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Removal of trace element by isolates of *Aspergillus brasiliensis* EPAMIG 0084 and *Penicillium citrinum* EPAMIG 0086 in biofilters

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Coffee beans processing generates a large volume of wastewater composed of trace elements which can be detrimental to human health. The present study aimed at evaluating the capacity of strains of *Aspergillus brasiliensis* and *Penicillium citrinum* in tolerating and removing trace elements namely: Cu, Mn and Zn from coffee wastewater. The use of fungi in the treatment of polluted wastewater has emerged as a viable alternative to conventional treatment. The fungi were isolated from polluted and unpolluted areas, which were tested on a laboratorial scale and on large scale (aerobic bioreactor) with immobilized biomass. As expected, the strains isolated from polluted areas (*P. citrinum* EPAMIG 0086 and *A. brasiliensis* EPAMIG 0084) were more tolerant to the elements studied than the strains isolated from unpolluted areas (*A. brasiliensis* IBT 26433 and *P. citrinum* INCQS 40011). As for the removal tests conducted on a laboratorial scale, it is worth mentioning that the fungal strains under study responded differently to the tested elements (regardless of their origin). In the tests conducted in bioreactors, the fungus *P. citrinum* EPAMIG 0086 presented a greater removal capacity of the elements in aerobic biofilters (44, 62 and 48% for Cu, Mn and Zn, respectively) than the *A. brasiliensis* EPAMIG 0084 (21.7, 51 and 41.6%, respectively), which indicates that this fungus is an efficient alternative to conventional treatments.

Key words: Coffee wastewater, bioreactors, fungi, immobilization, removal, trace elements.

INTRODUCTION

Increasing population and consumption are placing unprecedented demands on agriculture and natural resources. The productivity increase has led in the production of large quantities of agricultural waste and

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therefore in its disposal in soil and water bodies (Foley et al., 2011). Coffee production is no exception to this rule. According to Melo (2009), wet processing requires large volumes of water in the stages of husk removal, fermentation and washing. For every bag of coffee produced, 3,500 L of coffee wastewater is generated. These residue is rich in organic and inorganic matter, and among the inorganic compounds, there is a large number of trace elements from the use of fertilizers, agricultural lime and pesticides that accumulate in the fruit during development (Kiekens and Cottenie, 1985).

According to Wuana and Okieimen (2011), the predominant trace elements in agricultural areas contaminated with fertilizer and pesticide waste are lead, chromium, arsenic, zinc, cadmium, copper, mercury and nickel. The trace elements and their toxicity are of particular interest to public health, because these substances cannot be processed or chemically destroyed (Davis et al., 2001); are not biodegradable and tend to accumulate in living organisms and many heavy metal ions are known to be toxic or carcinogenic (Fu and Wang, 2011).

According to Ribeiro et al., (2009), however, in addition to the trace elements hazardous to the environment, coffee wastewater also contains N, P, K, Ca, Mg and micronutrients. Thus, if properly treated, it can be reused to supply part of the water and the nutrients demanded by crops. Conventional methods for removal of dissolved trace elements are chemical treatments (like hydroxide precipitation, sulfide precipitation, trace elements chelating precipitation and ion exchange), physical treatments like adsorption (activated carbon adsorbents and carbon nanotubes adsorbents), membrane filtration (ultrafiltration and nanofiltration), coagulation, flocculation, flotation and electrochemical treatment (Fu and Wang, 2011).

Even though conventional technologies adopted for removal of heavy metals from polluted environment tend to be efficient, they are generally expensive and produce huge quantity of toxic chemical products (Zaidi et al., 2011). Moreover, methods such as chemical precipitation and reverse osmosis for removal of trace elements present in low concentrations (below 100 mgL^{-1}, such as coffee wastewater) result in incomplete removal of trace elements and excessive use of reagents and energy (Brierley et al., 1985; Kapoor and Viraraghavan, 1995).

The use of biological materials including fungal biomass offers an economical, effective and safe option for removing heavy metals and, therefore, has emerged as a potential alternative method to conventional treatment techniques. Among the various remediation strategies, biosorption of heavy metals by metabolically active or inactive nonliving (dead) biomass of fungal origin is an innovative and alternative technology for removal of metals from contaminated sites (Zaidi et al., 2011). Adsorption is an effective and economic method for heavy metal wastewater treatment. The adsorption process offers flexibility in design and operation and in many cases will produce high-quality treated effluent. In addition, because adsorption is sometimes reversible, adsorbents can be regenerated by suitable desorption process (Fu and Wang, 2011).

The use of fungal isolates from environments contaminated by trace elements is a very promising alternative in wastewater treatment. As these microorganisms can proliferate in a contaminated environment, they probably have strategies for tolerating and growing in such situation (Lemos et al., 2008; Sprocati et al., 2006). Treatment of trace element contaminated wastewater through microorganisms can be optimized by means of their immobilization on supports inside biofilters. That is because the use of immobilized biomass facilitates its separation from the aqueous medium for subsequent retrieval of elements adsorbed and reuse of the biomass (Tsezos and Deutschmann, 1990).

The present study was conducted in order to assess the removal of the elements copper (Cu), manganese (Mn) and zinc (Zn) present in coffee wastewater by using *Aspergillus brasiliensis* EPAMIG 0084 and *Penicillium citrinum* EPAMIG 0086 isolated from contaminated environments and *A. brasiliensis* IBT 26433 and *P. citrinum* INCQS 40011 from uncontaminated environments. The aim was to compare removal efficiency based on prior exposure of the fungi to the pollutant, through the use of a laboratory-scale and larger scale experiments in aerobic biofilters.

MATERIALS AND METHODS

All tests were performed in triplicate, and all media were prepared with Milli-Q water (deionized water purified by a Milli-Q system supplied by Millipore Corporation).

Fungal isolates

The following fungal species were used: *A. brasiliensis* EPAMIG 0084 and *P. citrinum* EPAMIG 0086, both isolated from Zn, Cu, Pb and cadmium (Cd) contaminated soil from the municipality of Três Marias, MG, Brazil, as well as the same fungal species isolated from uncontaminated regions; *A. brasiliensis* IBT 26433 and *P. citrinum* INCQS 40011.

Collection of coffee wastewater samples: Qualification and quantification of trace elements

The water derived from the washing of coffee beans, coffee wastewater (CW), was collected from the Coffee Processing Center of Lavras Federal University (CEP - UFLA) for the pH and trace element evaluation. A 0.45 µm membrane was used to filter the water and sent for atomic absorption spectrophotometer (AAS) analyses to check for the presence of the following trace elements described in the literature by Gonçalves (2006) as normally present in the CW: copper (Cu), manganese (Mn) and zinc (Zn). The samples were digested in a digestion tube using nitric acid (HNO_3) according to the US Environmental Protection Agency (EPA) 3051A

method. Metal concentrations were determined through the use of an atomic absorption spectrophotometer (Perkin Elmer Analyst 300).

Trace element tolerance test

Tolerance of the fungi study to the trace elements Cu, Mn and Zn was tested at the following concentrations found in the CW by AAS: 0.5, 1 and 5 mgL^{-1} of each element. The isolates were transferred to Petri dishes containing malt extract agar medium (MA) with the addition of copper sulfate, manganese sulfate or zinc sulfate, each one separately. The isolates were also inoculated on dishes containing MA without the addition of any trace element as control. The culture media were adjusted to pH 5 because, according to Zafar et al. (2007), this pH is the best for obtaining the highest rate of metal ion removal by microbial biomass. Moreover, according to Gonçalves (2006), the pH found in CW varies from 4 to 5. The fungi were incubated at 25°C for seven days and the diameter of the colonies was measured. Isolates that exhibited growth at or above the growth of the control were considered tolerant (da Silva et al., 2003).

Trace element removal tests on a laboratory scale

The present methodology was adapted from da Silva et al. (2003). The isolates were inoculated in Petri dishes containing MA and incubated at 25°C for seven days. After fungal growth, five discs (0.5 cm diameter) were removed and transferred to 250 mL Erlenmeyer flasks containing 130 mL of 2% malt extract broth (MB) and a solution with trace element salts: copper sulfate, manganese sulfate and zinc sulfate (0.5, 1 and 5 mgL^{-1}). Controls were used by means of Erlenmeyer flasks containing the culture medium with the salt solution and without the inoculum, and the culture medium with the inoculum but without the salt solution. These flasks were incubated (100 rpm) at 25°C for 10 days.

After 5 and 10 days, the samples were filtered in 0.45 µm Millipore membranes, the trace element content quantified by AAS and the pH of the solution was evaluated. After 10 days of incubation, the biomass produced was filtered and the dry weight was evaluated. After this sampling period, an aliquot of the biomass was removed with the aid of a platinum loop for analysis in a scanning electron microscope (SEM) with x-ray microanalysis.

Trace element removal tests in aerobic biofilters

Three previously disinfected biofilters were used (glass batch reactors) with a 14.6 L capacity, built by Silva (2008). Along the reactors, four taps were installed to allow sampling of the effluent for trace element quantification analyses. The biofilter was continuously oxygenated with a Boyu model U-3600 air pump, with a 4.0 L min^{-1} flow rate. The fungal isolates from the region contaminated by trace elements *A. brasiliensis* EPAMIG 0084 and *P. citrinum* EPAMIG 0086 were transferred to plates containing MA and incubated for 20 days at 25°C.

After this period, 20 mL of sterile distilled water was added to the plate surface and the fungal biomass was scrapped to disaggregate the spores. The spores present in the suspension were counted in a Neubauer chamber and the concentration adjusted to approximately $5x10^6$ spores mL^{-1}. This suspension (20 ml) was inoculated in bottles containing 250 mL of MA medium (40°C), homogenized and transferred to trays containing support for fungal growth - corrugated pipes (conduits) - previously sterilized in an autoclave. The trays were incubated (25°C) for 20 days for the fungi to grow and colonize the surface and inside of the conduit. As mentioned at the introduction, this test was performed in a period of 20 days rather than seven days, to evaluate the behavior of the trace element against fungal biomass in a longer period of time on a higher retention time on enlarged scale.

After this period, the support material containing the attached fungal biomass was transferred to the biofilter. Subsequently, 10 L of a synthetic solution of distilled water with the addition of copper sulfate, manganese sulfate and zinc sulfate at a concentration of 5 mgL^{-1} Cu, Mn and Zn was added. The pH of the solution was adjusted to pH 6 in order to assess whether at this pH there was greater adsorption of trace elements than at pH 5, previously tested in Erlenmeyer flasks. This pH is also described by Saya et al. (2001) as ideal for the removal of some trace elements tested.

The biofilter was activated and 20 mL was removed from each tap, initially every 12 h, and after the first day, once a day for five days (120 h). The trace element concentration present in the samples was determined by AAS.

Scanning electron microscopy with X-ray microanalysis

The use of scanning electron microscope (SEM) helps elucidate the mechanisms involved in biosorption. The SEM can be attached to equipment energy dispersive system (EDS), which allows the determination of the qualitative composition of the trace elements present in the samples from the emission of characteristic X- rays (Calfa and Torem, 2007). In order to perform this test, aliquots of biomass formed in the flask tests with 0 (control) and 5 mgL^{-1} trace element concentration were removed. These were washed with distilled water and placed on aluminum supports (stubs) previously covered with carbon tape and observed in a scanning electron microscope (QUANTA 250 - FEI) coupled to a system of X-ray microanalysis (EDS) that uses the emission of X- rays to describe the elements found in the samples. The Genesis program was used for performing the microanalysis under the following conditions: 30,000 kV, spot ranging from 5 to 7 and high vacuum.

These analyses were performed in the Department of Biophysics at Rio de Janeiro Federal University - UFRJ). In order to draw the graphs of the X-ray microanalysis spectrum, a determination was made of the composition of samples per sample area under the following conditions: working distance (WD) of 10 mm, an increase of 1,000 X and voltage of 30 kV.

Statistical analyses

Statistical analyses were performed using SISVAR software. Analyses of variance (ANOVA) were conducted using a completely randomized factorial experimental design (DIC).

RESULTS AND DISCUSSION

Evaluation of trace elements present in the CW

Table 1 presents the results of the trace element concentrations (Cu, Mn and Zn) detected in CW obtained from the CEP (UFLA). The pH obtained in CW was 4.5. The pH obtained from CW in the present study corroborates the results presented by Gonçalves (2006). In studies conducted by Vasco (1999) and Gonçalves (2006) regarding the presence of trace elements in CW, concentrations of these elements were higher than those found in this study (Table 1); 5 mgL^{-1} Cu; 4 mgL^{-1} Zn (Vasco, 1999); 1.27 mgL^{-1} Cu; 1.33 mgL^{-1} Mn and 0.66 mgL^{-1} Zn (Gonçalves, 2006).

Table 1. Results of the Cu, Mn and Zn concentrations present in coffee wastewater obtained from Lavras Federal University (UFLA-MG).

Trace element present in the CW	Concentration obtained by atomic absorption spectrophotometry (mgL^{-1})
Cu	< 1.0
Mg	< 0.5
Zn	< 0.4

Numbers followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

Table 2. Trace element tolerance test: result of fungus growth (cm) in different Cu concentration. Test of means for the fungi factor within the Cu in each concentration level by *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011.

Fungal strain	Cu concentration (mgL^{-1})		
	0.5	1	5
A. brasiliensis EPAMIG 0084	2.60^{b}	1.63^{a}	1.57^{a}
A. brasiliensis IBT 26433	6.03^{d}	6.25^{d}	6.10^{d}
P. citrinum EPAMIG 0086	2.03^{a}	2.05^{b}	2.00^{b}
P. citrinum INCQS 40011	4.97^{c}	5.05^{c}	4.88^{c}

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

Table 3. Trace element tolerance test: Result of fungus growth (cm) in different Mn concentration. Test of means for the fungi factor within the Mn in each concentration level by *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011.

Fungal strain	Mn Concentration (mgL^{-1})		
	0.5	1	5
A. brasiliensis EPAMIG 0084	1.86^{a}	1.67^{a}	1.73^{a}
A. brasiliensis IBT 26433	6.5^{c}	6.10^{d}	6.43^{d}
P. citrinum INCQS 40011	5.28^{b}	5.02^{c}	5.00^{c}
P. citrinum EPAMIG 0086	2.03^{a}	1.97^{b}	2.10^{b}

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

Table 4. Trace element tolerance test: result of fungus growth (cm) in presence of Zn. Test of means for the fungus factor Zn element by *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011. For Zn, the trace element concentration was not significant.

Fungal strain	Fungus growth (cm)
A. brasiliensis EPAMIG 0084	1.67^{a}
A. brasiliensis IBT 26433	6.22^{b}
P. citrinum INCQS 40011	5.03^{ab}
P. citrinum EPAMIG 0086	4.07^{ab}

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

Trace element tolerance test

After variance analysis for the tolerance to trace element, it was concluded that the F test at 5% of significance level indicated that two-way interaction (fungi X concentration) was significant to Cu and Mn. Therefore, the level of fungi in each level of concentration was analyzed (Tables 2 and 3). For the Zn element, there was no significance level in the two-way interaction (fungi X concentration). Then for this element, an individual analysis for the factor fungus was done (Table 4). From Tables 2 to 4, for the most elements tested, it can be observed that fungal strains isolated from contaminated

Table 5. Trace element removal test on a laboratory scale: Cu final concentration (mgL^{-1}) in different Cu concentrations tested (0.5, 1 and 5 mgL^{-1}) after 5 days of incubation. Test of means, for the fungi factor within the concentration levels of Cu by *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011.

Fungal strain	Cu concentration (mgL^{-1})			
	0	**0.5**	**1**	**5**
Control	0.03^{a}	0.49^{bc}	0.95^{cd}	4.54^{d}
A. brasiliensis EPAMIG 0084	0.09^{a}	0.66^{c}	1.22^{d}	4.62^{e}
A. brasiliensis IBT 26433	0.10^{a}	0.38^{abc}	0.52^{ab}	1.34^{a}
P. citrinum EPAMIG 0086	0.01^{a}	0.08^{a}	0.33^{a}	2.40^{b}
P. citrinum INCQS 40011	0.00^{a}	0.08^{a}	0.40^{a}	3.06^{c}

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

environments *A. brasiliensis* EPAMIG 0084 and *P. citrinum* EPAMIG 0086 showed greater tolerance to all the trace elements in the concentrations evaluated, with one exception: the element zinc (Table 4). For this element, the two strains of *P. citrinum* INCQS 40011 and EPAMIG 0086 did not show significant differences regarding tolerance. It was also observed that *A. brasiliensis* IBT 26433 and EPAMIG 0084 had a better growth than *P. citrinum* INCQS 40011 and EPAMIG 0086. However, this fact does not indicate greater tolerance to *A. brasiliensis* strains, since it has a faster natural growth rate than *P. citrinum* strains.

According to Pan et al. (2009), tolerance to trace elements among strains of the same fungal species is due to different mechanisms of detoxification developed by them in response to environmental pressures. These strains promote the reduction of toxicity of trace elements through the complexation of these elements, precipitation or other mechanisms and therefore they can tolerate and grow under such conditions (Saxena et al., 2006).

The results of this study support the assumption that species isolated from environments contaminated with trace elements have a higher tolerance than species isolated from uncontaminated environments (Rouch et al., 1995). Other authors have also studied trace element tolerance of fungi isolated from areas contaminated with these elements. Ezzouhri et al. (2009) evaluated the tolerance of microorganisms isolated from areas contaminated by trace elements in Tangier (Morocco) against the elements Pb, Cr, Cu and Zn. Isolates of *Aspergillus* spp. and *Penicillium* spp. were the most tolerant to all trace elements tested and exhibited the greatest growth in the presence of these elements in relation to other fungi and their controls (medium without addition of trace elements).

Hemambika et al. (2011) also concluded that trace elements resistant isolates show no inhibition of growth for higher concentration of heavy metals, whereas trace elements sensitive isolates show inhibition of growth for higher concentration of heavy metals. These results were similar to that obtained in the present study, in which both species *P. citrinum* EPAMIG 0086 and *A. brasiliensis* EPAMIG 0084 from contaminated area showed great tolerance to the trace element evaluated.

Probably, this occurs due to the fact that the fungi from uncontaminated areas have no protection mechanisms against these elements as a result of environmental pressure that selects such characteristics. Thus, according to Gadd (1992) once inside the cell, the trace element may be located into organelles, or may be attached to proteins, shifting the ions suitable for cell function from their original positions, promoting cell damage or interfering with the metabolic functions.

Trace element removal test on a laboratory scale

Following the variance analysis for the removal of all trace elements tested by the fungi under study, after 5 and 10 days of incubation, it may be concluded that the three way interaction: time X fungus X concentration, was significant by the F test at the 5% significance level for the trace elements Cu and Mn. Therefore, the levels of concentration and time for the trace element Cu (Tables 5 and 6) and Mn (Tables 7 and 8) were analyzed.

When comparing the removal of the copper by the studied strains with their respective controls (Tables 5 and 6), it may be observed that the greatest removal rate was obtained after five days of incubation. Furthermore, it can be inferred that for both species, the greatest removal rate was achieved by strains isolated from uncontaminated areas. *A. brasiliensis* IBT 26433 showed 70.5% removal in a concentration of 5 mgL^{-1} for Cu and *P. citrinum* INCQS 40011 and 81% of removal in a concentration of 0.5 mgL^{-1} for Cu.

These results seem to contradict the results obtained in the initial tolerance tests when the fungi isolated from contaminated areas showed higher growth and tolerance compared to strains isolated from uncontaminated areas. Therefore, the tolerances to these trace elements are not directly related to the capacity of trace elements removal by these fungi. Shen et al. (2013) evaluated the hypothesis

Table 6. Trace element removal test on a laboratory scale: Cu final concentration (mgL^{-1}) in different Cu concentrations tested (0.5, 1 and 5 mgL^{-1}) after 5 days of incubation. Test of means, for the fungi factor within the concentration levels of Cu by *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011.

Fungal strain	Cu concentration (mgL^{-1})			
	0	0.5 mgL^{-1}	1 mgL^{-1}	5 mgL^{-1}
Control	0.001^{a}	0.47^{ab}	0.99^{d}	4.39^{d}
A. brasiliensis EPAMIG 0084	0.05^{a}	0.40^{ab}	1.00^{cd}	4.24^{de}
A. brasiliensis IBT 26433	0.07^{a}	0.41^{ab}	0.86^{bcd}	1.41^{a}
P.citrinum EPAMIG 0086	0.01^{a}	0.15^{a}	0.70^{abc}	2.12^{b}
P.citrinum INCQS 40011	0.01^{a}	0.32^{ab}	0.48^{a}	2.49^{c}

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

Table 7. Trace element removal test on a laboratory scale: Mn final concentration (mgL^{-1}) in different Mn concentrations tested (0.5, 1 and 5 mgL^{-1}) after 5 days of incubation. Test of means, for the fungi factor within the concentration levels of Mn by *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011.

Fungal strain	Mn concentration (mgL^{-1})			
	0	0.5	1	5
Control	0.05^{a}	0.52^{b}	1.67^{d}	4.55^{e}
A.brasiliensis EPAMIG 0084	0.00^{a}	0.35^{a}	0.73^{b}	3.88^{b}
A.brasiliensis IBT 26433	0.00^{a}	0.34^{a}	0.33^{bc}	4.40^{d}
P. citrinum EPAMIG 0086	0.03^{a}	0.28^{a}	0.66^{b}	3.51^{a}
P. citrinum INCQS 40011	0.07^{a}	0.42^{a}	0.74^{b}	4.03^{c}

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

Table 8. Trace element removal test on a laboratory scale: Mn final concentration (mgL^{-1}) in different Mn concentrations tested (0.5, 1 and 5 mgL^{-1}) after 10 days of incubation. Test of means, for the fungi factor within the concentration levels of Mn by *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011.

Fungal strain	Mn concentration (mgL^{-1})			
	0	0.5	1	5
Control	0.03^{a}	0.55^{bc}	1.04^{c}	4.45^{d}
A. brasiliensis EPAMIG 0084	0.01^{a}	0.35^{a}	0.73^{ab}	4.14^{b}
A. brasiliensis IBT 26433	0.00^{a}	0.36^{a}	0.75^{ab}	4.44^{bc}
P. citrinum EPAMIG 0086	0.03^{a}	0.29^{ab}	0.78^{ab}	2.96^{a}
P. citrinum INCQS 40011	0.11^{a}	0.40^{a}	0.88^{abc}	4.21^{bc}

that endophytic isolated from trace elements contaminated sites would enhance their host capacity to trace elements removal from environment; but, they observed that there was no significant difference in this capacity by endophytic isolated from contaminated and uncontaminated sites. Then, like the results obtained in the present research, they concluded that growth and heavy metal sorption/absorption and accumulation were not correlated with origin of the endophytic.

P. citrinum INCQS 40011 showed the best results for Cu removal. This phenomenon can be explained by the final pH media. Akar et al. (2009) evaluated Cu adsorption by *Tramaetes versicolor* ATCC 200801 at different pHs, and observed that the maximum removal rate of this element from the solution occurred at pH 5 and, below this pH, adsorption decreased, reaching zero at pH 1. The same phenomenon occurred in the present study, in which *A. brasiliensis* IBT 26433 and EPAMIG 0084 changed the pH of the solution to three, removing the element Cu less efficiently than *P. citrinum* INCQS

Table 9. Trace element removal test on a laboratory scale: Zn final concentration (mgL^{-1}) in different Zn concentrations tested (0. 5, 1 and 5 mgL^{-1}) after 5 days of incubation. Test of means, for the fungi factor within the concentration levels of Zn by *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011.

Fungal strain	Zn concentration (mgL^{-1})			
	0	0.5	1	5
Control	0.00^{a}	0.60^{c}	1.02^{bc}	4.57^{d}
A. brasiliensis EPAMIG 0084	0.00^{a}	0.19^{a}	0.73^{a}	4.28^{c}
A. brasiliensis IBT 26433	0.02^{a}	0.25^{ab}	0.71^{a}	4.06^{b}
P. citrinum EPAMIG 0086	0.10^{a}	0.47^{bc}	1.01^{bc}	3.34^{a}
P. citrinum INCQS 40011	0.03^{a}	0.42^{abc}	0.81^{ab}	4.47^{cd}

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

40011 and EPAMIG 0086, which maintained the solution pH close to 5. The increased removal rate observed at pH 5 is due to the release of negatively charged binding sites on the fungal cell wall, as a result of proteins denaturation that facilitates binding (Verma et al., 2013).

A higher removal rate observed within five days of incubation compared to 10 days (Tables 5 and 6) is probably due to fungi protection mechanisms that send back trace elements to the solution after long incubation periods. According to Melo and Azevedo (2008), the removal by adsorption process occurs until biomass saturation, in other words, when the active sites are not available anymore and the desorption process of trace elements to solution may occur.

Concerning the Mn, the results from Tables 7 and 8 demonstrate that the best performances were obtained from *A. brasiliensis* IBT 26433 (71% of removal in 1 mgL^{-1}, by five days of incubation), followed by *P. citrinum* EPAMIG 0086 (34.9% in 5 mgL^{-1}, 10 days of incubation). Therefore, for the Mn trace element, the same pattern was not observed regarding strains isolated of contaminated and uncontaminated areas as occurred previously. Osaizua et al. (2014) studied the trace elements (Al, Fe and Mn) accumulation capacity by fungi isolated of trace elements contaminated areas. They also concluded that the genus Aspergillus exhibited the highest bioaccumulation potential. In their study they observed that *A. oryzae* showed the best Mn (II) sorption rate (over 30%).

On the other hand, for the Zn, the F test at 5% of significance level was not significant for the three way interaction: time x fungus x concentration for the Zn removal test. Thus, an analysis of each of the three 2-way interactions was conducted (Table 9). Only the two way interaction: fungus x time was significant by the F test at 5% of significance level for this element. For Zn (Table 9), a greater removal rate after five days of incubation was observed for both species in the study. However, *A. brasiliensis* strains EPAMIG 0084 and IBT 26433 showed a significant percentage of this element ablation compared to *P. citrinum* strains. *A. brasiliensis* EPAMIG 0084 presented 68% of removal in Zn concentration of 0.5 mgL^{-1} while *A. brasiliensis* IBT 26433 showed 60% in the same concentration. For the fungus *P. citrinum* EPAMIG 0086, no significant Zn removal was observed and *P. citrinum* INCQS 40011 showed 26.8% of removal in the concentration of 1 mgL^{-1} of Zn; for Zn, the strain origin did not interfere in this element uptake but the species evaluated.

Akhtar et al. (2013) evaluated the zinc removal from industrial effluent by *Aspergillus* sp. They obtained approximate 50% of Zn removal by *Aspergillus flavus* NA9 and observed that the contribution of the functional groups and lipids to zinc biosorption as identified by chemical pretreatment was in the order: carboxylic acids > hydroxyl > amines > lipids. Probably these functional groups and lipids were also responsible by zinc uptake by the *A. brasiliensis* strains EPAMIG 0084 and IBT 26433 used in this study.

Faryal et al. (2006) observed that the optimum pH for Zn removal by *Aspergillus fumigates* RH05 is 5. In the present study, at the beginning of the experiments, the pH reached was 6, a fact that probably influenced significantly the removal of this element by most of the fungi evaluated.

The removal of these trace elements by the fungi under study may be due to biosorption and absorption mechanisms. The phenomenon of biosorption occurs as a result of physical phenomena (regardless of cellular metabolism) and the sorption of these elements to functional groups present on the fungal cell wall such as amino groups, carboxyl, thiol, sulfhydryl and phosphate by electrostatic attraction or bond formation (Pan et al., 2009; Purchase et al., 2009). The adsorption is carried out due to binding of trace element to functional groups present on the cell wall such as the carboxyl, amine, sulfate and phosphate. The absorption occurs when the trace element are transported into the cell and are volatilizated or accumulated by binding to organelles or proteins (Aparicio, 2000).

Thus, it may be inferred that different fungi species are capable of adsorbing trace element of solutions by

Table 10. Trace element removal test on a laboratory scale: Dry weight obtained from *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011 after 10 days of growth in malt extract plus a solution with trace elements Cu, Mn and Zn in different concentrations.

Fungal strain	Dry weight (g)											
	Cu (mgL^{-1})				Mn (mgL^{-1})				Zn (mgL^{-1})			
	0	0.5	1	5	0	0.5	1	5	0	0.5	1	5
A. brasiliensis EPAMIG 0084	0.66	0.63	0.69	0.68	0.66	0.63	0.69	0.68	0.66	0.63	0.69	0.68
A. brasiliensis IBT 26433	0.60	0.52	0.70	0.58	0.60	0.52	0.70	0.58	0.60	0.52	0.70	0.58
P. citrinum EPAMIG 0086	0.57	0.51	0.51	0.54	0.53	0.51	0.51	0.54	0.53	0.51	0.54	0.54
P. citrinum INCQS 40011	0.49	0.50	0.62	0.49	0.46	0.58	0.62	0.49	0.44	0.52	0.53	0.57

different removal mechanisms and therefore remove them in different quantities; a fact that was observed in this study, even by strains belonging to the same fungal species. These results corroborate those obtained by Adriano (2001), who concluded that species of microorganisms within the same genus, or even strains within the same species, may differ in their sensitivity to trace element.

According to Pan et al. (2009), the greater the variety of trace element present in the solution, the smaller the concentration of trace element adsorbed by the fungi biomass, due to the competition of these elements for active sites. Vivas et al. (2005) in a study of Cd and Zn adsorption by fungal biomass *Glomus mosseae* concluded that these elements have antagonistic effects when together in solution. However, this phenomenon was not observed in this study. Furthermore, the study of removal of the trace element separately would have no interest in the present study because it is not what actually occurs in CW, where there is a wide variety of these elements.

Ren et al. (2009) studied the removal of Cu, Cd, Pb and Zn by *Aspergillus niger* isolated from environments contaminated with such elements. After two days of incubation, there was 84.3% of Cu, 84.4% of Cd, 25% of Pb and 14.4% of Zn removal. In the present study, *A. brasiliensis* IBT 26433 also showed greater efficiency in removal of the element Cu (70.5% - Tables 5 and 6) than Zn (60% - Table 9) and *P. citrinum* INCQS 40011 also removed the element Cu (81% Tables 5 and 6) more efficiently than the element Zn (26.8% - Table 9), indicating that probably the components present on the cell wall of these strains have greater affinity with the trace element Cu.

With regard to dry weight, it was observed that for both fungal species studied, it remained approximately constant in relation to the control for all elements and concentrations tested (Table 10). These results demonstrate that trace elements concentrations are not toxic to the microorganisms assessed because they did not inhibit their growth.

The results allow us to infer that the strains of *A. brasiliensis* EPAMIG 0084 and *P. citrinum* EPAMIG 0086, isolated from contaminated region, and *A. brasiliensis* IBT 26433 and *P. citrinum* INCQS 40011, from and uncontaminated region, reacted differently regarding their removal capacity of the trace element Cu, Mn and Zn (Tables 5 to 9). These results indicate that the cell wall of the fungi studied may show a higher affinity to one or another specific trace biosorbent which showed higher affinity towards certain metal in mixed metals solution due to the selectivity of the biosorbent and/or the ambient conditions which may prefer the adsorption of such metal over the others (Sheng et al., 2008).

Trace element removal test in aerobic biofilters

An analysis of variance was performed on results of the response variable of trace element removal in aerobic biofilters. The removal of copper by *A. brasiliensis* EPAMIG 0084 and *P. citrinum* EPAMIG 0086 isolated from contaminated region was used as a response variable. The F test at a 5% significance level was significant for the interaction fungi X time. Thus, unfolding of the fungus factor within time levels was performed (Table 12). Table 12 reveals that the fungus *P. citrinum* EPAMIG 0086 showed the best results in Cu removal for all the times evaluated.

Concerning the removal of manganese and zinc, an analysis of variance was performed for both species studied. The F test at the 5% significance level was not significant for the two way interaction, fungus X time. Therefore, an individual analysis of the time factor and the fungus factor was carried out. Both were significant by the F test at 5% significance level (Table 13). Table 13 shows that the *P. citrinum* EPAMIG 0086 exhibited better results removing manganese and zinc compared with *A. brasiliensis* EPAMIG 0084.

The results of the pH into biofilters are presents in Table 11. It may be observed that in the biofilter inoculated with *A. brasiliensis* EPAMIG 0084, there was a decline of three points in relation to the initial value (pH 6 to 3) after 6 h of activation and maintenance of this pH value after 120 h (Table 11). This pH variation may have influenced Cu removal (Table 12) by this fungus, once Cu

Table 11. Trace element removal test in aerobic biofilters: changes in pH into biofilters inoculated with fungi isolated from contaminated area after 120 h of activation.

Fungal strain	pH					
	12 h	24 h	48 h	72 h	96 h	120 h
A. brasiliensis EPAMIG 0084	6	3	3	3	3	3
P. citrinum EPAMIG 0086	6	5	6.5	7	7	7

Table 12. Trace element removal test in aerobic biofilters: Cu Concentration after fungi treatment in biofilters (mgL^{-1}). Test of means for the fungus factor within time levels (12 to 120 h) for Cu removal by *A. brasiliensis* EPAMIG 0084 and *P. citrinum* EPAMIG 0086.

Fungal strain	Time					
	12 h	24 h	48 h	72 h	96 h	120 h
A. brasiliensis EPAMIG 0084	6.21^{b}	6.22^{b}	6.20^{b}	6.16^{b}	6.12^{b}	6.06^{b}
P. citrinum EPAMIG 0086	5.58^{a}	5.19^{a}	4.69^{a}	4.42^{a}	3.47^{a}	3.11^{a}

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

Table 13. Trace element removal test in aerobic biofilters: Mn and Zn concentration after fungi treatment in biofilters (mgL^{-1}). Test of means for the fungus factor for the elements Mn and Zn by *A. brasiliensis* EPAMIG 0084 and *P. citrinum* EPAMIG 0086.

Fungal strain	Mn concentration	Zn concentration
A. brasiliensis EPAMIG 0084	2.45^{b}	3.53^{b}
P. citrinum EPAMIG 0086	1.87^{a}	2.59^{a}

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

was not removed during the incubation period (five days, 120 h). A similar result was achieved in laboratory test, after five days incubation, in which only 21.7% of Cu removal was observed by this strain (Table 5). For Mn and Zn (Table 13), it was observed that time was not significant for the trace element variable removal. The aforementioned elements obtained respectively, 51 and 41.6% removal by *A. brasiliensis* EPAMIG 0084. A much lower rate was obtained for Mn and Zn in laboratory scale after five days incubation (11% of Mn and 6.3% of Zn). This occurred even with the final pH 3 for both tests using this strain. Presumably, the initial pH utilized in the biofilter test (pH 6) and the biomass immobilization process helped Mn and Zn removal in the solution. The pH has been identified as one of the most important parameter in metal biosorption study. It is directly related with competition ability of hydrogen ions to active sites on the biosorbent surface (Lodeiro et al., 2006).

According to Arica et al. (2003), the fungal cell immobilization in supports could also enhance fungal cell performance and adsorptive capacity of the biosorbent system for trace element because they were found to be far more stable during continuous operation in a bio-reactor than the fungal cells in free forms. Furthermore, Sayer and Gadd (2001) believed that the production of gluconic, citric and oxalic acids by *A. niger* ATCC 210373 helped the removal of trace elements, such as zinc, from solutions due to its complexation with these elements, followed by a connection to the fungal cell wall. This phenomenon may occur with the *A. brasiliensis* species, since it belongs to the same genus of the aforementioned fungus (Varga et al., 2007).

Kapoor et al. (1999) studied the removal of Pb, Ca and Cu by *A. niger* ATCC 11414 at different pHs and compared it with the removal obtained by traditional methods of ion exchange resin and activated carbon. They observed that at pH 5.5 the precipitation of the trace element did not occur, which seemed to indicate that the removal of the trace element obtained in this study was the result of the mechanisms of biosorption and bioaccumulation, and not a process of elements precipitation due to physical phenomena. The same authors concluded that trace element removal by fungal biomass is greater than that obtained with activated carbon, and the removal by ion exchange resins is not effective when the Ca^{2+}, Mg^{2+} and K^{+} ions are present, as occurs in CW. Thus,

treatment of these effluents with fungal biomass would be a very viable alternative. Likewise, the low removal rate of all elements by *A. brasiliensis* EPAMIG 0084 and *A. brasiliensis* IBT 26433 is due to a pH decrease, which makes it possible to obtain a positively charged biomass. This is due to a high proton concentration, which inhibits the trace element binding because of charges repulsion (Kapoor and Viraraghavan, 1995).

Tesekova et al. (2010) studied the Cu^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, Fe^{3+}, Pb^{2+} and Cd^{2+} removal from wastewater by *A. niger* B 77, immobilized in two different polymers: polyvinyl - alcohol hydrogel (PVA) and Ca - alginate. They also obtained a high removal percentage 72. 8% for Cu^{2+}; 55.4% for Zn^{2+} and 52.3% for Mn^{2+} by Ca - alginate immobilized biomass and by immobilized cubes PVA - biomass: 67.1; 58.5 and 44.6%, respectively. These results are similar than the results obtained in this research by *A. brasiliensis* EPAMIG 0084 (Tables 12 and 13).

Concerning the other fungus in the study, *P. citrinum* EPAMIG 0086 showed one point (pH 6 to 5) in pH decrease in the first 15 h, followed by an increase to 7, which was kept until the end of the experiment (120 h) (Table 11). Probably, the pH variation helped the Cu removal by this fungus (Table 12) (44% Cu removal after 120 h), since the removal of this element was significant with time variable (directly related to the pH variation). For the other elements, this relationship was not observed.

For Mn and Zn, there was no significance between these two variables. However, a significant removal of Mn (62%) and Zn (48%) was also obtained by this fungus (Table 13). These results did not corroborate those obtained in removal tests of trace elements on a laboratory scale after 5 days of incubation, with initial concentration of 5 mgL^{-1} (same condition of biofilters removal test). Thus, the following results were found: 21.7% removal of Cu, 6.5% Mn, and 1.6% of Zn (Tables 5, 7 and 9). Probably, this difference is due to the influence of the initial pH used in both tests, pH 5 on a laboratory scale and 6 in the aerobic biofilters. According to Tay et al. (2010), as initial pH increases, the active sites are being deprotonated and strengthened the charge attraction, thus leading to significant increase in Cu (II) biosorption uptake.

The above mentioned results were also obtained by Verma et al. (2013). They observed that the biosorption potential of immobilized biomass was higher than the biosorption potential of free biomass. When compared Cu Biosorption potential of immobilized and free biomass of fungus *P. citrinum* immobilized they obtained 76.2% of cu removal by immobilized biomass and 74% by free biomass. They also showed that the biosorption increased with rise in pH but can be concluded that no significant change in the amount of Cu (II) removal was observed after pH 5.0 (Verma et al., 2013).

Hemambika et al. (2011) studied the trace elements biosorption by *Aspergillus* sp. and Penicillium sp. isolated from effluent collected from an electroplating industry at India that uses copper, cadmium and lead for plating. They compared the trace elements removal by immobilized cells in sodium alginate and free cells. They achieved 60.94 and 46.91% of Cu removal by Aspergillus sp. immobilized and free cells respectively. For the *Penicillium* sp., it showed 97.21% of Cd removal by immobilized cells and 95.27% by free cells. Relative to the best pH to trace elements removal, the authors observed that to *Penicillium* sp. growth and trace elements removal which is pH 6; the same pH value reached in this research (Table 11). Then, it is evidence that the pH medium obtained with the *P. citrinum* EPAMIG 0086 cultivation facilitated the trace elements removal.

Fourest and Roux (1992) also related a greater trace element removal percentage (mainly Zn) by the filamenttous fungus Rhizopus arrhiizus in pH near neutrality, as well as a decrease of the removal proportional to pH decrease. These authors explain that this phenomenon happens due to the competition by protons and trace elements to the binding sites present on the fungal cell wall. The same was previously observed by Galun et al. (1987) regarding the Zn^{2+} uptake by *Penicillium digitatum*. In this study, removal of Zn was highly sensitive to variations in pH, being inhibited at a pH below 3. Probably, this phenomenon occurs also in the present work with fungus *A. niger* EPAMIG 0084, since this fungus changed the media pH from pH 6 to 3 (Table 11) and showed only 29.4% of Zn removal (Table 13) in aerobic biofilters removal test.

Another study evaluated Cu^{2+} biosorption by *Penicillium cyclopium*. Removal was considered strongly dependent on pH, on the amount of biomass and on the concentration of Cu ions in the solutions. The biosorption process was fast and, in the first 5 min, up to 75% of the total Cu ions were deposited on the surface of the fungus under study (Tsekova et al. 2006).

After evaluation of the present results (Tables 12 and 13), it may be concluded that throughout the whole experiment, the fungus *P. citrinum* EPAMIG 0086 more efficiently removed most of the trace elements studied as compared to *A. brasiliensis* EPAMIG 0084. Probably, the trace elements studied are sensitive to pH variation, which occurred in the biofilter inoculated with the fungus *A. brasiliensis* EPAMIG 0084 (Table 11).

Scanning electron microscopy with X-ray microanalysis

This method of assessing the presence of trace elements detects only those elements that are attached to the fungal cell wall, thus, exhibiting the trace elements that were removed from solution by the biosorption process. However, this analysis, in spite of providing quantitative

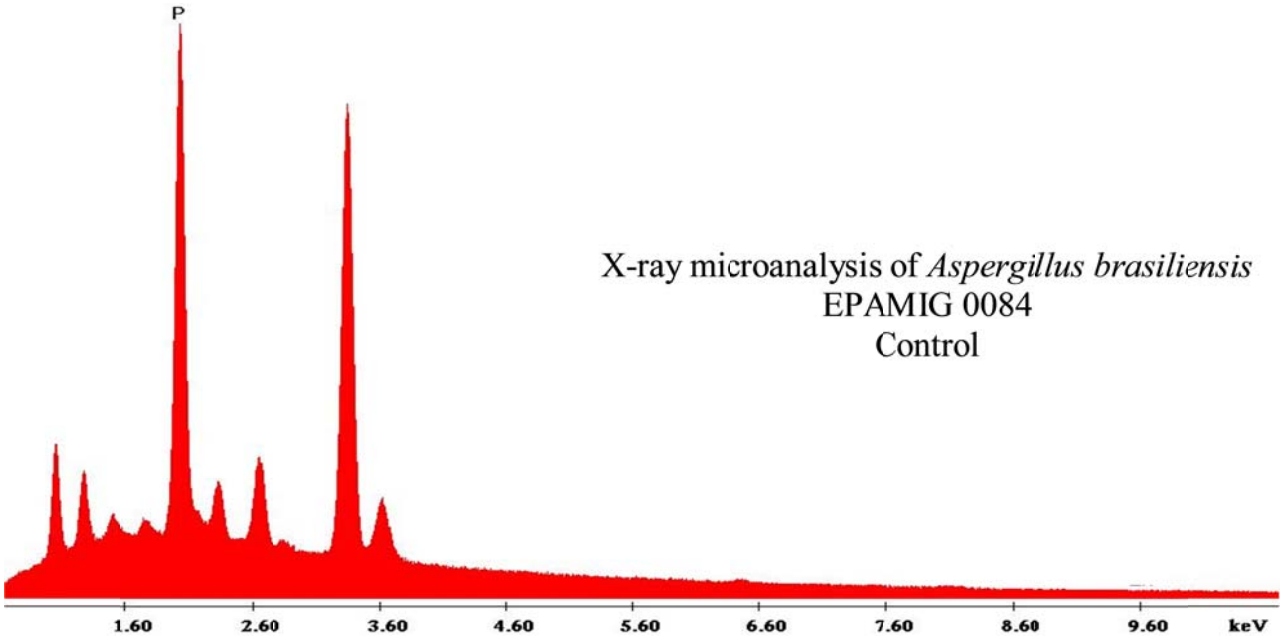

Figure 1. Scanning electron microscopy with X-ray microanalysis of the fungus *A. brasiliensis* EPAMIG 0084: control sample - Fungus cultivated without trace elements.

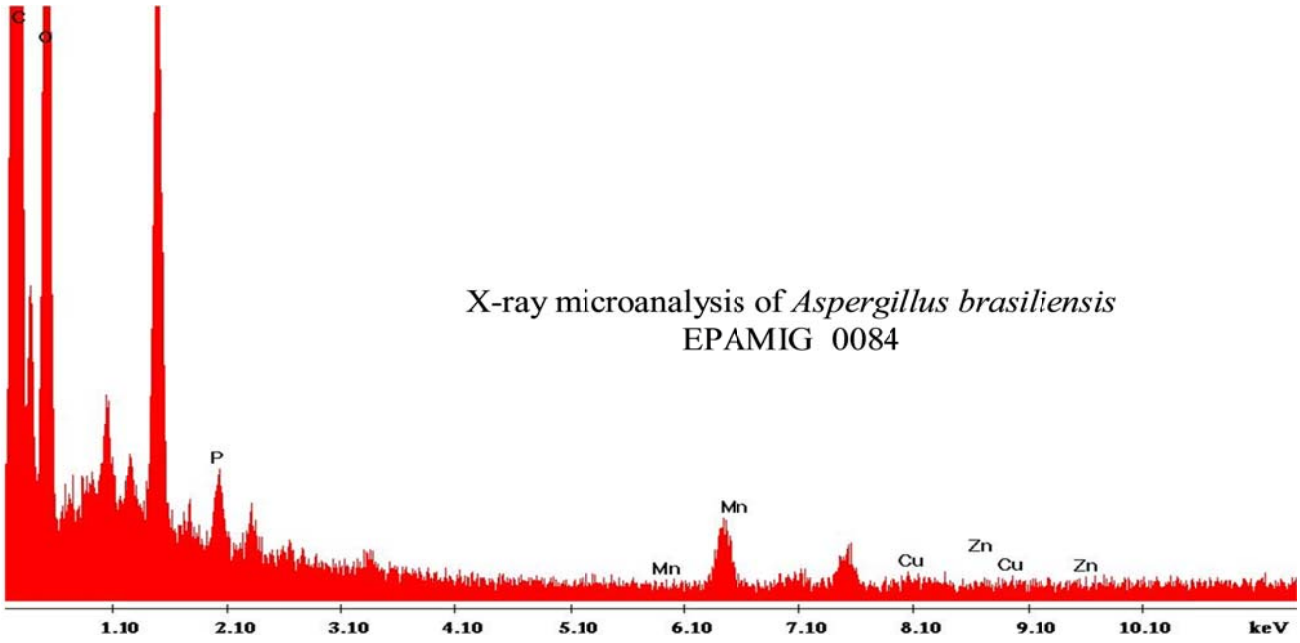

Figure 2. Scanning electron microscopy with X-ray microanalysis of the fungus *A. brasiliensis* EPAMIG 0084 isolated from the contaminated region cultivated in the presence of trace elements (Cu, Mn and Zn; 5 mgL^{-1}).

data near the real values, cannot be regarded as a quantitative analysis but only a qualitative one. Figures 1 to 6 present the results of scanning electron microscopy with X-ray microanalysis of *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011 after being cultivated in the presence of a trace element solution (5 mgL^{-1}) for 10 days in 100 rpm at 25°C. An evaluation of Figures 1 to 6 shows that there was the formation of peaks of the elements Cu, Mn and Zn for the four strains studied, indicating that probably the high

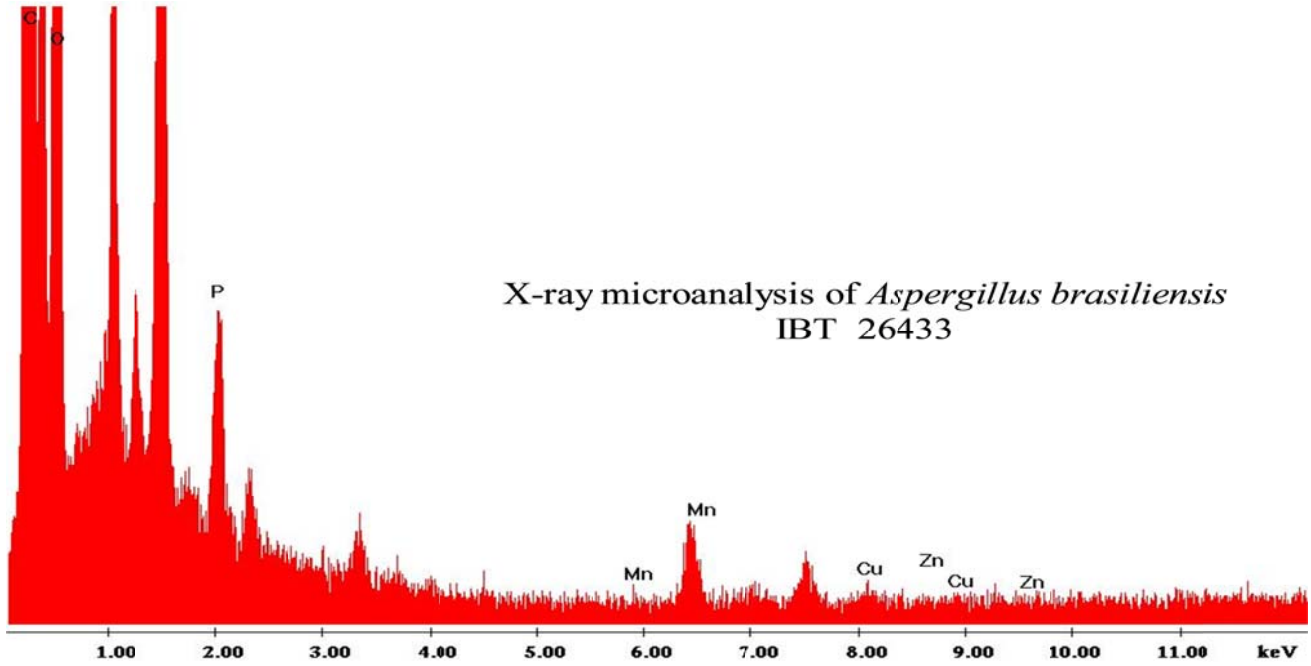

Figure 3. Scanning electron microscopy with x-ray microanalysis of the fungus *A. brasiliensis* IBT 26433 isolated from uncontaminated region cultivated in the presence of trace elements (Cu, Mn and Zn; 5 mgL^{-1}).

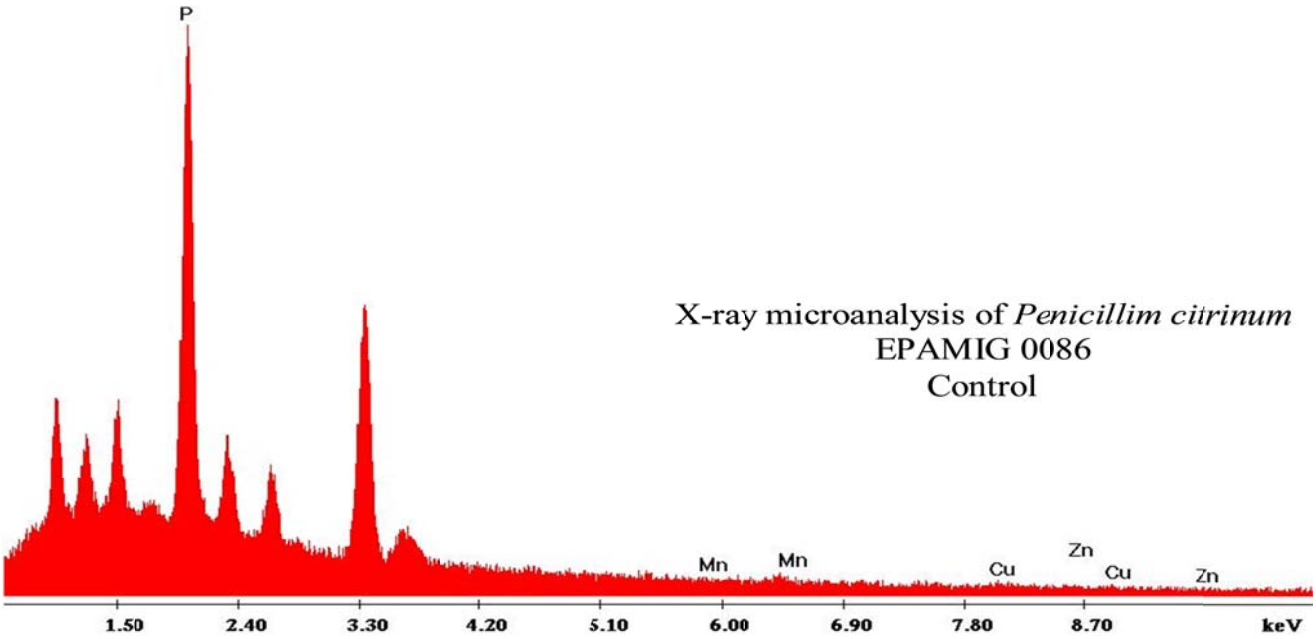

Figure 4. Scanning electron microscopy with X-ray microanalysis of the fungus *P. citrinum* EPAMIG 0086: control sample - without trace elements.

percentage trace element removal observed in previous tests could be due to adsorption process of these elements to the fungal cell wall (2008).

Purchase et al. (2009) and Velmurugan et al. (2010) also used scanning electron microscopy with X-ray microanalysis to show Pb adsorption by the fungus

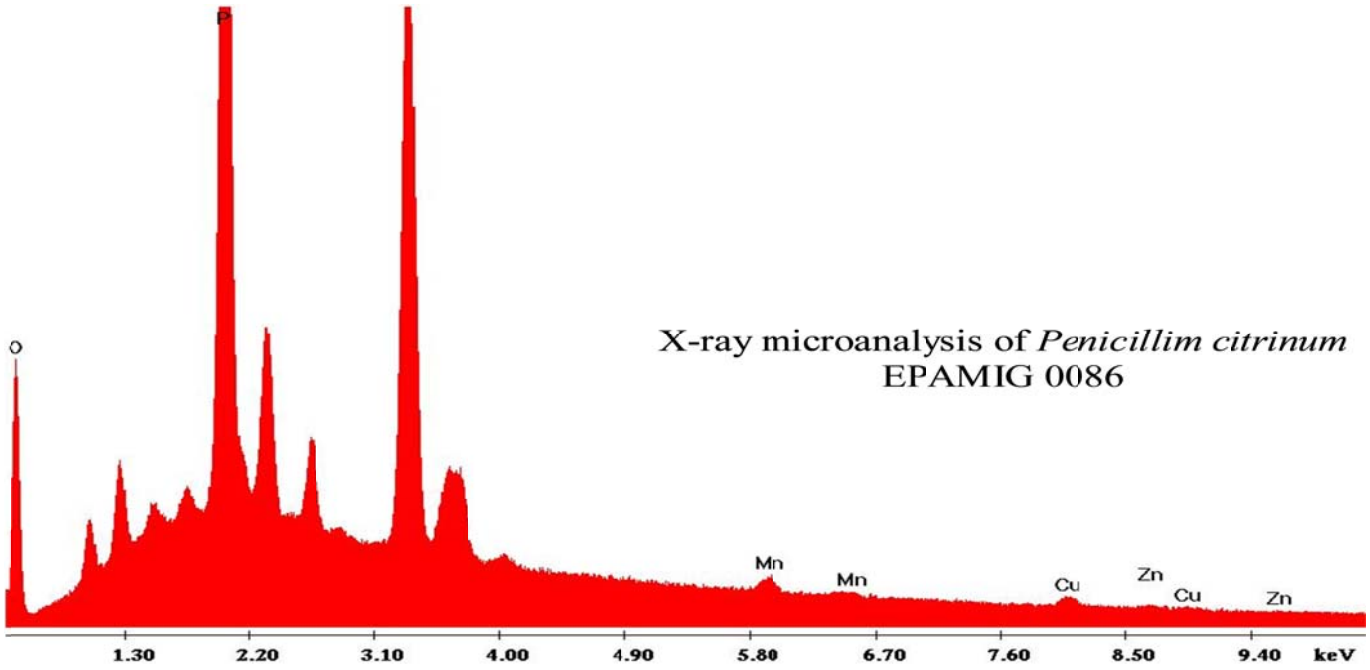

Figure 5. Scanning electron microscopy with X-ray microanalysis of the fungus *P. citrinum* EPAMIG 0086 isolated from a contaminated region cultivated in the presence of trace elements (Cu, Mn and Zn; 5 mgL^{-1}).

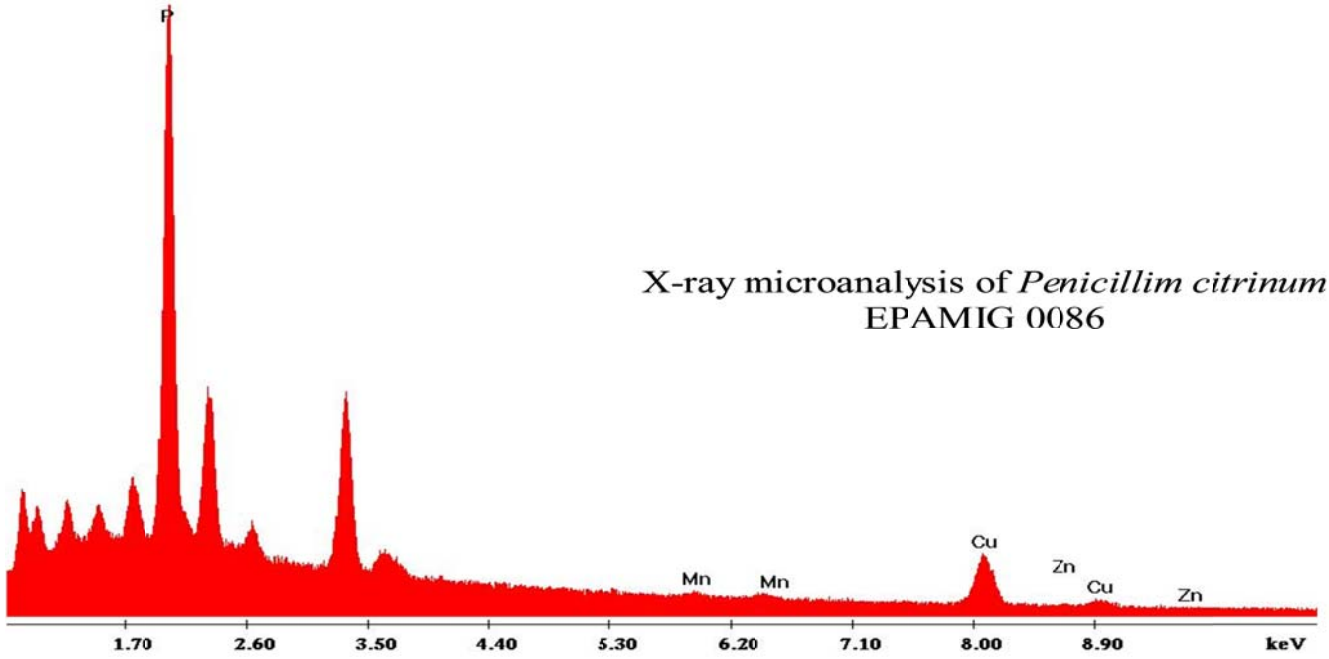

Figure 6. Scanning electron microscopy with X-ray microanalysis of the fungus *P. citrinum* EPAMIG 0086, isolated from an uncontaminated region cultivated in the presence of trace elements (Cu, Mn and Zn; 5 mgL^{-1}).

Beauveria bassiana and Zn by the biomass of *Fusarium* spp. They concluded, through this technique, that the adsorption process is one of the mechanisms responsible for removal of environment Pb and Zn by these fungi.

Although the results from X-ray microanalyses do not provide qualitative, but quantitative findings, it may be

observed that the peak formed for the Zn element, for all strains tested, was very small in relation to the other trace elements evaluated. This result is in apparent contrast with the previous tests (atomic spectrophotometry) in that Zn was one of the elements removed with greatest efficiency by the fungus being studied (Tables 9 and 12). However, this element must have been absorbed by fungal cells, since it is very important in various enzymatic functions, thus showing that both adsorption and absorption processes play important roles in removal of trace elements from the environment. The same process may have occurred with the other trace elements. However, as can be seen in the Figures 2 to 6, many of these were attached to the fungal cell wall.

Conclusion

It can be concluded from the present results, that removal is probably related to medium pH. In addition, based on the excellent aerobic biofilters test results, *P. citrinum* EPAMIG 0086 and *P. citrinum* INCQS 40011, regardless of their origin, demonstrated to be very efficient and viable alternatives for solution trace element removal. Therefore, they may be used for coffee wastewater treatment. Since the trace elements are toxic, bioaccumulative and carcinogenic, this study can benefit both the environment close to agricultural regions and public health. Moreover, as a pilot scale project, its applicability in real scale becomes a very close reality, thereby, ensuring environmental and human health quality.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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