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Comparative Analyses on Heavy Metals Immobilisation Ability of *Aspergillus niger*, *Penicillium* and *Trichoderma* Fungi in Soil

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Abstract

The heavy metals immobilisation ability of *Aspergillus niger*, *Trichoderma*, and *Penicillium* fungi was assessed by analysing leaching of selected heavy metals in soil. This was done by amendment of soil obtained from an irrigation site in Kaita, Katsina State with 10 mg/L of Cadmium (Cd), Chromium (Cr), Manganese (Mn), Nickel (Ni), Lead (Pb), and Zinc (Zn). This was then divided into four equal parts: one as control, and the other three with 10 ml of 4.0×10^6 CFU/ml *Aspergillus niger* (An), *Penicillium* (Pn), and *Trichoderma* (Tr) added separately in polythene bags. The setup stood for 14 days, with water added every 3 days. Samples were collected on days 7 and 14, and leaching was analysed using AAS. Results showed a sharp decrease in metal concentrations by day 7, with further reductions by day 14. However, variation was observed - some fungi-treated samples had higher or lower concentrations than controls, indicating differences in immobilisation. Notably, *An* showed strong retention for Mn, Cr, Pb (week 1), and Cd, Zn, Ni (week 2); *Tr* performed best for Cd, Zn, Ni (week 1) and Pb (week 2); *Pn* was more effective in week 2. All fungi showed potential for soil bioremediation.

Keywords: Fungi, Heavy metals, Soil, Leaching, Immobilisation

INTRODUCTION

Soil serves as a reservoir for essential trace elements like zinc and cadmium, necessary for plant growth. However, external factors can increase their concentration, reducing soil fertility and agricultural productivity. If these metals accumulate in the food chain at levels above normal, they can have adverse effects on human and animal health [1]. There is also the risk of heavy metals leaching into groundwater, potentially contaminating water sources for human consumption. Industrialization and urbanization have increased heavy metal pollution from effluents containing metals like lead, cadmium, chromium, nickel, and mercury [Heavy metals are a major [2, 3] concern due to their toxicity, reactivity, and mobility in soil. Emissions of these pollutants pose a severe threat to human health. Human exposure to heavy metals can occur through inhalation, skin contact, and ingestion [4,5]. To mitigate the environmental impacts of heavy metals, several methods are being adopted, including chelation, precipitation, adsorption, ion exchange, and biosorption.

Fungi possess great ability for heavy metal remediation because of their prevalence of large biomass in the soil and longer life cycle [6, 7]. Metals can be removed or fixed via the myco-remediation process where the fungi are used to degrade or sequester heavy metals in contaminated area by using its enzymatic activities [8] The fungal cell wall has specific metal-binding peptides, proteins, and polysaccharides that contain hydroxyl (OH), carboxyl (R-COOH), phosphate (PO_3), sulfate (SO_4), and amino (NH_2) groups that bind metal ions which are used for myco-remediation purpose [9]. Heavy metals can produce reactive oxygen species (ROS), leading to oxidative stress, alteration of calcium homeostasis, and DNA damage. ROS production causes toxicity and damage to essential biomolecules such as proteins, nucleic acids, and lipids. Fungal resistance to metals is linked to their ability to eliminate ROS. Intracellularly, heavy metal ions can be stored and/or detoxified by binding with metallothionein due to their high thiolate sulfur content. Various biomolecules, including thiol compounds like protein-bound sulfhydryl groups (PB-SH), glutathione disulfide (GSSG), and nonprotein sulfhydryl groups (NP-SH), as well as cysteine-rich peptides like phytochelatins, bind metal ions and scavenge ROS [10].

In fungi, antioxidant enzymes play a crucial role in cellular responses to metal exposure and detoxification. Enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), glutathione S-transferases (GSTs), and glutathione reductase (GR) protect cells from metal-induced stress [11]. The biosynthesis of PB-SH, NP-SH, GSH, and GSSG, along with the activation of these antioxidant enzymes, are significant in responding to copper (Cu) exposure and detoxification [12]. These enzymes and biomolecules act together to neutralize ROS and repair damage caused by oxidative stress.

With the presence of mycelia, fungi can diversify expeditiously in soil because of their large filaments surface area [13]. The toleration of elevated concentration of heavy metals is also supported by the ability of fungi to produce extracellular degradative enzymes that reduce the toxicity of the heavy metals when taken inside the cell. The secretion of enzymes coupled with the mechanical support through the penetration of fungi hypha into the soil help to facilitate and maximise the transformation of

contaminants in contaminated soils. The unique composition of the fungal cell wall offers the advantage of providing excellent metal-binding properties [14] Fungal cells can also use entrapment in extracellular capsules and precipitation of metals as means to bind the pollutants to their cell wall. In addition, intracellular accumulation and sequestration can be a part of the strategies to immobilise metals [15]. The membrane transport systems can play a part in suppressing cellular influx or active efflux of metals When metals are present in the cells, they can be sequestered via intracellular precipitation, chelation, localisation in organelles; or be biotransformed to less toxic form [16]. The mechanisms such as intracellular (binding to compounds like proteins) and extracellular (chelation and cell wall binding) sequestration of heavy metals have been offered as mechanisms for heavy metals resistance in fungi [17]. Numerous species of fungi use their spores and mycelium to absorb specific heavy metals like Cu, Cd, Pb, Hg, and Zn. Amongst all microbes, fungal biomass, predominantly species of *Aspergillus niger, Penicillium chrysogenum, Rhizopus nigricans, Saccharomyces*, etc., have a great percentage of cell wall content, which exposes metal-binding features through biosorption mechanism [18, 19].

The fungal hyphae can physically encapsulate soil particles containing metals, effectively immobilising them and reducing their availability to plants. By these mechanisms fungi act as natural biofilters in contaminated soils, reducing metal toxicity in plants and the broader ecosystem [20]

The main aim of this comparative study is to determine whether *Aspergillus niger*, *Penicillium* and *Trichoderma* fungi which possess multiple metal binding features and protective mechanisms as mentioned by [21]; [22]; [23]; [24], etc. can be able to inhibit the mobility of Cd, Cr, Mn, Ni, Pb and Zn metals in soil.

MATERIALS AND METHODS

The materials include soil sample from Kaita irrigation site; Spore suspension of *Aspergillus niger,Penicillium* and *Trichoderma* fungal species, which were obtained from Microbiology Department, UMYU); Standard solutions of Zn, Pb, Cd, Cr, Mn and Ni which were prepared by dissolving 4.398 g of Zinc Sulphate, 1.570 g of Lead ethanoate, 6.896 g of Cadmium Sulphate, 7.542 g of Chromium Sulphate, 2.894 g of Manganese Sulphate and 4.478 g of Nickel Sulphate in 1000 cm^3 of distilled water to obtain 1000 mg/L; 1 M standard solution of $MgCl_2$ which was prepared by dissolving 59.76 g in 1000 ml of distilled water; Laboratory shaker and filtration apparatus.

Sample treatment

The collected soil from Kaita irrigation site (KSS) was prepared by removing stones and debris, sieved using 8 mm pore laboratory sieve then allowed to remain on a clean dry surface. 10 mg/L solution of Cd, Cr, Mn, Ni, Pb and Zn metal salts was prepared from the stock solution through serial dilution in which 10 cm^3 of the metal solution was measured using measuring cylinder and added to the soil. These was then mixed thoroughly to obtain a homogenous sample. The sample was then divided into 4 equal parts of 500 g each, which were transferred to separate polythene bags. One bag was taken as the control, while the other 3 had 10 ml of ~4.0 x 10^6 CFU/mL *Aspergillus niger (An), Penicillium (Pn) and Trichoderma (Tr)* fungi

added. These were allowed to stand for a period of 2weeks in which water was being added on 3 days interval [25]. Samples were collected after the 1st (7th day) and 2nd (14th Day) week of soil amendment with metal solutions and fungal suspension. Leaching test was conducted on the collected samples to determine extent of the heavy metals leaching and the effect of the fungi on the leaching which will further determine the effectiveness of the fungi in the heavy metal immobilisation.

Leaching test

The soil samples collected on 7th and 14th day after introduction of metal solutions and fungal suspension will be used for the leaching test. Soil extracts were prepared by weighing out 10 g portion of the soil into 250 mL conical flasks. To the flasks 100 mL deionised water was added and then mantled to a laboratory shaker where they were shaken for a period of 1hr at the rate of 130 rpm. The flasks were removed and leached with $5cm^3$ of 1.0 M $MgCl_2$ then returned to the shaker to continue shaking at the rate of 120 rpm for 6 hours at 25 °C. The flasks were then removed and the samples filtered three times using Whatman No. 4 filter paper. [26]. The filtrates (leachates) were transferred to small plastic sample bottles and transported to Soil laboratory at Ahmadu Bello University, Zaria for Cd, Cr, Mn, Ni, Pb and Zn metals determination using Atomic Absorption Spectrometer (AAS).

Atomic absorption spectroscopy (AAS)

This is a widely used analytical technique for determining the concentration of specific elements in a sample. It is based on absorption of light by free ground- state atoms. Before analysis the leachates were acidified with HNO_3 to preserve and prevent precipitation. The instrument was then calibrated with standard solution of known metal concentration. A blank was used to zero the instrument and a calibration curve was generated. The sample was then introduced into the machine .by aspirating it through a nebuliser. This is converted into an aerosol and directed into a flame for atomization. The sample is then heated to a high temperature to break it into free atoms using the air -acetylene or nitrous oxide-acetylene flames. A Hollow Cathode Lamp (HCL) or an Electrodeless Discharge Lamp (EDL) specific to the elements being analysed emits light of a precise wavelength which corresponds to the required energy level to exile the electrons of the target element. As the light passes through the atomised sample, atoms of the target element absorb light at specific wavelength. The amount of light absorbed is directly proportional to the concentration of the elements in the sample (Beer Lambert's Law). A monochromator isolates the wavelength of interest and a detector measures the intensity of the absorbed light and the signal is processed to determine the elements concentration. The concentration values were read directly from the calibration curve in mg/L [4].

Quality control and statistical analyses

To ensure accuracy and reliability of data samples were analysed in triplicates with mean values of concentrations presented. Certified reference materials were analysed and sample blanks were also used to identify contamination. Analysis of variance (ANOVA) to determine significant difference or otherwise between the fungal treatments effect on immobilising heavy metals were carried out.

RESULTS AND DISCUSSION

Table 1. Heavy metals concentrations in leachates at 7th day of soil amendment

Treatment	Mn	Cd	Cr	Pb	Zn	Ni
KSS CSi	0.871	0.460	1.828	5.824	0.142	1.817
KSS PnSi	0.767	0.442	1.980	5.364	0.125	1.720
KSS AnSi	0.958	0.464	3.166	6.498	0.136	1.794
KSS TrSi	0.792	0.461	1.825	6.182	0.147	1.925

KSS C is the heavy metal amended soil with no fungi (control); KSS Pn is the heavy metal amended soil with *Penicillium* (Pn) fungi; KSS An is the heavy metal amended soil with *Aspergillus niger* (An) fungi; KSS Tr is the heavy metal amended soil with *Trichoderma* (Tr) fungi

Table 2. Heavy metals concentrations in leachates at 14th day of soil amendment

Treatment	Mn	Cd	Cr	Pb	Zn	Ni
KSS CSi	0.316	0.142	2.312	0.483	0.046	0.471
KSS PnSi	0.380	0.185	2.228	0.663	0.157	0.751
KSS AnSi	0.384	0.238	2.949	1.314	0.058	0.814
KSS TrSi	0.332	0.213	2.364	0.997	0.054	0.730

KSS C is the heavy metal amended soil with no fungi (control); KSS Pn is the heavy metal amended soil with *Penicillium* (Pn) fungi; KSS An is the heavy metal amended soil with *Aspergillus niger* (An) fungi, KSS Tr is the heavy metal amended soil with *Trichoderma* (Tr) fungi

Figures 1 and 2 below are graphical representations of leaching in the tested heavy metals (Mn, Cd, Cr, Pb, Zn and Ni) at 1st week and 2nd week of soil amendment which determine the immobilisation ability of the fungi when compared with control.

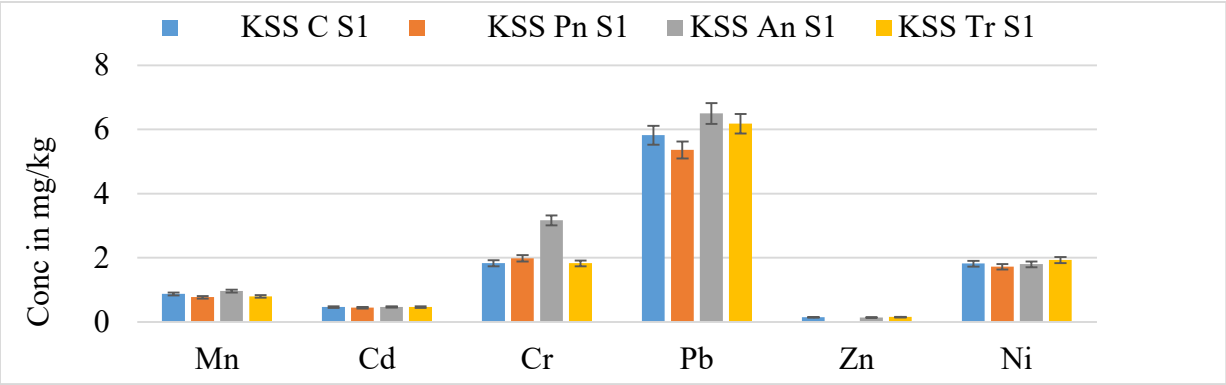

Figure 1. Leaching of heavy metals tested after 7 days t(S1)

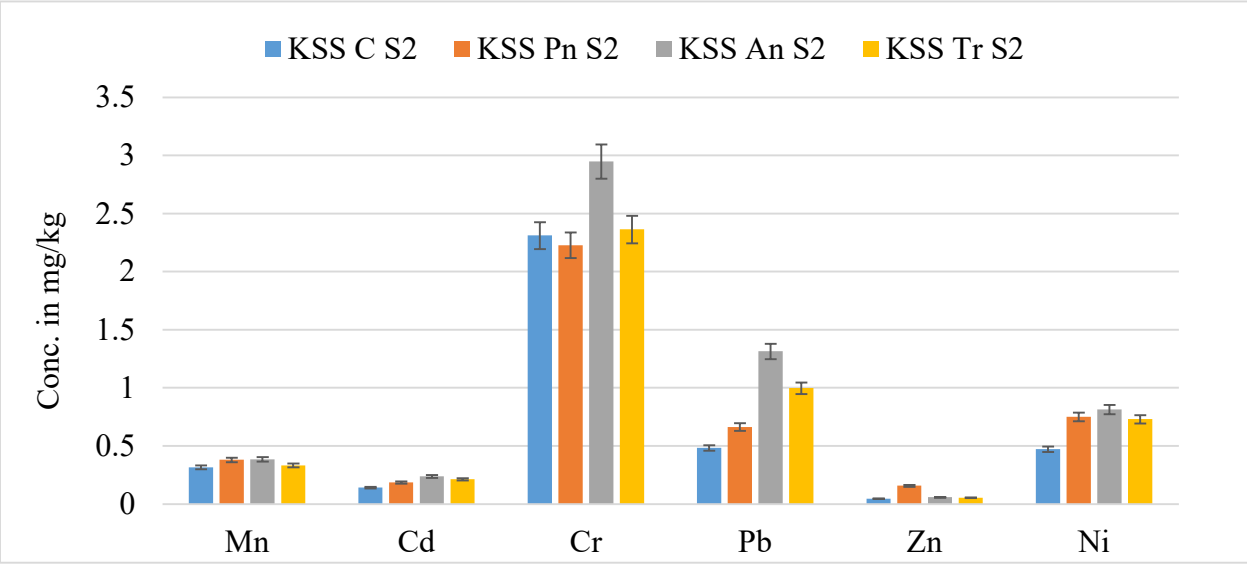

Figure 2. Leaching of heavy metals tested after 14 days t(S2)

Table 3 and 4 below are Analyses of variance (ANOVA) statistically carried out to determine whether there is a significant difference between the fungal treatments effect on immobilising the heavy metals in soil.

Table 3. ANOVA for Mean concentration of heavy metals Leached after 7 days

Sources	Df	Sum sq	Mean sq	F-value	Pr(>F)
TR	3	0.64	0.213	2.313	0.118
MC	5	91.74	18.349	199.546	3.63e-12
RESIDUALS	15	1.38	0.092		

Table 3. ANOVA for Mean concentration of heavy metals Leached after 14 days

Sources	Df	Sum sq	Mean sq	F-value	Pr(>F)
TR	5	24.705	4.941	101.663	5.04e-11
MC	3	0.393	0.131	2.695	0.0832
RESIDUALS	15	0.729	0.049		

Table 1 and 2 above presents the result of Heavy metals leaching analysed at 7th day (S1) and 14th day (S2). Heavy metals leaching analysed at 7th day showed a massive decrease of the metals concentration from the initial in-put (10 mg/L) indicating strong sorption of the metals in soil; the concentration decreased further after 14th day as a result of continued mobility of the soluble metals through the soil. Moreover, variations were observed in the concentration values where some of the samples with fungi incorporated were higher than controls (indicating effectiveness of the fungi to immobilise the metals and reduce their sorption through the soil) while some are lower (indicating that the fungi had no positive effect on the heavy metals mobility in the soil).

In Mn amended soil An has a value of 0.958 mg/kg at 7th day, this was higher than the control value (0.871 mg/kg); in Cd amendment Tr has higher value (0.461 mg/kg) than the control (0.460mg/kg), this was in contrast to the work of [20], where they reported *Trichoderma* as having no any immobilisation effect on Cd but on Pb when leaching test was conducted. In Cr amended soil An (3..166 mg/kg) > Pn (1.980 mg/kg) > C (1.818 mg/kg); In Pb amended soil, An (6.498 mg//kg) > Tr (6.182 mg/kg) > C (5.824 mg/kg), this was in agreement with the report by [1] where they found *Aspergillus* to be tolerant to high concentrations of Pb and Zn in soil matrix; In Zn and Ni amended soils, Tr has values 0.14 mg/kg and 1.925 mg/kg > C(0.14 mg/kg and 1.81 7 mg/kg), this has also tallied with the report by [15] on biosorption ability of *Penicillium* and *Trichoderma* to Pb and Zn .

The result of Heavy metals leaching analysed at 14th day showed further decrease in metal concentration from the 1st week. In Mn amended soil An value had decreased further to 0.384 mg/kg, Pn value also decreased to 0.380 mg/kg but it exceeds the control (0.316 mg/kg). in Cd amended soil, An (0.238 mg/kg) > Tr (0.213 mg/kg) > Pn (0.185 mg/kg) > C (0.142 mg/kg); in Cr amended soil, An (0.949 mg/kg) > Tr (0.364 mg/kg) > C (0.312 mg/kg); in Pb amended soil, Tr (0.997 mg/kg) > Pn (0.663 mg/kg) > C (0.483 mg/kg); in Zn amended soil, Pn (0.157 mg/kg) > An (0.058 mg/kg) > Tr (0.054 mg/kg) > C (0.046 mg/kg); in Ni amended soil, An (0.814 mg/kg) > Pn (0.75 mg/kg1) > Tr (0.730 mg/kg) > C (0.471 mg/kg). [23 and 24], reported independently on utilization of fungal biomass to immobilise heavy metals from contaminated soils and they all proved successful with varying degrees of immobisation between the heavy metals.

Aspergillus niger (An) showed strongest immobilising effect, likely due to acid production, as suggested by [27] in a study of organic acid production in contrasts between *Penicillium* and *Aspergillus*. In this study *An* showed higher retention of the metals in Mn (0.958 mg/kg), Cr (3.166 mg/kg) and Pb (6.498 mg/kg) amendments at first week, while at the 2nd week Cr and Pb still remain highest suggesting longer -term immobilisation and the retention extends to Cd, Zn and Ni amendments.

Trichoderma (Tr) also showed great ability for metal immobilization as it has highest values in Cd, Zn and Ni amendments, Pb also rose to 6.182 mg/kg while, Ni increased to 1.925 mg/kg at 1st week and in 2nd week Cr rose to 2.364 mg/kg, Pb to 0.997 mg/kg and

Ni to 0.730 mg/kg, less than *Aspergillus niger* but more than control- showing intermediate behaviour. In a report by [20], *Trichoderma* was found to immobilise Pb in heavy metal contaminated soil but has no effect on Cd mobility.

Penicillium (Pn) on its part showed slight decrease in all metals compared with the control at 1st week, for instance, Pb dropped to 5.364 mg/kg but Cr increased slightly to 1.980 mg/kg. It shows modest immobilisation effect on the 2nd week with concentration values higher than controls in Mn, Cd, Pb, Zn and Ni amended soil, for example Pb increased to 0.663 mg/kg, Zn also significantly increased to 0.157 mg/ kg. These findings coincide with the report by [28], who tested immobilisation ability of *Penicillium* fungi in soil with Calcium phosphate amendment and found a massive decrease in Zn, Cr, Cd, Pb, Fe and Ni mobility in soil

These results proved that *Aspergillus niger*, *Trichoderma* and *Penicillium* fungal species can be used for heavy metals remediation in soil as they were found to reduce the sorption of the metals in the soil by binding them to their cell walls and therefore reducing their bioavailability to plants.

The ANOVA result presented in Table 2 showed that the probability value (P-Value) for treatment is 0.118, which is higher than the conventional significance level (0.05), indicating that there is no significant difference among the different fungal treatments in terms of their effect on leaching heavy metals after 7 days. However, the value is relatively close to 0.05, suggesting a potential trend or emerging effect that might become more pronounced over time or with a larger sample size. The low residual variance (mean sq = 0.092) suggest that the model fits the data well, and most of the variation is accounted for by the treatment and metal type.

The ANOVA result presented in Table 3 showed that the probability value (P-Value) for treatment is 5.04e-11 which is extremely low indicating a very strong statistically significant effect of fungal treatments on immobilizing heavy metals after 14 days. This shows that overtime, the treatments became much more effective at reducing metal leaching. The p-value (0.0832 observed in metal concentration is not statistically significant at the 0.05 level but is close. This might suggest a possible variation among metals, but not enough to confidently state there is a significant difference after 14 days. It contrasts with the results at 7 days, possibly indicating that the fungal treatments exert a more uniform effect across metals overtime. There is an even lower residual variance than in table 2 (mean Sq = 0.049), supporting a strong model fit.

CONCLUSION

The result shows that the immobilisation process improved with time due to the enhanced effect of the fungi leading to significant decrease in leaching concentration between the 7th and 14th day, with Pb exhibiting the most substantial reduction in mobility indicating a strong response to the fungal treatments. Cr and Ni also showed notable leaching levels, while Zn has the lowest concentration across all treatment levels. *Aspergillus niger* enhances leaching causing the highest leachate concentration of Cr and Pb at day 7 and day 14. This suggests it promotes metal immobilisation, likely via organic acid production making it more suitable for

bioremediation. *Trichoderma* showed moderate immobilisation potential in Pb, Ni and Cr which suggests balanced potential for bioremediation where controlled metal immobilisation is desired without excessive leaching. *Penicillium* on the other hand showed minimal effect suggesting its role may be more in metal stabilization.

As recommendation, further studies are needed to compare different treatment methods to identify the most effective for each metal; Periodic leaching tests can be conducted to assess the stability of the immobilized metals; Additional measures, such as organic amendments like compost or biochar could be used to enhance the immobilisation effect.

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CONFLICT OF INTEREST

Authors declared no conflict of interest.

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ETHICAL STATEMENT

This work required no ethical statement.

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