosphate metabolism. Int J Mol Sci 2013;14:7180–92. http://dx.doi.org/10.3390/ijms14047180.
[51] Seufferheld MJ, Alvarez HM, Farias ME. Role of polyphosphates in microbial adaptation to extreme environments. Appl Environ Microbiol 2008;74:5867–74. http://dx.doi.org/10.1128/AEM.00501-08.
[52] Rao NN, Gómez-García MR, Kornberg A. Inorganic polyphosphate: Essential for growth and survival. Annu Rev Biochem 2009;78:605–47. http://dx.doi.org/10.1146/annurev.biochem.77.083007.093039.
[53] Ito H, Inouhe M, Tohoyama H, Joho M. Effect of copper on acid phosphatase activity in yeast Yarrowia lipolytica. Z Naturforsch 2007;62:70–6. http://dx.doi.org/10.1515/znc-2007-1-213.
[54] Umrania VV. Bioremediation of toxic heavy metals using acidothermophilic autotrophes. Bioresour Technol 2006;97:1237–42. http://dx.doi.org/10.1016/j.biortech.2005.04.048.
[55] Bennett JW. An overview of the genus *Aspergillus*. In: Machida M, Gomi K, editors. *Aspergillus* molecular biology and genomics. Linton, UK: Caister Academic Press; 2010. p. 1–16.
[56] Adrio JL, Demain AL. Fungal biotechnology. Int Microbiol 2003;6:191–9. http://dx.doi.org/10.1007/s10123-003-0133-0.
[57] Kanaly RA, Kim IS, Hur HG. Biotransformation of 3-methyl-4-nitrophenol, a main product of the insecticide fenitrothion, by *Aspergillus niger*. J Agric Food Chem 2005;53:6426–31. http://dx.doi.org/10.1021/jf050679w.
[58] Kapoor A, Viraraghavan T. Heavy metal biosorption sites in *Aspergillus niger*. Bioresour Technol 1997;61:221–7. http://dx.doi.org/10.1016/S0960-8524(97)00055-2.
[59] Kapoor A, Viraraghavan T, Cullimore DR. Removal of heavy metals using the fungus *Aspergillus niger*. Bioresour Technol 1999;70:95–104. http://dx.doi.org/10.1016/S0960-8524(98)00192-8.
[60] Magyarosy A, Laidlaw R, Kilaas R, Echer C, Clark DS, Keasling JD. Nickel accumulation and nickel oxalate precipitation by *Aspergillus niger*. Appl Microbiol Biotechnol 2002; 59:382–8. http://dx.doi.org/10.1007/s00253-002-1020-x.

[61] Liu YG, Fan T, Zeng GM, Li X, Tong Q, Ye F, et al. Removal of cadmium and zinc ions from aqueous solution by living *Aspergillus niger*. Trans Nonferrous Metals Soc China 2006;16:681–6. http://dx.doi.org/10.1016/S1003-6326(06)60121-0.

[62] Santhiya D, Ting YP. Use of adapted *Aspergillus niger* in the bioleaching of spent refinery processing catalyst. J Biotechnol 2006;121:62–74. http://dx.doi.org/10.1016/j.jbiotec.2005.07.002.

[63] Amiri F, Yaghmaei S, Mousavi SM, Sheibani S. Recovery of metals from spent refinery hydrocracking catalyst using adapted *Aspergillus niger*. Hydrometallurgy 2011;109: 65–71. http://dx.doi.org/10.1016/j.hydromet.2011.05.008.

[64] Gabriel J, Baldrian P, Hladíková K, Hladíková M. Copper sorption by native and modified pellets of wood-rotting basidiomycetes. Lett Appl Microbiol 2001;32: 194–8. http://dx.doi.org/10.1046/j.1472-765x.2001.00888.x.