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Full Length Research Paper

Tolerance for heavy metals by filamentous fungi isolated from a sewage oxidation pond

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Metal pollution of the environment, especially at elevated concentrations, is known to adversely affect microbial activities. However, microorganisms are a versatile group, as they can adapt and grow under various extreme conditions including high metal concentrations. In the present study, fungal strains isolated from the oxidation pond of the University of Nigeria, Nsukka sewage treatment plant were evaluated for their tolerance for different concentrations of metal salts, namely, Cu, Zn and Mn. The fungi isolated and tested belonged to the genera *Aspergillus*, *Penicillium* and *Cladosporium.* The results revealed that metal tolerance by the isolates was highest for Zn, followed by Mn and then Cu and the isolates tolerated up to 1500 µg/ml of the salt. Tolerance for the metal ions was concentration as well as time dependent and also on the isolate tested. *Aspergillus niger* and *Aspergillus fumigatus* were the most tolerant of the metals and exhibited good growth at all metal salt concentrations tested. These fungal isolates showed a high level of tolerance for the metals tested, which makes them attractive potential candidates for further investigations regarding their ability to remove metals from contaminated wastewaters.

Key words: Metal tolerance, biomass, bioremediation, waste water, fungi.

INTRODUCTION

Rapid industrial development has led to an increased discharge of industrial effluents, which may contain metal salts in concentrations well beyond the permissible limits, into the environment (Ahuja et al., 2001). The metal pollutants of serious concern, due to their carcinogenic and mutagenic nature, include lead, chromium, mercury, uranium, selenium, zinc, arsenic, manganese, cadmium, gold, silver, copper, nickel, etc. (Ahalya et al., 2005). These toxic materials may be derived from mining operations, refining of ores, sludge disposal, fly ash from incinerators, the processing of radioactive materials, metal plating, or the manufacture of electrical equipment, paints, alloys, batteries, textile dyeing, leather tanning, pesticides or preservatives (Ahalya et al., 2005).

Copper (Cu) is a ubiquitous metal present in the environment and is the most common contaminant of industrial effluents such as those produced by mining and metal processing (Anand et al., 2006) or those used in vineyards, ranging from the application of fertilizers to dumping of agricultural and municipal wastes. Copper is an essential element for all living organisms as a co-factor for a variety of enzymes; however, an excess of this element can be mutagenic and can cause the appearance of highly reactive oxygen radicals (Zapotoczny et al., 2006). Experimental studies in humans suggest that ingestion of drinking water with > 3 mg Cu/l will produce gastrointestinal symptoms, including nausea, vomiting and diarrhea (Pizzaro et al., 1999).

Zinc (Zn) is an environmentally ubiquitous heavy metal. It is an essential trace element that is needed for the normal metabolism of living organisms. However,

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anthropogenic inputs could cause elevated Zn concentrations in the environment due to man-induced activities (Yap et al., 2005). High exposure to Zn in humans can cause nephritis, anuria and extensive lesion in the kidney. The metal can be dissolved in aquatic ecosystems and transported by water and taken up by aquatic organisms or can be stored and transported in sediments (Yap et al., 2005).

Manganese is an essential element and is a co-factor for a number of enzymatic reactions, particularly those involved in phosphorylation, cholesterol and fatty acid synthesis. The metal is present in all living organisms (Keen and Zidenberg-Cherr, 1996). The industrial use of manganese has expanded in recent years as a ferroalloy in the iron industry and as a component of alloys used in welding and also in the application of manganese pesticides (Apostoli et al., 2000). Manganese from anthropogenic sources can contaminate surface water, ground water and sewage water.

Conventional methods for removing particulate and dissolved metal salts from aqueous solution have been studied in detail, such as chemical precipitation and sludge separation, chemical oxidation or reduction, ion exchange, electrochemical treatment, membrane technologies, reverse osmosis, filtration, adsorption on activated carbon and evaporative recovery (Lopez and Vazquez, 2003). However, these methods have problems associated with their application and are not economically feasible (Volesky, 2001; Okoronkwo et al., 2007). Biological process like biosorption has acquired due attention owing to a number of advantages, which have engaged scientists all over the world to identify the potent biomass type (Khan et al., 2009; Al-Masri et al., 2010).

Environmentally ubiquitous fungi are structurally unique organisms that contribute to significant removal of metal ions from wastewater than other microbes. This is because of their great tolerance towards metals and other adverse conditions such as low pH, and their intracellular metal uptake capacity (Gadd, 1987). Fungi biomass, both living and dead, has been used as suitable biosorbent for metal uptake (El-Sayed and Morsy, 2004; Subudhi and Kar, 2007).

There are two modes of metal ion uptake, the first mode is independent of cell metabolic activity, and is referred to as biosorption or passive uptake. It involves surface binding of metal ions on the cell wall. The functional groups involved in the binding of metals to microbial cells are phosphate, carboxyl, and hydroxyl groups (Karna et al., 1999). This mode is common to both living and dead cells. The second mode of metal uptake into the cell across the cell membrane is dependent on the cell metabolism, and is referred to as intracellular uptake, active uptake or bioaccumulation, which occurs in living cells only (Garnham et al., 1992).

The main purpose of the present study was to evaluate the potential of some fungal isolates from a sewage oxidation pond to tolerate different concentrations of some metal ions, namely, Cu, Zn, and Mn.

MATERIALS AND METHODS

Collection of samples

The sewage water samples used for the study were collected from the sewage treatment plant of the University of Nigeria, Nsukka, Southeast, Nigeria. The samples were collected with sterile containers from the oxidation ditch and transported to the laboratory immediately for analysis.

Isolation and identification of organisms

The samples were serially diluted (10-fold) before surface-plating on sterile potato dextrose agar (PDA) plates. All the inoculated plates were incubated at room temperature (30 ± 2℃) for five days according to Lopez and Vazquez (2003). A number of morphologically different colonies were randomly selected and sequentially sub-cultured for purification on the same medium. Pure cultures of the isolates were characterized and identified using standard mycological techniques of George (1971) and Beneke and Rogers (1969), which included lactophenol cotton blue staining and observing under the microscope with low power (x10 magnification) for screening in low intensity for septation in mycelium, shape and structure of the conidia and also the morphological characteristics of the isolates on the medium.

Tolerance of fungal isolates for metals

The fungal isolates obtained from PDA were tested for their ability to tolerate different concentrations of the metal salts incorporated into potato dextrose broth. The ability of the isolates to tolerate different concentrations of metal salts, namely, $ZnSO_4$ $CuSO_4$, and $MnSO_4$, was determined by incubating the isolates in 50 ml broth contained in 250 ml Erlenmeyer flasks. The medium was amended with the appropriate aliquot of the stock solution of the metal salt to give the final concentrations of each metal salt of 100, 200, 500, 1000, and 1500 µg/ml. The inoculum was introduced into the flask with a sterile cork borer (6 mm diameter). The flasks were incubated at room temperature (30 ± 2℃) for seven days af ter which the growth of the fungal strains was determined. The fungi biomass was harvested by filtration and dried until a constant weight was obtained. The set-ups were prepared in duplicates.

Data analysis

The ANOVA test was used on the generated data from the tolerance study using GENSTAT discovery edition 3. Separation of means for statistical significance was done using F-LSD at 5% probability level according to Obi (2002).

RESULTS

Tolerance of fungal isolates to the metal salts

The results displayed in Figures 1, 2 and 3 shows the level of tolerance and biomass production at the different metal salt concentrations by the fungi isolates.

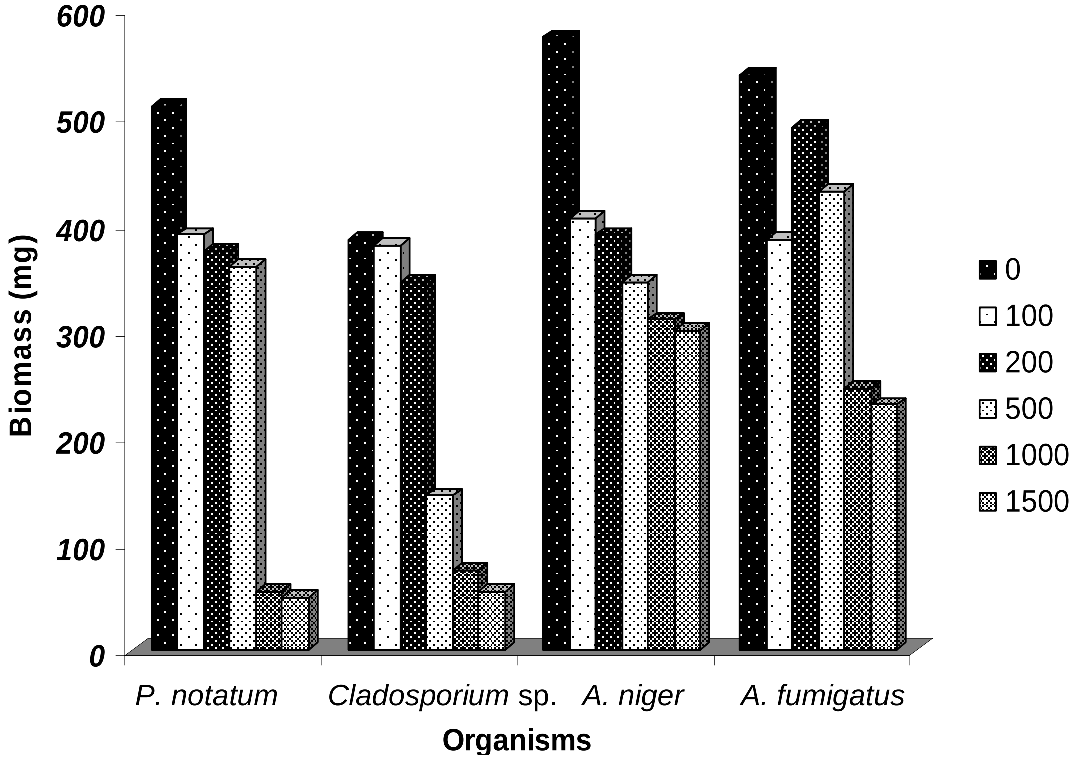

Figure 1. Tolerance of the fungi to different concentrations of $CuS0_4$.

Figure 1 shows the tolerance of the isolates for different concentrations of $CuSO_4$. In this study, the highest biomass yield was observed, for all the isolates, in the medium with no metal ion amendment, which served as the control. In the absence of the metal ion, the amounts of biomass dry weight produced within the incubation period was 575 mg for *Aspergillus niger*, 540 mg for *Aspergillus fumigatus*, 510 mg for *Penicillium notatum* and 385 mg for *Cladosporium* sp. For all the isolates except *A. fumigatus*, biomass yield decreased as metal ion concentration increased from 100 to 1500 µg/ml. There was an initial decrease in biomass yield for *A. fumigatus* at 100 µg/ml, which slightly increased at 200 µg/ml and decreased continuously to 1500 µg/ml. At the highest concentration of 1500 µg/ml, *A. niger* biomass yield was 300 mg, while it was 230 mg for *A. fumigatus*, 55 mg for *Cladosporium* sp. and 53 mg for *P. notatum*.

In Figure 2, the biomass yields of the fungal isolates in the presence of different concentrations of zinc ion are shown. The biomass yield for all the isolates, namely, *Penicillium notatum*, *Cladosporium* sp, *A. niger* and *A. fumigatus*, in medium without metal salt was highest, similar to what was obtained for $CuSO_4$. These isolates had their biomass decreased as the metal salt concentration increased. The least biomass yield was, therefore, obtained at the highest concentration of 1500 µg/ml. However, for *P. notatum* and *A. niger*, there was an increase in biomass yield as metal salt increased from 100 to 200 µg/ml. The highest biomass yield in presence of metal salt was 490 mg and was obtained at 1000 µg/ml, which was still lower than 510 mg obtained without metal salt while the lowest biomass yield of 370 mg was obtained at 100 µg/ml concentration of $ZnSO_4$.

The ability of the isolates to tolerate different concentrations of $MnSO_4$ is shown in Figure 3. There was observed a stepwise decrease in biomass yield for all the isolates from zero concentration of metal salt to the highest concentration of 1500 µg/ml. The biomass yield in the absence of metal salt was 385 mg for *Cladosporium* sp., 510 mg for *P. notatum*, 540 mg for *A. fumigatus* and 575 mg for *A. niger*. The values of the biomass were obtained as 85 mg for *P. notatum,* 130 mg for *Cladosporium* sp., 255 mg for *A. fumigatus* and 345 mg for *A. niger* at 1500 µg/ml.

The statistical analysis of the data obtained in the interactions between the organisms, and the respective metal ions yielded the results shown in Tables 1, 2 and 3. Table 1 shows the mean effect of the concentrations of the metal ions on the tolerance of the organisms translated into biomass production. The results showed that as the metal ion concentration increased, biomass production decreased. Thus at 100 µg/ml concentration, the organisms produced the highest mean biomass of 389.20 mg, which is statistically different ($p \le 0.05$) from

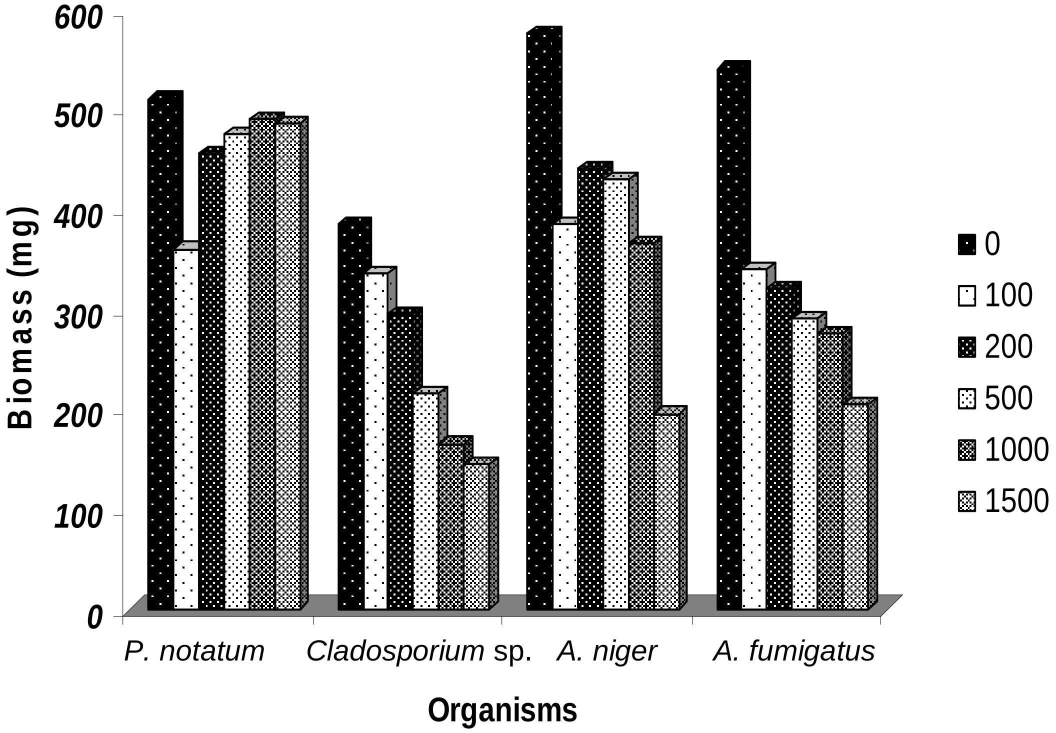

Figure 2. Tolerance of the fungi to different concentrations of $ZnS0_4$.

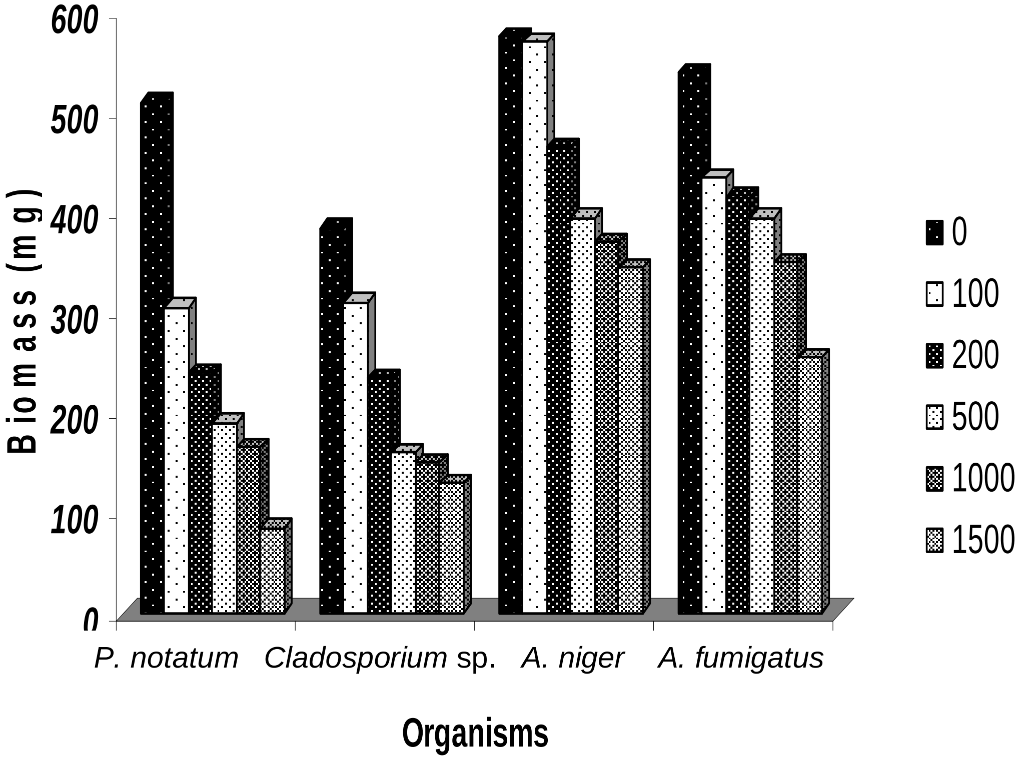

Figure 3. Tolerance of the fungi to different concentrations of $MnS0_4$.

Table 1. Mean effect of metal ion concentration on biomass production.

Concentration (µg/ml)	Biomass (mg)
100	389.20
200	372.10
500	319.20
1000	251.20
1500	206.70
F-LSD $_{(0.05)}$	9.02

Table 2. Mean effect of metal ion on biomass production.

Metal ion	Biomass (mg)
Zn^{2+}	336.80
Mn^{2+}	298.30
Cu^{2+}	288.00
F-LSD (0.05)	6.98

Table 3. Mean effect of organism on biomass production.

Organism	Biomass (mg)
Aspergillus niger	385.30
Aspergillus fumigatus	337.30
Penicillium notatum	298.70
Cladosporium sp.	209.30
F-LSD $_{(0.05)}$	8.07

that produced at 200, 500, 1000, and 1500 µg/ml with biomass of 372.10, 319.20, 251.20 and 206.70 mg, respectively.

The mean effect of the metal species on biomass production by the different organisms is shown in Table 2. The results showed that the organisms exhibited greater tolerance for Zn with mean biomass value of 336.80 mg, which is significantly different ($p \leq 0.05$) from Mn and Cu with mean biomass values of 298.30 and 288.00 mg, respectively.

Among the four organisms used in the present study, Table 3 showed that *Cladosporium* sp. exhibited the least tolerance for the metals with a mean biomass production of 209.30 mg. On the other hand, *A. niger*, with mean biomass production of 385.30 mg, significantly ($p\leq 0.05$) showed better tolerance than the rest organisms. The interaction effects were significant among the three factors considered in the study.

DISCUSSION

Metals such as copper and zinc are important trace nutrients for some microorganisms, but they can also be toxic when concentrations are too high. However, whether metals have a harmful effect depends essentially on the availability of the metals for the organisms. Microbial systems for regulating trace-metal uptake can be important factors in competition with other microbes when the metal ions are either limiting (Kloepper et al., 1980) or present at toxic levels (Silver and Walderhuag, 1992). The understanding of microbial tolerance and adaptation to the presence of metal in the environment is critical in determining the management and potential long-term effect of that part of the environment receiving metal contamination.

In the present study, four fungal isolates were obtained which exhibited different growth patterns in the presence of different metal salts, namely, copper, zinc and manganese. The isolates showed decrease in growth (measured in terms of biomass production) upon increasing metal concentration at any given time interval compared to the control without metal amendment. The reduction in biomass values revealed that the fungi were affected due to the presence of metal in the growth medium. The reduction in growth in the presence of increased concentrations of the metals was evident throughout the experiment compared to the control without metal. However, for the three metals used in this study, growth was least affected in the presence of Zn^{2+}. A similar finding was reported elsewhere (Hassen et al., 1998).

Generally, all the isolates showed the highest sensitivity to Cu^{2+} followed by Mn^{2+}, based on the mean biomass produced in presence of the metals. The isolate, *A. niger* followed by *A. fumigatus*, were the most tolerant of the metals. It has been reported by Faryal et al. (2007) that strains of *A. niger* in a study showed the highest resistance to lead warranting them to be successful candidates for metal detoxification. Sayer et al. (1995), observed that *A. niger* could solubilize Zn when grown on malt extract with 4% (w/v) $Zn_3(PO_4)_2$. The levels of metal tolerance among the fungal isolates were different from one to the other showing different levels of reduction of their growth in the presence of same concentration of a given metal.

This finding revealed that when tolerance to the three metals was compared with respect to each fungus, it was evident that the fungi were more sensitive to Cu^{2+} than the other two metals. All the fungal isolates were more tolerant of Zn^{2+} than the other two metals. Zinc is known to be associated with enzymes, particularly metallo-enzymes, which are essential for fungal growth (Ross, 1994). Sintuprapa et al. (2000) reported of tolerance to Zn at 1000 µg/ml for ectomycorrhizal fungi and *Penicillium* species. However, at higher concentrations, zinc could become toxic to the organism (Aronson, 1982) which could explain the reason for the reduction of biomass at 1500 µg/ml.

The reduction in biomass production by the fungi as metal concentration increases for the different metals might be due to the fact that at higher concentrations, the metal interacts with nucleic acids and enzyme active site, which can lead to rapid decline in membrane integrity and cell death (Cervantes and Gutierrez-Corana, 1994; Stohs and Bagchi, 1995). However, the differences in tolerance reported for the fungi to the metals may result from the diverse ability of the different fungi (Lopez and Vazquez, 2003).

Conclusion

The current work demonstrated that the tolerance for metals varied between fungi, even though they were isolated from the same soil. All the fungi were more sensitive to Cu^{2+} than the other two metals, while being more tolerant to zinc than the other two metals. The fungal isolates obtained in the present study have the ability to tolerate metals at high concentrations. *A. niger* exhibited the greatest ability to tolerate the metal salts than the other fungi which makes the organism a better candidate for further studies in the removal of metals from aquatic environment in bioremediation.

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