



## Research Article

# Proteomic analysis of a copper mine isolated fungus *Rhizopus microspores* IOC 4686 when exposed to copper sulfate

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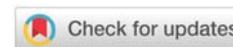
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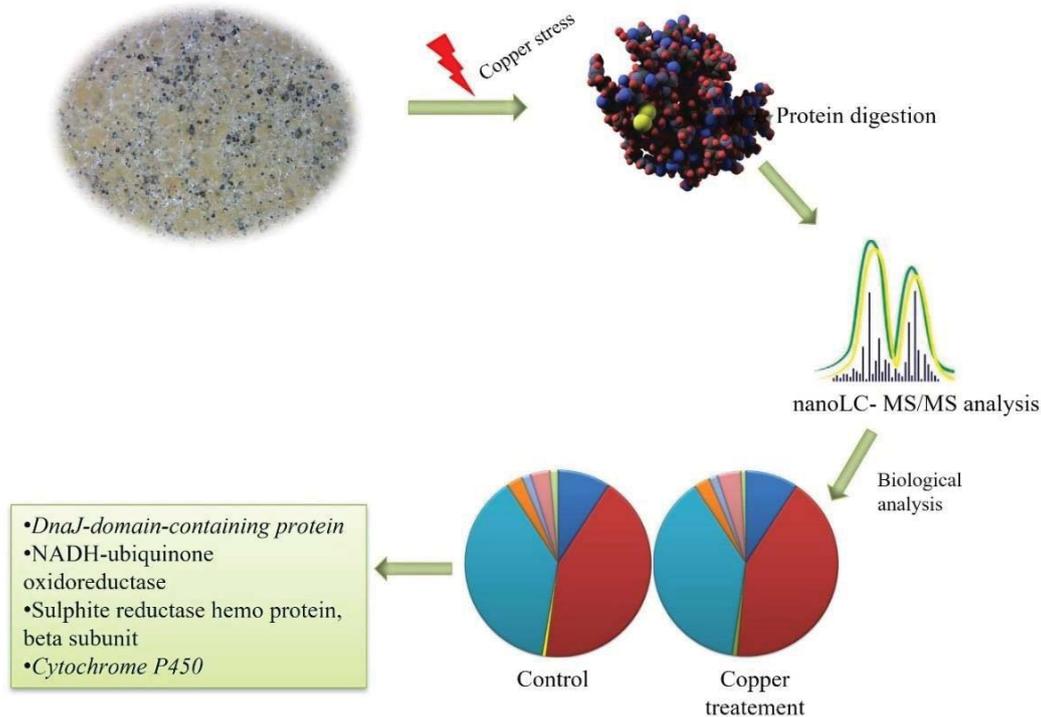
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## Abstract

The fungus *Rhizopus microsporus*, known for its absorption capacity for toxic metals was used to understand the green metal recovery via analysis of its physiology under metal stress conditions through proteomic methods. To investigate the effects of copper stress on fungus, *R. microsporus* IOC 4686, isolated from the mine environment, was exposed to copper ions (50mgL<sup>-1</sup>) for 48 h. This study was performed only on copper exposure. Tryptic and chymotryptic extracts of proteins were analyzed by nano- liquid chromatography-tandem mass spectrometry, identification was performed by PEAKS Studio 8.5. Proteins were classified according to their molecular function and biological process. Enzymes, such as catalase, superoxide dismutase, and cytochrome c peroxidase were found in the presence and absence of copper ions. However, only in presence of copper ions, was observed the presence of heat shock proteins (HPS 20, HPS 70, and HPS 78) and metalloproteins (GrpE protein homolog and cytochrome P450). These classes of proteins have been produced by cells in response to stress conditions. The control group (absence of copper ions) also presented antioxidant enzymes suggesting that the fungus isolated from the mine environment already has adapted to the copper. The presence of these proteins suggested a physiological response of *R. microsporus* IOC 4686 to oxidative stress induced by copper.

*Rhizopus microsporus* IOC 4686

## Graphical abstract

## Introduction

Oxidative stress is the unbalance in the regulatory system that causes physiological alteration of microorganisms to compensate for the changing environment by generating molecules to neutralize radical species through oxygen metabolism [1]. The oxidative stress of microorganisms has aroused great interest due to the relationship between the physiological responses and the damage suffered by cellular metabolism.

Toxic metals, when exceeding the resistance capacity of organisms, induce physiological damage that interferes in biological processes, such as enzymatic activity, DNA transcription, and translation or cell integrity. In general, metals such as copper and chromium, despite having an important role in biochemistry, can be toxic at high concentrations [2].

The expansion of mining processes has quantitatively increased the complexity of toxic waste released into the environment. In this sense, the extraction of copper, mainly in Brazil, increased by 90% between 1998 and 2015 due to primary and secondary copper production. Thus, it was estimated that in 2030, national metal production will reach up to 374,000 tons/year, and Brazilian reserves were estimated at 21 million tons [3]. Screening of processes to reduce the environmental damage produced by toxic metals has intensified in recent years. Some studies have investigated the application of microorganisms, such as bacteria, microalgae, and fungi, in bioremediation processes, which have been adapted to reduce toxic metal concentrations [4,5]. In general, compared with conventional methods such as precipitation, reverse osmosis,

or ion exchange, 50–65% of the costs could be saved by applying bioremediation to treat the soil Pb- polluted [2].

Filamentous fungi are a versatile group in bioremediation processes, as they can grow under extreme temperatures, nutrient availability, toxic metal conditions, and pH variations (Anand, et al. 2006). Filamentous fungi react differently when exposed to pollutants, producing upregulation or downregulation responses through organic compounds, mainly proteins, although their use requires a complete understanding of response mechanisms [6]. Protein response, such as heat shock and Superoxide dismutase is used as conventional biomarkers due to the well-characterized presence in oxidoreductive intracellular alterations [7]. *Rhizopus* is a fungus genus known for its ability to sequester and precipitate metals, producing intracellular and extracellular enzymes which can be used for metal recovery from contaminated soils [8,9].

Proteomics is one of the most recent techniques used in the identification of new biomarkers, which can be proteins or other cellular components that react specifically to the presence of a pollutant, indicating environmental damage [10]. Therefore, proteomics approaches provide remarkable information on protein expression in different environments (e.g., polluted and unpolluted), enabling the use of proteins as biomarkers [7,10]. The present work studied the proteomic profile from *R. microsporus* strain IOC 4686 grown in the presence of copper. NanoLC-MS/MS system was used to identify proteins Cu<sup>2+</sup>-response. This study aimed to use proteomic profiles to better understand of bioremediation process and improve the in-situ bioremediation.

## Materials and methods

### *R. microsporus* fungus

*R. microsporus* strain IOC 4686 was isolated from a mine environment (local copper concentration of about 80mgkg<sup>-1</sup>) located in Pará, Brazil. Fungal identification was performed using the MALDI-TOF Score: 2.216) BioTyper 3.1 database (Bruker Daltonics, Germany) [11]. Isolated fungal strains were morphologically identified by FioCruz Institute and kept in its filamentous fungus collection.

### Fungal growth and inhibition

The fungus was inoculated and maintained in Potato Dextrose Agar (PDA), Sigma Aldrich solution (42gL<sup>-1</sup>). The inoculum was performed using the spores collected after 10 days at 25°C, from solid culture. The spores (6.7×10<sup>6</sup>/ mL<sup>-1</sup>) were suspended in glycerol solution (20% v/v), and stored at -80°C [4]. These inoculums were used in the fungal growth and inhibitory concentration tests.

Fungal growth inhibition was determined by exposing *R. microsporus* IOC 4686 to a PDB medium containing copper ions (5–120mgL<sup>-1</sup>) [12,13], at 30°C for 80h under 150rpm.

Biomass was determined by sampling 5ml every 8h, filtering in 0.45-µm pore membranes (Merck Millipore). After filtration, the membranes were microwaved at 228W for 15min and weighed to obtain the fungal mass [14]. The weight obtained for each sampling was used to calculate the growth rate. It was plotted the time of reaction by the weight of biomass.

To determine stress induced by copper, the fungus was grown in a PDB medium in the absence (control group) and presence of 50mgL<sup>-1</sup> of copper ions (treated group). The experiments were carried out in triplicate.

### Determination of copper biosorption

To determine metal ion absorption, the supernatants of cultures were collected after 48 and 60 h of growth in a medium containing 50mgL<sup>-1</sup> of copper ions. Elemental composition was determined directly by the EDX instrument (Epsilon 3-XL-PANalytical) operated in 50kV, 99µA, standard detector, air drag medium configuration. Samples and standards were dispensed in 32 mm diameter vessels fitted with a 6µm polypropylene (PP) film.

### Proteomic analysis

Fungus biomass was collected and lyophilized for protein extraction. Proteins were extracted by cell disruption by adding 50 µL of 70% formic acid (MS grade) to each sample (0.2mg) and shaken for 2min. Then, 50µL of acetonitrile was added, and samples were sonicated (Ultronique-Q30140A) for 15min at room temperature. The extracts were centrifuged (Mini Spin-Eppendorf) at 17,400×g for 30min at 6°C, and supernatants were collected for protein quantification. Protein concentrations were determined by measuring the absorbance at 595nm in a spectrophotometer (UV2600, Shimadzu) using the Bradford assay (Sigma-Aldrich) with bovine serum albumin as standard [15].

## Identification of proteins by nanoLC-tandem mass spectrometry

Protein extract samples (2mg) were digested in a 1:50 (enzyme: substrate) ratio using two enzymes, trypsin (Sigma-Aldrich) and chymotrypsin (Roth). Trypsin was prepared in 400µL of NH<sub>4</sub>HCO<sub>3</sub> solution (50mM) to reach a final concentration of 0.05µg µL<sup>-1</sup>, according to the manufacturer's instructions. Subsequently, 5µL of the trypsin solution was added to each sample and incubated at 37°C for 24h. Chymotrypsin digestion of protein was performed by adding 50 µL of enzyme solution to each sample, incubating at 25°C for 24h. The digestion was stopped by adding 10µL of 10% (v/v) trifluoroacetic acid (TFA- Sigma-Aldrich) solution for 90min, at 37°C and 30°C for trypsin for chymotrypsin, respectively.

The peptides solution of each digestion was analyzed separately, using a nanoLC System (Thermo Fisher Scientific), containing a PepMap column (15cm×75µm; Thermo Scientific) by using a gradient from 2 to 98% (v/v) acetonitrile with 0.1% TFA for 180 min [5,16] and ESI-Q-TOF mass spectrometry (Impact II mass spectrometer Bruker Daltonics) The nanoLC-ESI-Q-TOF system was operated in extracted ion mode, and chromatograms and full- scan MS spectra were acquired at a rate of 0.5Hz. MS precursors and MS/MS product ions were acquired over a 50–3000 m/z range, and the collision-induced dissociation energy ranged from 7 to 70eV.

### Database searching and statistical analysis

Data file (.d) analysis was performed in PEAKS Studio 8.5 software (Bioinformatics Solutions Inc., Waterloo, Canada), and MS/MS spectra were submitted for *de novo* analysis and database search using peaks B, PTM, and Spider tools [17]. Considering that *R. microsporus* has a low number of curated proteins in the database, *de novo* sequenced peptides with average local confidence scores ≥ 50% were compared with the *R. microsporus* UniProt/TrEMBL database (53,362 sequences, downloaded in January 2021). Parameters used were precursor mass tolerance of 20 ppm; fragment mass tolerance at 0.025 Da; trypsin or chymotrypsin was set as the specific enzymes and up to two missed cleavages were required; carbamidomethylation (Cys) as fixed modification and oxidation (Met) set as variable modifications, with maximal 3 modifications per peptide in SPIDER outcomes. The combination of multiple runs (trypsin and chymotrypsin) in a single project and a database search were performed for each experimental group. A false discovery rate (FDR) threshold of 1% on the peptide-spectrum match (PSM) and at least one unique peptide was applied to protein identifications. The UniProt classification system was used to analyze all the identified proteins by specifically selecting *R. microsporus*, followed by ontology relations, such as molecular function, and biological process.

## Results

### Effects of copper on the fungal growth profile

IOC 4686 was obtained from a collection of fungi isolated from the Sossego mine environment, during the screening of the copper recovery assay. Dendrogram (Figure 1) showed

genetic relation between IOC 4686 strain and other *Rhizopus* species cataloged in the Biotyper database.

*R. microsporus* showed resistance to 50mgL<sup>-1</sup> of copper ions, close to the IC50 value (28 mg L<sup>-1</sup>). The cell growth density of 4.66gL<sup>-1</sup> when exposed to a concentration of 50mgL<sup>-1</sup> copper ions (Figure 2), decreased to 0.11gL<sup>-1</sup> in the higher tested concentration (125mgL<sup>-1</sup>). Based on this, 50mgL<sup>-1</sup> of copper ions was considered to induce a non-lethal stress level for proteomic study.

In absence of copper (control), the fungus showed a rapid adaptation phase, and accelerated growth (log phase) followed by a stationary phase occurred after approximately 80h. Cultures treated with copper showed a cell growth rate 65.14% lower than the control group (Figure 3).

### Copper absorption

The results showed that the *R. microsporus* IOC 4686 fungus was able to remove metal from the medium culture. Figure 4 shows the copper concentration determined at two points of fungal growth by EDX analysis, indicating that two processes were occurring. During the first 48h copper absorption process, after 60h copper desorption process.

Cells exposed to 50mgL<sup>-1</sup> of copper ions showed the highest copper absorption (48.6% of recovery) occurred after 48h of growth, then, after 60h (Figure 4), a metal ion desorption process was observed the fungus released 6,7% of metal. The lower copper concentration in the culture after 60 h of cultivation observed suggested that the fungus releases the absorbed copper to the medium.

### Protein profile of *R. microsporus* IOC 4686 after copper exposure - Gene ontology (GO) functional enrichment.

To compare the proteins profile in both groups (with and without copper), these proteins were assembled in a list. The protein set showed 1071 proteins in the control and 1232 proteins in the culture containing copper. The Venn diagram (Figure 5) showed that 843 proteins were common to both, mostly proteins related to the basal metabolism of the fungus.

Proteins involved in the oxidative process such as ATP-citrate (pro-S)-lyase, pyruvate decarboxylase isozyme, Hsp71-

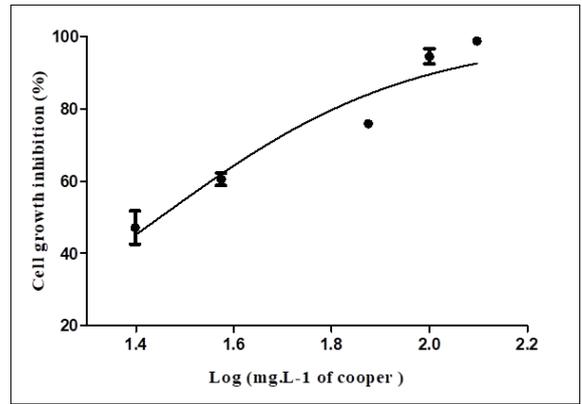


Figure 2: Inhibition growth of *R. microsporus* IOC 4686 after 80 h induced by copper ions.

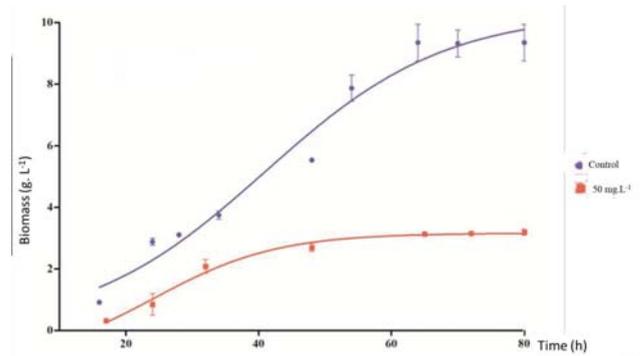


Figure 3: Growth profile of *R. microsporus* IOC 4686 in the absence and presence of copper ions (50 mg. L<sup>-1</sup>).

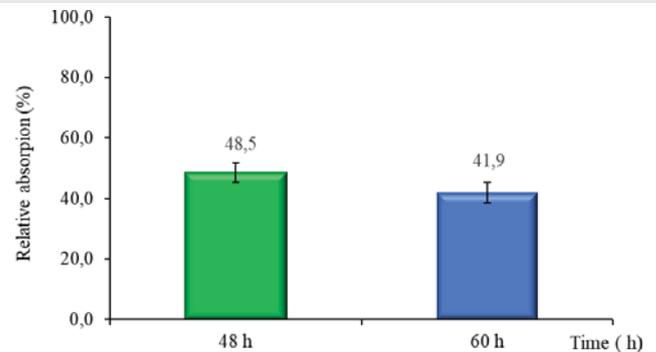


Figure 4: Copper ions absorption by *R. microsporus* IOC 4686 after 48 and 60 h of the bioprocess.

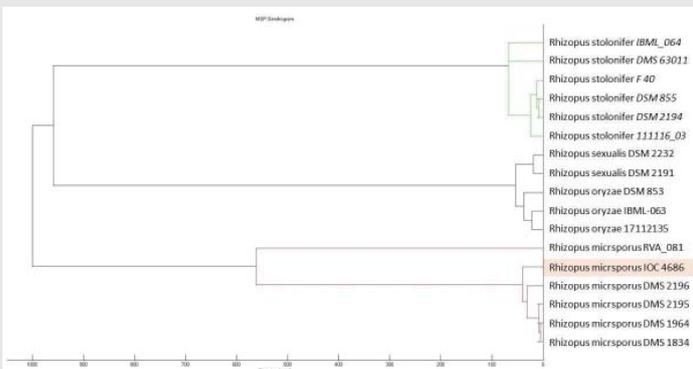


Figure 1: Phylogenetic tree showing the similarity of fungal species of *Rhizopus* genus based on the Biotyper database.

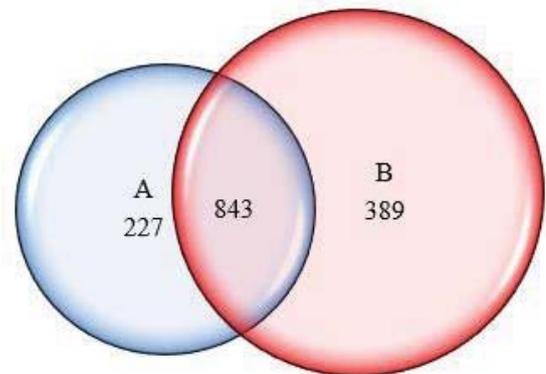


Figure 5: Venn diagram showing proteome profile of *R. microsporus* IOC 4686 in the absence (A) and presence (B) of copper ions (50 mg l<sup>-1</sup>) after 48h.

like protein, glyceraldehyde-3-phosphate dehydrogenase, elongation factor 1- $\alpha$  fructose-bisphosphate aldolase and ATP synthase subunit  $\alpha$  were observed in fungi treated (Table 1).

HSPs proteins were identified in both groups, however, the HSPs, such as G- $\alpha$  protein, HSP (7, 70, 78, 90, and SSB1), and Small COP II coat GTPase were identified only in the culture containing copper (Table 1). Considering only proteins

**Table 1:** Identification of proteins present in *R. microsporus* IOC 4686 in the absence (control, marked "0" in the "Condition" column) and presence (marked "1") of copper.

Access n°	Protein	Score	Molecular function	Condition
A0A0A1MR01	<i>HSP20-like chaperone</i>	7692	Metal ion binding	(0)
A0A1X0S4U4	Policy-diphosphooligosaccharide-protein glycosyltransferase	7512	Metal ion binding	(0)
A0A1X0SDF9	Aspartate carbamoyltransferase	4964	Metal ion binding/ ATP bindi	(0)
A0A1X0S9G4	N(6)-L-threonylcarbamoyladenine synthase	5935	Metal ion binding	(0)
A0A1X0RUW6	Phosphoglycerate mutase	15435	Metal ion binding	(0)
A0A1X0SDI6	Purple acid phosphatase	15774	Metal ion binding	(0)
A0A1X0SOW2	ATP-citrate (pro-S)-lyase	31214	Metal ion binding/ATP bindi <sup>n</sup>	(0)
A0A1X0RYN4	Alpha-mannosidase	5144	Metal ion binding	(0)
A0A1X0RMA2	DNA polymerase epsilon catalytic subunit	5664	Binding	(0)
A0A1X0RUE4	<i>Heme peroxidase</i>	19543	Peroxidase activity	(0)
A0A1X0RUE0	3-isopropyl malate dehydratase	9525	Metal ion binding	(1)
A0A0A1NGC3	G- $\alpha$ protein	8386	GTP binding	(1)
A0A1X0SGK7	Cytochrome c domain-containing protein	24886	Metal ion binding	(1)
A0A1X0RL43	<i>Heat shock protein Hsp90</i>	11698	ATP binding	(1)
A0A0A1NWB8	<i>Putative heat shock protein 70</i>	41161	ATP binding	(1)
A0A0C7BLY5	<i>Putative heat shock protein 78, mitochondrial</i>	6608	ATP binding	(1)
A0A0A1PGE3	Putative 70 kDa heat shock protein 3	31996	Metal ion binding	(1)
A0A0C7AYE6	Putative Heat shock protein SSB1	44266	Metal ion binding	(1)
A0A0C7BJB6	Putative heat shock 70kDa protein 4	26951	Metal ion binding	(1)
A0A0C7CBA1	Putative Hsp7-like protein	13146	ATP binding	(1)
A0A1X0S643	<i>DnaJdomain-containing protein</i>	7116	Heat shock proteins binding	(1)
A0A1X0SDV8	<i>ATPase GET3</i>	6497	ATP binding	(1)
A0A0A1PEG2	NADH-ubiquinone oxidoreductase	6045	Metal ion binding	(1)
A0A1X0RQT6	<i>Cytochrome P450</i>	9498	Integral component of membrane	(1)
A0A1X0RTY9	Sulfite reductase hemoprotein, beta subunit	4349	Metal ion binding	(1)
A0A1X0RLV2	1,4- $\alpha$ -glucan-branching enzyme	14599	Cation binding	(1)
A0A1X0S8S2	Cytochrome b-c1 complex subunit Rieske, mitochondrial	11893	Metal ion binding	(1)
A0A0A1PEG2	NADH-ubiquinone oxidoreductase	6045	Metal ion binding	(1)
A0A0A1PCJ5	Acetohydroxy-acid reductoisomerase	24719	Metal ion binding	(1)
A0A1X0S221	Small COPII coat GTPase	17065	GTP binding	(1)
A0A0A1NP16	Succinate-CoA ligase subunit beta	15731	Metal ion binding/ ATP binding/ ligase activity	(0,1)
A0A0A1P8W9	COX5A-domain-containing protein	20723	Metal ion binding	(0,1)
A0A1X0RTE9	Peptide hydrolase	18098	Metal ion binding	(0,1)
A0A0A1NN97	Pyruvate carboxylase	30204	Metal ion binding	(0,1)
A0A0A1NJK0	Cytochrome c	24360	Metal ion binding	(0,1)
A0A1X0SC07	<i>Hsp7-like protein</i>	29292	ATP binding	(0,1)
A0A1X0RRK5	<i>Heat shock protein 70</i>	30048	ATP binding	(0,1)
A0A0A1PIV1	<i>Heat shock protein 90-1</i>	26970	ATP binding	(0,1)
A0A0A1NFK8	<i>Hsp60-like protein</i>	17266	ATP binding	(0,1)
A0A0A1NFK8	<i>HSP70-domain-containing protein</i>	14461	ATP binding	(0,1)
A0A1X0RP71	<i>Hsp71-like protein</i>	31992	ATP binding	(0,1)
A0A0C7BYC4	<i>Catalase</i>	12324	Metal ion binding	(0,1)
A0A1X0SGK7	Cytochrome c domain-containing protein	14393	Metal ion binding	(0,1)

identified under stress conditions induced by copper, could highlight anti-oxidative process proteins, increasing 27.3% transporter activity protein (Cytochrome b-c1 complex subunit Rieske, mitochondrial and V-type proton ATPase subunit G), and 17.6% binding activity proteins (Ribonucleoprotein and Aconitate hydratase, mitochondrial).

GO analysis (Figure 6) showed the presence of proteins belonging to molecular functions such as antioxidant, transporter, enzymatic regulator, translation factor (RNA binding), binding, structural molecular, ATPase, SNAP receptor, peroxidase, and catalytic. Proteins related to catalytic activity, binding, translation factors, structure, transporter, and enzyme regulator were found in both groups. In another way, SNAP receptor activity was present only in stress conditions.

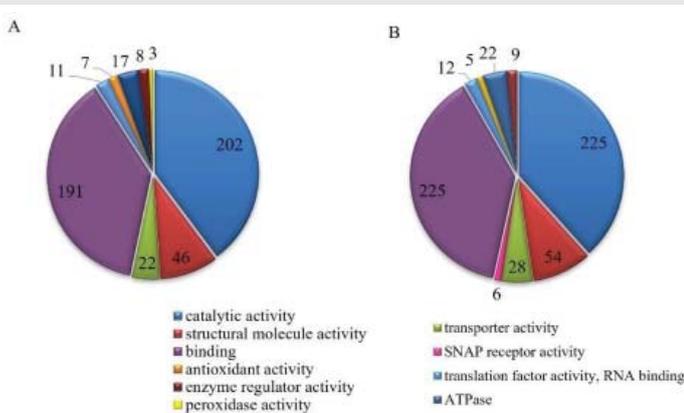
The presence of selective stress-induced binding enzymes, such as calreticulin, cell division/GTP binding protein, tyrosine-tRNA ligase, Peptidase M18, amino-peptidase I, arginyl-tRNA synthetase, and ATP-dependent metalloproteinase Hfl, in both groups suggests that the fungi were adapted to stress environment.

In the copper-containing culture were observed an increase in the number of proteins related to transporter activity (27.3%), structural molecule, binding (17.5%), catalytic, translation factor (9.1%), and enzyme regulator (12.5%). Peroxidase function proteins were found only in the control group.

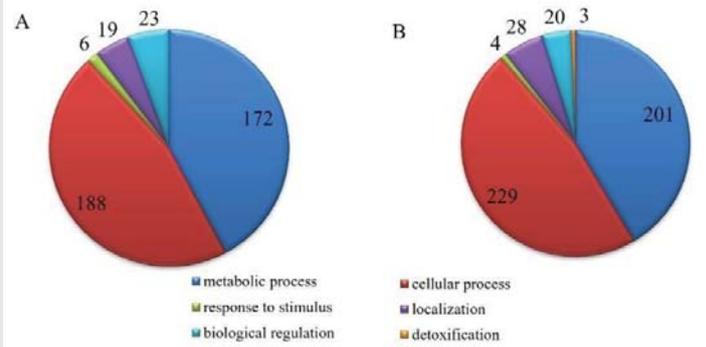
The different biological process of proteins is shown in Figure 7. Metabolic, cellular, biological regulation, response to stimulus, and localization process were observed in both groups, however, the copper treatment also presented a detoxification process, this is the only difference observed concerning the biological process.

In *R. microsporus* strain IOC 4686, the pyruvate dehydrogenase, and pyruvate kinase-carboxylase complex was identified in the copper-treated group.

Fungus under copper stress, showed an increased number of ATPase proteins isoforms, such as ATP-citrate (pro-S)-



**Figure 6:** Molecular functions of proteins identified in *R. microsporus* strain IOC 4686 in the absence (A) and presence of 50 mg L<sup>-1</sup> copper ions (B). Using UniProt gene ontology.



**Figure 7:** Biological Processes of proteins identified assessed by GO search (UniProt gene ontology) of *R. microsporus* strain IOC 4686 in the absence (A) and the presence of copper ions (B), (50 mg L<sup>-1</sup>).

lyase, ATP-dependent metalloproteinase Hfl, and C-terminal domain of alpha and beta subunits of F1 ATP synthase.

Induction of the expression of metalloproteins such as leukotriene A hydrolase, AFG3 family protein, and ATP-dependent metalloproteinase Hfl was observed in *R. microsporus* IOC 4686 in the presence of copper (Table 2).

## Discussion

The results observed during the grown experiments showed that two processes occurred, the absorption and desorption processes. The second one occurred about 60h after, the fungus released 6.7% of copper ions recovered, this process occur probably due to the decrease of pH values caused by the metabolites secreted by the fungus [18]. Copper is an essential metal for several enzymatic and protein functions in the cell, contributing to the maintenance of homeostasis. However, when the limit of tolerance is exceeded, cell growth, metabolism, and protein expression can be modified [10,19,20].

The influence of copper ions on biological processes, such as blocking functional groups or denaturing enzymes, could explain the variation in *R. microsporus* IOC 4686 growth, mainly in the highest concentration of metal. Cell growth reduction has usually been used as a heavy metals tolerance indicator as well as in the determination of the metabolic effects on microorganisms [20]. To evaluate the metabolism of fungi under copper stress, samples were collected at the maximum copper absorption conditions (48h) to identify the proteins involved in the absorption and resistance to the metal.

Fungi expressed different proteins isoform in presence of copper, which suggested differences in cellular metabolism represented by alteration in enzymes involved in oxidative stress. Proteomic analysis of *R. microsporus* IOC 4686 identified proteins involved in basal metabolic pathways, such as carbohydrate consumption, protein biosynthesis, catabolism of secondary metabolites, energy production, and conversion. Mitochondrial pyruvate carrier and putative acetyl-CoA hydrolase/transferase, proteins responsible for changes in the tricarboxylic acid cycle (TCA) which are involved in the degradation of amino acids were observed in presence of copper.

**Table 2:** Metalloproteins identified in *R. microsporus* IOC 4686 in the absence ("0" in the "Condition" column) or presence ("1") of Cu<sup>2+</sup>.

Access n°	Protein	Score	#Peptides	Classification	Condition
A0A1X0S1W9	Transcription initiation factor TFIID subunit 2	11862	1	Metalloprotease	(0)
A0A0A1PB24	Mitochondrial import inner membrane translocase subunit	13945	2	Chaperone	(0)
A0A1X0S1W9	Transcription initiation factor TFIID subunit 2	11862	1	Metalloprotease	(0)
A0A1X0RM32	Peptidase M18, aminopeptidase I	6231	1	Metalloprotease	(0)
A0A1X0S9G4	<i>N(6)-L-threonyl carbamoyladenine synthase</i>	5935	1	Metalloprotease	(0)
A0A1X0S8L0	Zincin	23161	7	Metalloprotease	(1)
A0A1X0RX01	<i>ATP-dependent metallopeptidase Hfl</i>	7174	1	Metalloprotease	(1)
A0A1X0RM32	Peptidase M18, amino-peptidase I	6924	1	Metalloprotease	(1)
A0A1X0SD88	26S proteasome regulatory subunit RPN11	5813	1	Metalloprotease	(1)
A0A1X0SA92	T-complex protein 1 subunit alpha	12801	1	Chaperone	(1)
A0A0A1P552	<i>Uncharacterized protein</i>	4340	1	Chaperone	(1)
A0A1X0RY47	Superoxide dismutase [Cu-Zn]	18795	3	Superoxide dismutase activity	(1)
A0A1X0S1F7	Leukotriene A (4) hydrolase	4909	1	Metalloprotease	(1)
A0A1X0S6X0	Creatinase/aminopeptidase	10084	2	Metalloprotease	(0,1)
A0A1X0RKB1	CNDP dipeptidase	22842	9	Metalloprotease	(0,1)
A0A1X0SF98	Aminopeptidase	25326	16	Metalloprotease	(0,1)

To carry out the proteomics studies, we chose to use two different types of digestive enzymes: trypsin and chymotrypsin, since these enzymes breakdown in different amino acid residues, increasing the number of identified proteins, by generating different sets of peptides, improving protein sequence coverage helping the understood of the metabolism involved in the fungi metal recovery.

The results showed that the oxidative stress induced by copper in fungus isolated from the mine environment modified the protein profile. However, there is a lack of knowledge about the genes of *R. microsporus* involved in the absorption of copper in bioremediation processes, in the present work was established that proteins closely related to this process were induced by the presence of copper [21].

The proteomic analysis showed that some proteins related to copper response increased in several isoforms, and it was also observed the presence of proteins, that were not observed in the control (Table 1), these proteins were considered as possible biomarkers present in *R. microspores* which given to the fungus the ability to survive in a harmful environment. These proteins overexpression indicated modification in fungal metabolism as a response to the metal present in the medium.

Copper stress reduced the protein isoforms related to the mitochondrial respiration process in *R. microsporus* IOC 4686. Considering that ATP production is the main function of mitochondria [22], alteration in this function also causes alteration in the expression of NADH, FADH<sub>2</sub>, and cytochrome (P450, b<sub>5</sub>, c), since copper ions blocked the enzymes that compose the electron carrier chain. Proteins such as cytochrome P450, malic enzyme, acyl-CoA desaturase, among other proteins with oxidoreductase activity in the presence of NADPH were expressed in the presence of an oxidizing agent [23].

FMN-linked oxidoreductase (FMN) was expressed under copper stress. As the copper ions are blocking the electronic carriers the fungal cells must produce FMN to replace the blocked carrier. Additionally, FMN acts as the protein which maintains the electronic chain of cells, protecting the fungi of the osmotic lysis [10].

In the presence of copper, glycolytic pathway proteins were affected by metal, reducing the formation of ATP and its intermediates [24]. In contrast, proteins related to glycolysis and gluconeogenesis, such as pyruvate kinase and enolase, as well as glutamine synthetase and adenosylhomocysteinase, are proteins associated with the assimilation of copper by the cell, were identified in the absence and presence of copper ions. These proteins are essential to energetic metabolism, present in all organisms, their presence in the group under copper stress indicated that copper ions inhibit the electronic chain, but they do not affect the ATP production, suggesting that isoforms stress-induced (present in the electronic chain) maintained cell energy production. The synthesis of isoforms allowed the production of pyruvate through the glycolytic pathway helping aerobic respiration activity. All these proteins play an important key role in *R. microsporus* strain IOC 4686 fungus homeostasis, being responsible to transport copper across the intracellular and cytoplasmic membrane using ATP [25].

Proteins related to the mitochondrial cytochrome b-c<sub>1</sub> complex, which is activated by the Fe-S cofactor in the mitochondria, also participate in the transfer of electrons from ubiquinol to cytochrome during oxidative phosphorylation [22].

The presence of oxidizing agents in the mining environment, in which the fungus was isolated, induced metabolism adaptation. Among the metabolic responses to oxidative stress, the expression of catalases, DnaJ- protein,

and ATPase\_ protein (Table 1), which were physiological responses induced by the metal, can be highlighted [26]. It is well known that under copper stress occur reduction in the expression of mitochondrial-related proteins [26]. However, increases in HSPs and catalase proteins were observed, conserving the cell homeostasis. This protein can be expressed or overexpressed under stress conditions, as shown in Table 1. Therefore, proteins identified in the presence of the metal, including binding proteins and cytochromes, led to a new structural composition in the fungal wall [23]. In this way, the expression of HSPs may be responsible for resistance to toxicity or absorption of the metal.

Small COPII coat GTPase is related to transmembrane copper transport and cellular copper tolerance (Figure 8). To avoid the oxidant metal effect during the cell depuration process, the copper-binding to SOD is transported until storing Get3, Cox, and HSP (Figure 8). In presence of metal, fungal metabolism showed ATPase/Get3 expression associated with cell uptake of copper ions. HSP protein was described as indicative of increased sensitivity to heat and oxidative stress [27]. Copper treatment increased the isoforms of HSP-7, HSP60, HSP70, and HSP78 proteins compared to control, indicating a metabolic response through HSPs expression, suggesting that copper stress modified the process of cellular modeling and adhesion in *R. microspores*. In another way, the presence of HSP90 only in the copper-treated group was suggested to maintain the protein expression stability. This adaptation is triggered by environmental stress to heat or cell wall damage, activating key transcription factors and intracellular signal transduction proteins [28].

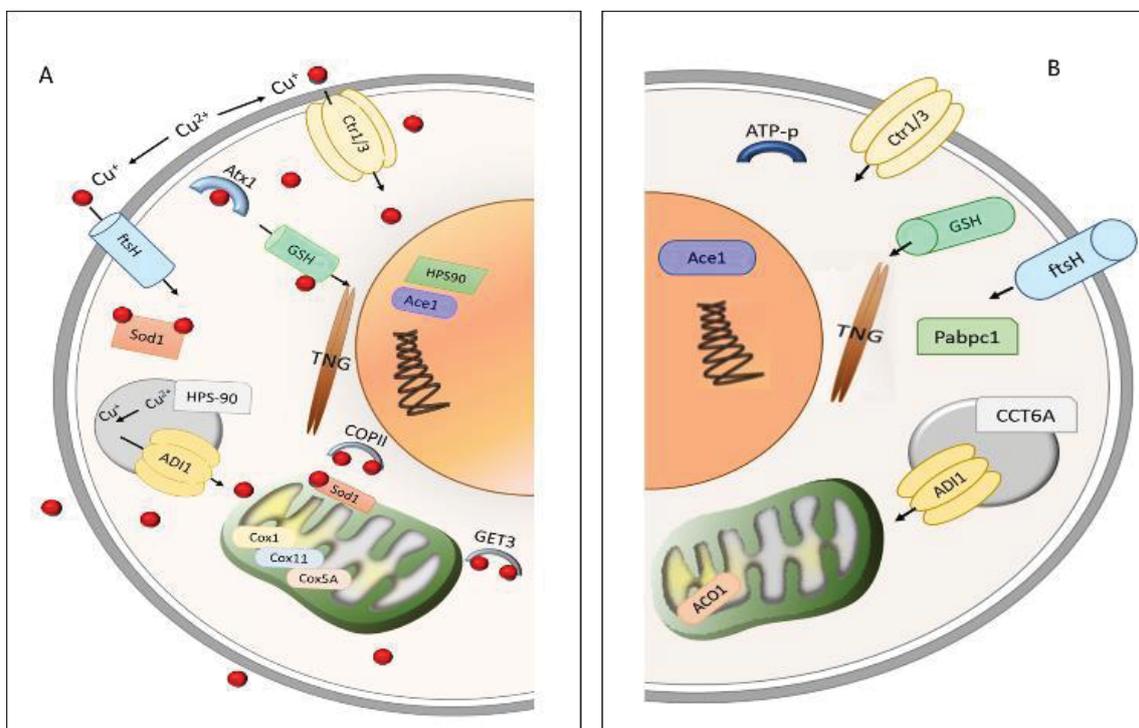
Further, Cox protein as a final receptor of transporting metal from membrane to mitochondria was associated with SOD, suggesting that intracellular transport of copper ions was performed to complex Cox-SOD. Copper presence associated with the Cox1 subunit in mitochondria was measured by the copper joint action of the proteins Cox11 and Cox5A [29].

The overexpression of Get3, Cox, and HSP proteins was related to copper concentration in the cells. These proteins support the copper transport (absorption and desorption) between the cell and the environment. The desorption process observed in *R. microsporus* was also observed in *A. niger* IOC 4687 [18].

Therefore, the expression of new isoforms of HSPs in *R. microsporus* IOC 4686 suggests a cellular adaptation response to stress caused by exposure to copper. Changes in cell metabolism and metalloproteins expression must be by metal catalytic oxidation, which links divalent ions to a polypeptide chain and generates reactive oxygen species causing damage in adjacent amino acid chains [7,30,31].

Seven metalloproteins were identified in both, control and copper-treated, in the presence of copper, metalloproteins such as ATP-metalloproteinase Hfl, cytochrome P450, methionine aminopeptidase, and cell division protease fish were observed (Table 2, Figure 8). Changes in the metabolic pathways induced by the metals (stress) cause alteration in the cellular activity of the organisms, which results in disorders in cell homeostasis [30].

Considering genetic proximity from other species from *Rhizopus* genera (Figure 1) was observed that *R. oryzae* fungus



**Figure 8:** Scheme describing proteins involved in copper transport and absorption during the metal recovery process, highlighting Cox, Get3, COPII and HSP90 identified in cells in presence of copper ions (red spot). Source: Modified from Nevitt [29].



in aqueous copper solution removed copper ions by viable and NaOH treated hyphae, being maximum copper loading capacity of the viable and pretreated biomass according to Langmuir isotherm was 19.4 and 43.7mgg<sup>-1</sup>, respectively [32].

In another way, the *R. oryzae* fungi consortium used for bioremediation of metals and dyes of textile industry effluents showed copper ions absorption of 25mgL<sup>-1</sup> [33]. Further, *R. stolonifer* consortium in a medium contaminated by a mixture of Ni, Pb, and Cd showed high uptake efficiency of Pb (541.5mgkg<sup>-1</sup>) and Ni (501.05mgkg<sup>-1</sup>) [34]. Despite, copper recovery by *R. microsporus* from solution was not reported, this study showed that maximum copper recovery by IOC 4686 was higher than *R. oryzae* consortium [26]. Thus, intracellular and extracellular proteins conferring to fungus metal sequester and precipitation ability suggest that these fungi could be used for metal recovery from the contaminated environments [8,9].

The copper absorption process by *Rhizopus arrhizus* showed absorption of copper ions (64.0mgL<sup>-1</sup>), being weakly influenced by temperature ranging from 4 to 25°C, and showed directly related metal absorption with biomass concentration [35]. Same fungi specie treated with Cd (II), Pb (II), and Cu (II) ions at different temperatures and pH, showed a maximum absorption concentration of 7.32mgg<sup>-1</sup> Cu (II) in 72 h of cultivation at 30°C and pH 4.5 [36]. *R. arrhizus* also was studied in bioremediation processes in different copper concentrations, pH and temperature, showing 97.32% of maximum efficiency in culture containing 100mgL<sup>-1</sup> of copper in pH 7.0 and 35°C [37]. Despite reports showing that the pH and temperature influenced metal recovery, this research does not fix temperature and pH, using similar conditions concerning the local sampling environment and attempting to lower bioremediation process costs.

Proteomic analyses of *R. microsporus* IOC 4686 incubated in a copper solution showed an increase in translocation membrane, oxidative molecular reaction, and metal-binding proteins compared to control. P450 was the main protein responsible for conferring resistance to the fungus under stress induced by copper. These proteins involved in the defense mechanism were found in different isoforms in copper-treated samples. Based on these results, *R. microsporus* IOC 4686 has the potential for metal bioremediation with a resistance mechanism capable of neutralizing oxidative stress induced by copper. The purpose of this study was to understand the mechanism of the fungus, *R. microsporus* to absorb and accumulate metal from contaminated sites. This work was performed only with copper ions, but it could help to understand the physiological changes induced by other metals, such as Cr and Pb.

The Challenge of this work was to understand the changes in the metabolism of *R. microsporus* IOC 4686 induced by copper allowing environmental adaptation.

### Author contribution

MD, ER, CN, and MM conceived and designed research. MD and LA conducted experiments. JL analyzed the data. MD, LA, and MM wrote the manuscript. All authors read and approved the manuscript.

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### Data availability Sequence

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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