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RESEARCH PAPER

Synergistic and Antagonistic Effects on Metal Bioremediation with Increasing Metal Complexity in a Hexa-metal Environment by *Aspergillus fumigatus*

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Abstract

In the present study, *Aspergillus fumigatus* (Genbank accession no. KX365202), was used for heavy metal removal in a hexa-metal system containing mixture of six heavy metals (Cu, Cr, Cd, Ni, Pb and Zn). The total concentration of the heavy metals was kept at 30 mg L^{-1}. The experimental sets were designed based on the relative abundance of the heavy metals present in the wastewater of Delhi-NCR region. Toxicity of the heavy metals to the fungus varied with different metal combinations. Combination of Pb and Cr proved to be most toxic followed by that of Pb, Cr, Cu, Zn and Ni. Biomass production of 2.90 g L^{-1} was found in control whereas the combination Pb and Cr produced the lowest biomass (1.59 g L^{-1}). In the presence of six metals, heavy metal removal pattern was Ni = Cd > Cu > Pb > Zn > Cr. SEM studies showed broken fungal hyphae in presence of hexa-metal stress. TEM–EDX studies showed that among the six heavy metals, Cu, Pb and Cd were adsorbed on the cell surface whereas Ni, Cr and Zn were accumulated inside as well outside of the cell. This system could be useful in treating water with multiple heavy metal contaminants.

Article Highlights

- **Combination of six heavy metals used to test toxicity effect on fungus**
- **Metal removal pattern in presence of six metal followed order Ni = Cd > Cu > Pb > Zn > Cr**
- **Combination of Pb and Cr produced lowest biomass proving toxic to the fungus**
- **Hexa metal stress caused broken hyphae in fungus**
- **Heavy metals partitioned by either adsorbing on the surface or going intracellular**
- **Present study approximates natural conditions containing mixtures of heavy metals**

Keywords Heavy metals · Bioremediation · Fungus · Multi-metal · Toxicity

Introduction

Fungi has been widely used for bio-remediating heavy metals from soil and water (Iskandar et al. 2011; Mishra and Malik 2012; Hassan et al. 2020; Zapana-Huarache et al. 2020). However, most of the studies reported were done in a single-metal system which rarely resembles the actual scenario. Ample studies have been done using *Aspergillus* for bioremediation of heavy metals owing to its high metal tolerance (Tsekova et al. 2010; Vardon et al. 2011; He et al. 2012; Oladipo et al. 2016; Bano et al. 2018). Very few studies have been done on fungal bioremediation in a multi metal complex system as recently reviewed by Gola et al. (2016b).

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Such studies with multi metal containing water could mimic the actual environmental conditions more closely where multiple contaminants are present. Multi-metal containing systems have been limited to binary, tertiary or at the most quaternary combinations of heavy metals (Pakshirajan and Swaminathan 2009; Pan et al. 2009; Guo et al. 2010; Mishra and Malik 2012; Flouty and Estephane 2012). An entomopathogenic fungi *Beauveria bassiana* was used to treat water containing six heavy metals (Gola et al. 2016a, 2019). A three metal system using *Fucus vesiculosus* was used for removal of Hg, Cd and Pb from salt water (Henriques et al. 2017). In a recent study, fungi isolated from metal-contaminated soil were able to bioremediate Pb Cd and Cu up to 50 mg L^{-1} (Albert et al. 2019). Another study with *A. flavus* have shown high tolerance of the fungus to metals such as As (2000 mg L^{-1}), Ni (1600 mg L^{-1}), Mn (1600 mg L^{-1}), Pb (1200 mg L^{-1}), Cr (800 mg L^{-1}), Cu (mg L^{-1}) and Cd (100 mg L^{-1}) in synthetic wastewater (Kumar and Dwivedi 2020).

With the increase in metal complexity, the toxicity of the system increases due to synergistic or antagonistic interactions between the individual metal ions (Gola et al. 2016b). Further, most of the studies done with multi-metal are batch studies. There has been no report on the metal removal efficiency and toxicity with increasing metal complexity in a hexa-metal system that could indicate exactly which metal combinations are more toxic. In addition to this, very limited studies are available that investigated the intracellular distribution of heavy metals under a hexa-metal stress, which could shed some light on the possible cause of toxicity with combinations of metals. Previous studies with *A. fumigatus* have shown its ability to tolerate multi-metal stress efficiently (Dey et al. 2016). Recently, it was seen that *A. fumigatus* had high tolerance of heavy metals found in mining sites (Oladipo et al. 2016). In the present study, the toxicity effects and metal removal pattern of *A. fumigatus* were studied in a hexa-metal system with increasing metal complexity. Hence, this study could be useful for treating multiple metal-contaminated wastewater using a fungal species.

Materials and Methods

Microorganism Isolation and Growth Conditions

Aspergillus fumigatus (GenBank accession no: KX365202) isolated from Yamuna river bank was used for the bioremediation experiments (Dey et al. 2016). Soil sample (1 g) was mixed with sterile 10-mL distilled water in a laminar air flow cabinet. From the mixture different dilutions (10^3, 10^4, 10^5) were plated on PDA (potato dextrose agar) plates and incubated at 28℃ till colonies appeared. Colonies were purified by repeated streaking till single type of colonies were observed under microscope. The fungus was maintained in PDA slants at 28 ℃. For experimental purpose, fungus was grown in sterilized composite medium (composition in g L^{-1}): K_2HPO_4 (0.5), $MgSO_4$ (0.1), NH_4NO_3 (0.5), NaCl (1.0), yeast extract (5.0) and glucose (10.0) at 28 °C and 150 rpm overnight.

Metal Solution Preparation

Heavy metal stock solution of 10,000 mg L^{-1} was prepared by dissolving the respective salts $K_2Cr_2O_7$, $CuSO_4{\cdot}5H_2O$, $Ni(NO_3)_2$, $Cd(NO_3)_2{\cdot}4H_2O$ and $Zn(NO_3)_2{\cdot}6H_2O$ in deionized water. Solution of Pb was prepared by dissolving required amounts of Pb metal granules in concentrated HNO_3 and making up the volume with deionized water. The amount salts used for preparing the stock solutions are mentioned in Table 1. The stock concentration was verified by analyzing it in MP-AES after preparation before use.

Effect Of Toxicity with Increment in Metal Complexity

Composite growth medium (Gola et al. 2019) was used for the experiments. Six flasks containing total initial metal concentration of 30 mg L^{-1} were prepared. Complexity of metal concentration (in mg L^{-1}) for each flask was changed as: Set 1 = Pb (30), Set 2 = Pb(25)Cr(5), Set 3 = Pb(20)Cr(5)Cu(5), Set 4 = Pb(15)Cr(5)Cu(5)Zn(5), Set 5 = Pb(10) Cr(5)Cu(5) Zn(5)Ni(5) and Set 6 = Pb(5)Cr(5)Cu(5) Zn(5)Ni(5)Cd(5). A control set was run in composite medium without any heavy metal. The experimental sets were designed based on the relative abundance of the heavy metals present in the wastewater of Delhi-NCR region. The average concentrations of the six heavy metals varied between 0 and 3 mg L^{-1}. Hence, 5 mg L^{-1} of each heavy metal was chosen to account for any variation. In the presence of six heavy metals, the total metal concentration would be 30 mg L^{-1}. Hence, total metal concentration was kept at 30 mg L^{-1} in each experimental set. Pb was found to be the most abundant metal

Table 1 Amount of salts used for making stock solution of metals of 10,000 mg L^{-1} concentration

Compounds	Amount used to make 10,000 mg L^{-1} stock solution (per 100 ml) in g
$K_2Cr_2O_7$	2.82
$Zn(NO_3)_2$	2.89
$Cd(NO_3)_2$	2.10
$Cu(NO_3)_2$	2.95
$Ni(NO_3)_2$	3.11
Pb (powder)	1.00

in the wastewater samples whereas Cd was the rarest. The metals were introduced into the experimental sets according to their relative abundance in the wastewater as reported in previous studies (Bhattacharya et al. 2015; Gola et al. 2020). Fungal spore suspension (10^6 spore mL^{-1}) was inoculated in 100 ml sterilized composite medium along with metals and incubated at 28 ℃ and 150 rpm. For the estimation of residual metal concentration in medium, 10 mL of the samples from the flask were taken out aseptically after every 24 h for 3 days, and centrifuged at 5000 rpm for 10 min. Supernatant was digested with nitric acid in the ratio 9:1 (supernatant:acid) in a microwave digester (Anton Paar Multiwave Pro, Anton Paar Germany) according to standard methods (Eaton and Franson 2005) and analyzed for residual metal concentrations using Microwave plasma-atomic emission spectroscope (MP-AES, Agilent Technologies, USA). Glucose estimation of the medium was done using DNS method (Miller 1959). Fungal biomass was filtered through a pre-weighed Whatman no. 1 filter paper and dried at 60 ℃ overnight in hot air oven. Amount of biomass produced was calculated from the difference in weights of the filter paper. Fungal biomass was collected after 72 h for set 6 and analyzed for morphological change and metal distribution in the cells using SEM (scanning electron microscopy) and TEM–EDX (transmission electron microscopy–energy-dispersive X-ray spectroscopy), respectively.

SEM Analysis Of Fungal Pellets

The fungal pellets were first washed with phosphate buffer saline (pH 6.8) and then fixed with 1% glutaraldehyde for 4 h at room temperature. The samples were centrifuged, and the fixative was discarded. Samples were then lyophilized for SEM analysis. SEM analysis of the normal algal cells and algal cells incubated with fungi for 2.5 h were done using a ZEISS EVO 50, Germany under the following analytical condition: EHT = 20.00 kV, WD = 9.5 mm, Signal A = SE1 (Bhattacharya et al. 2019).

TEM–EDX Analysis of Fungal Pellets

Fungal pellets from Set 6 were washed with phosphate buffer saline (pH 6.8) and subsequently fixed in 1% glutaraldehyde and 2% paraformaldehyde for 12–18 h at 4 °C. After fixation, pellets were washed again using PBS buffer. Post washing fixation was done using osmium tetra oxide (1%) for 2 h at 4 °C. Pellet was then dehydrated using acetone and embedded in epon–araldite. Ultrathin sections of 60–80 nm thickness were cut using an ultramicrotome (Leica EM UC 6). The sections were stained in alcoholic uranyl acetate and lead citrate for 10 min, before examining the grids in a transmission electron microscopy and recorded on Philips transmission electron microscope (CM-10) on carbon-coated copper grids with an accelerating voltage of 70 kV.

Statistical Analysis

All experiments have been performed in triplicates and the results have been represented as mean ± SD. Student's *t* test ($p < 0.05$) has been done wherever applicable to test the significance of the data.

Results and Discussion

Effect Of Increasing Metal Complexity On Biomass Production And Glucose Utilization

Different combinations of heavy metals (Sets 1–6) were used to assess the toxicity effect on *A. fumigatus* with increasing metal complexity. Complexity of metal concentration (in mg L^{-1}) for each flask was changed as Set 1 = Pb (30), Set 2 = Pb (25) Cr (5), Set 3 = Pb (20) Cr(5) Cu(5), Set 4 = Pb(15) Cr(5) Cu(5) Zn(5), Set 5 = Pb(10) Cr(5) Cu(5) Zn(5) Ni(5) and Set 6 = Pb(5) Cr(5) Cu(5) Zn(5) Ni(5) Cd(5). Figure 1 shows the biomass generated in different sets. Highest biomass (2.90 g L^{-1}) was produced in control (no metals). The glucose utilization pattern in case of the biotic control showed that the glucose was utilized within 24 h. There was a significant decrease in biomass ($p < 0.05$) with the addition of metals in subsequent sets. Although there was a gradual decline in biomass with increasing metal complexity, the pattern did not follow a linear relation. Set 1 containing only 30 mg L^{-1} Pb had biomass production of 2.71 g L^{-1} after 72 h. The glucose utilization rate (Fig. 1) showed a considerable lag compared to the control. When Cr was introduced in Set 2, the biomass production decreased to 1.57 g L^{-1}. A decrease in biomass suggests that this combination was toxic to the fungus. Glucose utilization pattern also showed that there was a lag in utilizing the glucose. The residual glucose in Set 1 became zero at 48 h whereas it took 54 h in Set 2 to completely utilize the glucose. Surprisingly, when Cu was added along with Pb and Cr (Set 3), the biomass increased to 2.15 g L^{-1}. In Set 4, the overall biomass production further increased to 2.39 g L^{-1}. It was interesting to note that a combination of Pb and Cr caused a lower biomass production, whereas the addition of Cu and Zn somewhat lowered the toxicity and an increase in biomass was observed. Addition of Cu has been reported to promote fungal growth in soil (Fernández-Calviño and Bååth 2016). In Set 5 with the addition of Ni, there was again a sharp decrease in the biomass production. After 72 h, the biomass produced was 1.67 g L^{-1} comparable to the biomass produced in Set 2. In the final set (Set 6), which

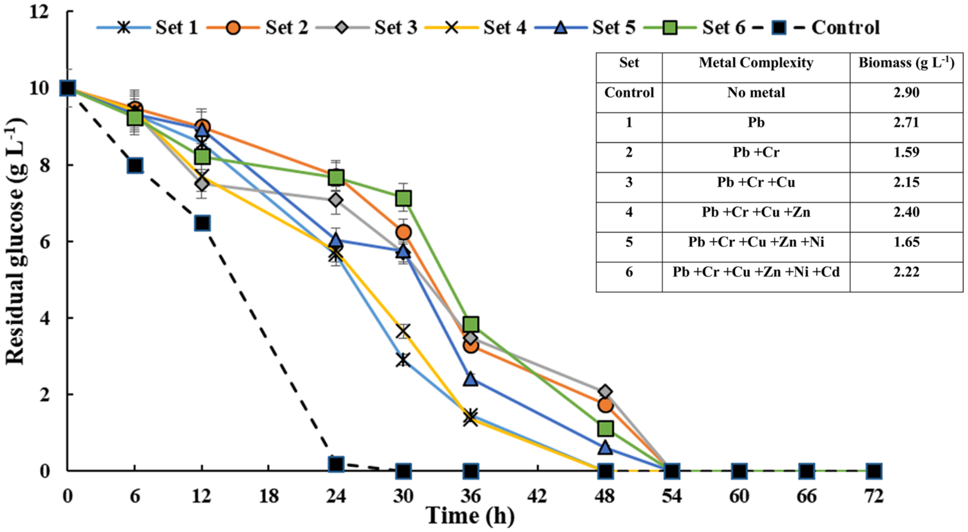

Fig. 1 Glucose utilization rate of *A. fumigatus* under different metal complexity. Complexity of metal (in mg L^{-1}) for each flask was changed as Set 1 = Pb (30), Set 2 = Pb(25)Cr(5), Set 3 = Pb(20)Cr(5)Cu(5), Set 4 = Pb(15)Cr(5)Cu(5)Zn(5), Set 5 = Pb(10)Cr(5)Cu(5)Zn(5) Ni(5) and Set 6 = Pb(5)Cr(5) Cu(5) Zn(5)Ni(5)Cd(5). Error bars represent mean ± S.D of triplicate results

contained the six heavy metals in equal concentrations (5 mg L^{-1} each), the overall biomass produced after 72 h was 2.22 g L^{-1}. From the biomass data, it was observed that set 2 (Pb and Cr) and set 5 (Pb, Cr, Cu, Zn and Ni) caused major inhibitions to the biomass production. These results indicated that the different combinations of metals induced different levels of toxicity to the fungus. It also suggested that in a system with multi metal, the toxicity effect does not depend on the number of metals rather it depends on the specific metal combinations and its metal dynamics. As seen from the above results, binary mixture of Pb and Cr proved to be more toxic than hexa-metal system containing Pb, Cr, Cu, Zn, Ni and Cd. Dey et al. (2016) observed that a fungal strain *A. terreus* AML02 that was able to uptake a high concentration of individual metals (Cr: 95.57%; Cu: 65.77%), was unable to uptake total chromium and Cu at a low concentration of 5 mg/L each metal in a 30 mg/L multimetal concentration.

Metal Removal Pattern With Increasing Metal Toxicity

The objective of this study was to first look at metal removal pattern critically (amount of metal removed in each set with time) to understand how the metal complexity impacts this parameter. As shown in Fig. 2, maximum amount of Pb was removed during the first 24 h, irrespective of the complexity. The removal of Cr showed a different pattern. In Sets 2 and 3, Cr was removed gradually over 72 h but after the introduction of Zn, the removal of Cr decreased significantly ($p < 0.05$). This may be because, in a multimetal environment, there is competition for binding between Zn and Cr (Nguyen et al. 2015). Similarly, Cu removal was the highest

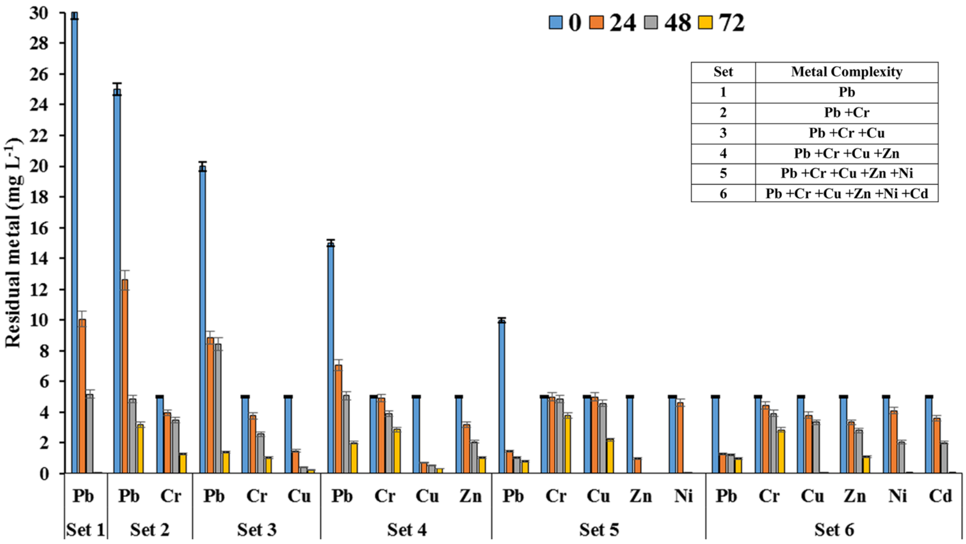

Fig. 2 Heavy metal removal by *Aspergillus fumigatus* in batch studies with increase in metal complexity. Error bars represent mean ± SD of triplicate results

in the first 24 h in Sets 3 and 4. However, as Zn was introduced in Set 5, the removal of Cu was negatively affected. In the presence of equal concentrations of all the six heavy metals in Set 6, there was slight removal of Cu after 48 h. However, complete removal took place after 72 h unlike the previous set. The removal of Zn did not follow a definite pattern. In Set 4, Zn was removed progressively with time. However, in the presence of Ni in Set 5, maximum Zn was removed within 24 h but the removal was not significant ($p > 0.05$). Again, in the presence of six heavy metals, gradual removal of Zn was seen with time. Similarly, for Ni, maximum removal occurred after 48 h in Set 5 whereas in the presence of Cd, gradual removal of Ni took place in Set 6. Removal of Pb was consistent and was not affected by the addition of metals. The removal of Pb has been reported to be mediated by extracellular polymeric substances secreted by the fungus (Dang et al. 2018). Hence, consistent removal of Pb was seen in all sets.

Individual Metal Removal After 72 H Under Different Metal Combinations

The metal removal pattern showed a distinct variation with increasing metal stress. To further observe the effect of metal complexity on the total removal efficiency of the fungus, residual metal present after 72 h was observed for each set. The total metal removed after 72 h is shown in Fig. 3. In Set 1, the residual Pb present after 72 h was 0.10 mg L^{-1} indicating that almost 99% of the metal had been removed. In the presence of only Pb, the removal was 99% but in presence of Cr, 87% Pb was removed whereas Cr was itself removed by 75% in Set 2. Gola et al., (2019) had observed similar affinity of Pb and Cr towards chitin. Since fungal cell wall contains chitin, it was possible that there was competition for binding sites among Pb and Cr. The metal removal data for Set 3 showed that after 72 h Pb was removed by 93%, Cr by 79% and Cu by 95%. Previously, it was reported that Cu ions inhibit the uptake of Pb ions (Gola et al. 2019; Zhang et al. 2019) when present in equal concentrations. However, in the present case, the concentration of Pb was 20 mg L^{-1} compared with 5 mg L^{-1} each of Cr and Cu. Hence, no such inhibition was observed. In Set 4, the amount of metal removed was 86% Pb, 42% Cr, 93% Cu and 79% Zn. The addition of Zn caused a significant ($p < 0.05$) decrease in Cr removal which occurred may be due to the competitive binding of Zn and Cr. The metal removal data for Set 5 showed that 92% Pb and 100% of Zn and Ni were removed. However, only 55% Cu and a mere 24% Cr was removed. Addition of Ni negatively impacted the removal of Cu and Cr which suggested that there might be competition of binding sites among Ni, Cu and Cr. Gola et al. (2019) employed analytical techniques like FTIR and TEM-HADFF that indicated similar bio-sorption and bioaccumulation sites for Ni, Cu and Cr in fungus when exposed simultaneously to multiple metals. The metal removal data in Set 6 showed removal of 80% Pb, 43% Cr, 98% Cu, 78% Zn, 99% Ni and 99% Cd. Greater than 80% removal of Pb was observed irrespective of the increase in metal complexity (number of metals in the mixture). Similarly, removal of Ni (99–100%) and Zn (79–100%) was not influenced significantly ($p > 0.05$) by the metal complexity. Gola et al. (2016a) observed a similar trend for Ni and Zn in the presence of multi-metal ion, where only 3% and 1% decrease in metal removal was observed for Ni and Zn in multi-metal ion medium, respectively. On the other hand, Cr removal declined with the increase in metal complexity. Aksu and Donmez (Aksu and Dönmez 2006) also reported competitive binding of Zn and Cd on algal biomass which affected each other's removal. This might be because of the

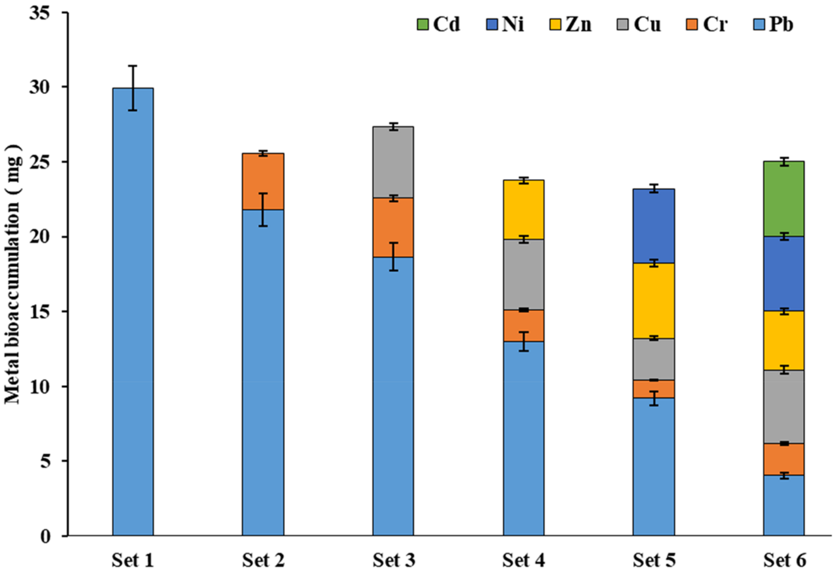

Fig. 3 Bioaccumulation of heavy metal by*Aspergillus fumigatus* with increasing metal complexity. Complexity of metal (in mg L^{-1}) for each flask was changed as: Set 1 = Pb (30), Set 2 = Pb(25)Cr(5), Set 3 = Pb(20)Cr(5)Cu(5), Set 4 = Pb(15)Cr(5)Cu(5)Zn(5), Set 5 = Pb(10)Cr(5)Cu(5)Zn(5) Ni(5) and Set 6 = Pb(5)Cr(5) Cu(5) Zn(5)Ni(5)Cd(5). Error bars represent mean ± S.D of triplicate results

ionic radius of Zn and Cd are comparable. It has been seen in the case of a macro-fungus *Pleurotus ostreatus* that the individual binding capacity of metals (Ni^{2+}, Cu^{2+}, Zn^{2+} and Cr^{4+}) decreases when they are in combination rather than when single (Javaid et al. 2011).

The metal removal efficiency in a multi-metal system is dependent on the atomic weight, atomic or ionic radius and electronegativity of the individual ions (Pakshirajan and Swaminathan 2009; Gola et al. 2017). Also, the bio-sorption of every metal is dependent on the temperature, concentration and pH of the solution (Gola et al. 2016a). Gadd (1993) had reported the relative affinity of heavy metals is of the order $Mn^{2+} < Zn^{2+} < Ni^{2+} < Fe^{2+} \sim Co^{2+} < Cd^{2+} < Cu^{2+} < Pb^{2+}$. In another study, order for metal removal followed ionic radius trend, i.e. Cr > Pb > Cd > Cu > Ni > Zn (Gola et al. 2019). Among the six heavy metals used, Cr had the smallest ionic radius. The ionic radii follow the order Cr < Cu < Ni < Zn < Pb < Cd. In the present study, in the presence of six heavy metals, the metal removal trend was Cr < Zn < Pb < Cu < Cd = Ni (Fig. 3). With the increase in metal complexity (number of metals), removal of Cr decreased consistently as it had the smallest ionic radius. However, in the case of other metals, they do not strictly follow the ionic radius trend. This is because the inter-ionic interactions between the metal ions may cause a synergistic or antagonistic effect on the metal removal process (López Errasquín and Vázquez 2003; Bishnoi and Garima 2005). This was evident from the different removal pattern of the same metal under different combinations. From Fig. 3, it was seen that in Set 5, the removal of Cr decreased with the addition of Ni, but it again increased after the addition of Cd in set 6.

SEM and TEM–EDX Studies of *A. fumigatus* Treated with Multi-metal

The SEM analysis of *A.fumigatus* showed intact hyphae in control (Fig. 4a) whereas broken hyphae were observed under hexa-metal stress (Fig. 4b). To further investigate the mechanism of the metal removal under multi-metal stress, TEM–EDX studies of fungal biomass obtained after 72 h in Set 6 were performed. The TEM–EDX image of *A. fumigatus* under hexa-metal stress is shown in Fig. 5. It was seen that the metals Cr, Ni and Zn accumulated inside as well as outside of the cell whereas Cu, Cd and Pb were adsorbed on the surface. Accumulation of Cr by fungus has been previously reported (Zafar et al. 2007; Sharma and Adholeya 2011). The mechanism of Cr tolerance in fungus has been attributed to the reduction of Cr^{6+} to Cr^{3+} form and then bioaccumulation (Dey et al. 2016). In case of set 2, Pb accumulation increased with the addition of Cr unlike other sets. This could be attributed to the fact that Cr gets accumulated inside the cells whereas Pb gets adsorbed on the surface. Hence, there is no competition for binding due to which Pb remediation did not decrease. However, this set caused a decrease in biomass production indicating its toxicity to the fungus. This may be due the fact that in Set 2, the concentration of Pb was very high in comparison to Cr. Hence, maximum amount of Pb adsorbed on the surface forcing Cr to be accumulated inside the cell. Cr induces a lot of reactive oxygen in the cell causing toxicity which could be the reason for low biomass productivity (Li et al. 2017). When Cu was added along with Cr and Pb, there was a slight decrease in Pb removal while Cr removal remained unchanged. Also, the biomass produced was greater than Set 2. This indicated that the toxicity effect due to introduction of Cr in the previous set has somewhat been lowered with the addition of Cu. The TEM–EDX images showed that Cr got adsorbed on the surface as well as accumulated inside the cells.

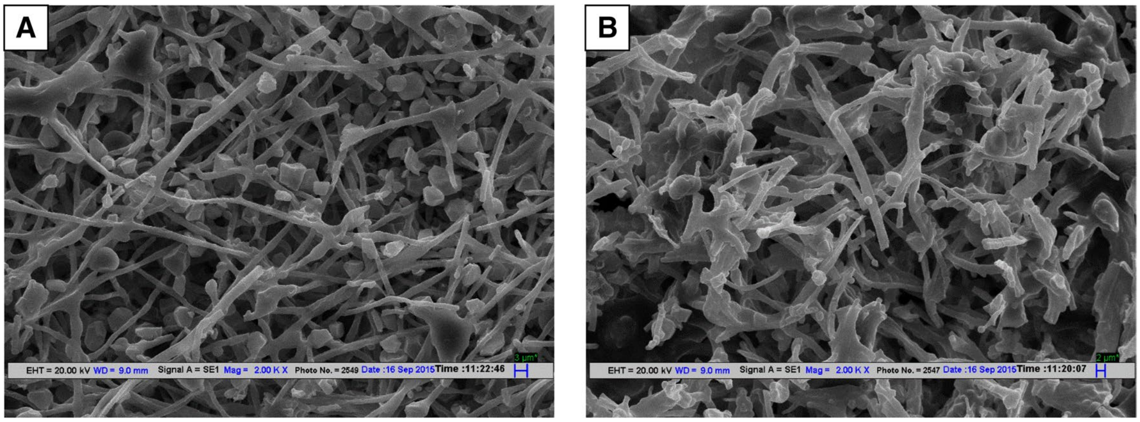

Fig. 4 SEM images of Aspergillus fumigatus (**a**) without metal (**b**) in presence of hexa metal stress

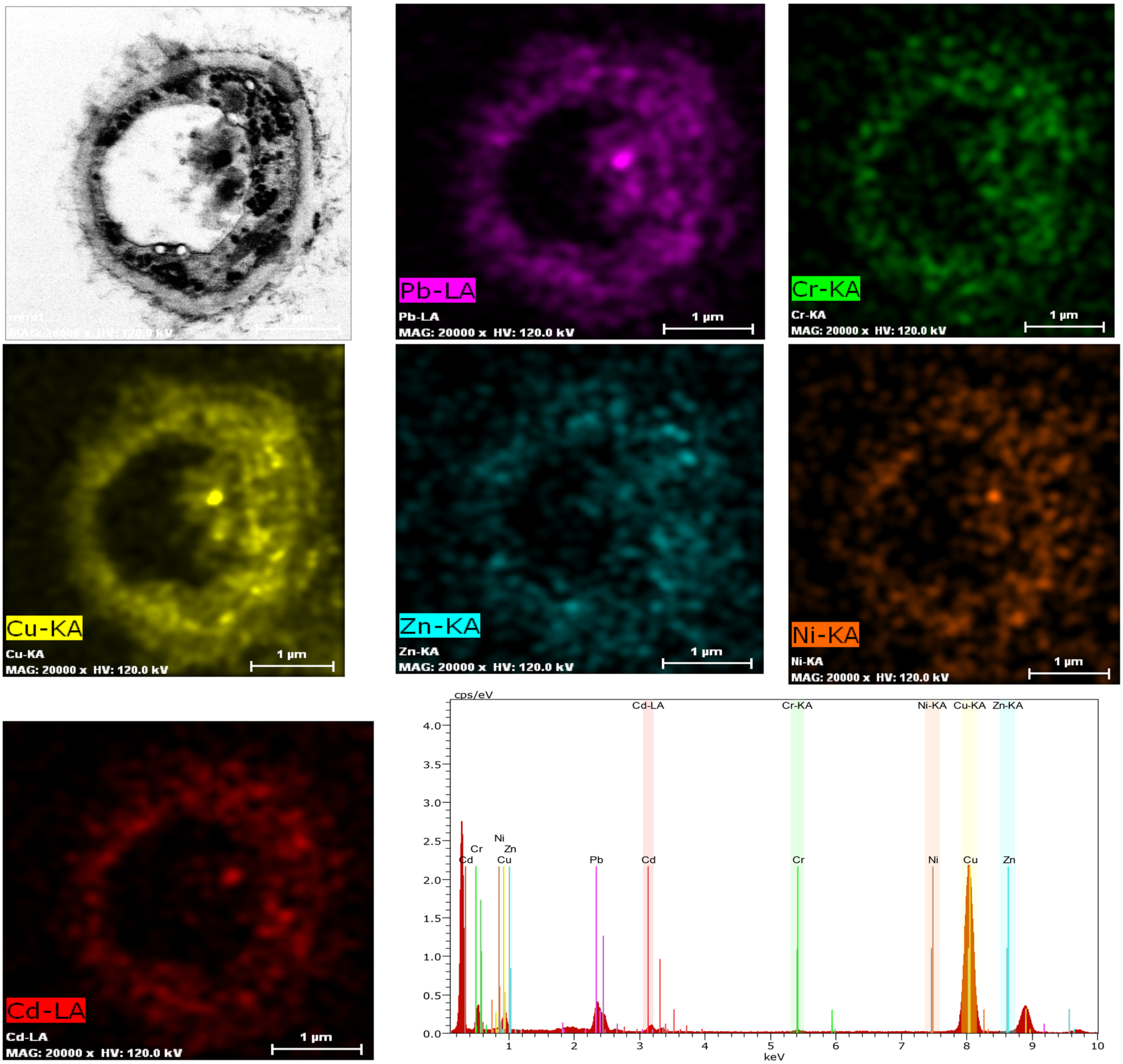

Fig. 5 TEM-EDX analysis of A.fumigatus treated with 5 mg L-1 each of six heavy metals (Cr, Cu, Cd, Ni, Pb and Zn)

However, this partitioning depended upon the concentration of the metals present in the solution (Ahmad et al. 2006). The concentration of Pb was decreased to 15 mg L^{-1} in Set 3. Hence, both Cu and Cr might have adsorbed on the cell surface lowering toxicity. Similarly, in Set 4, the addition of Zn decreased Cr removal as both the metals accumulated inside the cells. Also, the ionic radius of Cr is lesser than Zn; hence, Zn accumulated more than Cr (Fig. 2). This was because since Cr has a smaller ionic radius and higher charge than Zn, Cr gets more rapidly hydrated with OH^- ions before competition with other metals becomes a factor (Srivastava et al. 2015). Zn is an essential micro-nutrient in various enzymes which may also be a reason for its preferential accumulation (Gola et al. 2017; Teng et al. 2018).

Analogous trend was observed in set 5 with the addition of Ni. Cr was removed the lowest whilst Ni and Zn were removed completely. Since the ionic radii of Zn and Ni are comparable, no preference was seen in their removal. Also, it has been reported that there is an intense competition of binding between Cr and Ni which could also be the reason for low Cr removal (González-Costa et al. 2018). Here again, the biomass produced was comparable to that in Set 2 indicating that this combination of metals was also toxic to the fungus. In the presence of equal concentrations of six heavy metals (5 mg L^{-1} each of Cr, Cu, Cd, Ni, Pb and Zn), the removal of Cr was the least (43%) owing to its least ionic radius among the six metals. Similar results were also obtained by Dey et al. (2020) with multimetal exposure to *A. fumigatus*. Interestingly, although previous studies had reported preferential removal of Pb in the presence of Cd (Li et al. 2004; Henriques et al. 2017), the opposite was seen in Set 6. The probable reason could be that Pb adsorbed more efficiently at pH 3 whereas Cd adsorbed at pH 5.5 (Abdel-Aty et al. 2013) which was the pH of the medium after fungal growth.

Overall, the metal removal pattern with increasing metal complexity varied with the number of metals. Introduction of some metals caused toxicity (Set 2 and Set 5) whereas some alleviated the effect. Fungi use different strategies like vacuole production, extracellular polymer secretion, chelation, active transport of metals, etc. to alleviate heavy metal stress (Khan et al. 2019). A single metal stress triggers a specific response while the presence of multimetal stress elicits a number of defense pathways. Pb removal occurs in *Aspergillus* sp. by reducing the metal ion to oxalate (Tian et al. 2019). Cr on the other hand is reduced to Cr^{3+} by reductase and then remediated by the fungus (Kumar and Dwivedi 2019). Hence, it is seen that the presence Pb and Cr causes two different metabolic pathways to trigger simultaneously in the fungus causing lower biomass production. Cu and Zn removal in *A. fumigatus* occurs through metallothione in controlled by a single transcription factor (Cai et al. 2018). Also it has been seen that Cu and Zn compete with Cr. Hence, it was seen in Sets 3 and 4 that the introduction of Cu and Zn, respectively, did not affect the biomass production but Cr removal decreased significantly ($p < 0.05$). Ni has been reported to be toxic to fungus and has caused low biomass production *Trametes pubescence* (Enayatizamir et al. 2020). Cd competes with Ni (Remelli et al. 2016) and hence it was seen that in Set 6, the biomass productivity increased and Ni removal decreased. It is seen that metal removal in a multi-metal system is a complex interplay between physical and chemical properties of the elements. Also, the concentration of heavy metals was a crucial factor determining the amount of metal removed. Previous studies with multi-metal complexes have cited multiple factors such as electronegativity, pH, atomic and ionic radii to be crucial factors affecting the removal rates. Further, no study could be traced showing the precise location of heavy metal accumulation sites in *A. fumigatus* using TEM–HAADF micrograph. Hence, this study provides a new insight into the sequestration patterns for six heavy metals.

Conclusion

The present study showed that the combinations of metals affected its tolerance by *A. fumigatus*. Highest biomass was produced in the abiotic control. Lowest biomass was produced in the presence of Pb and Cr suggesting its toxicity to the fungus. With increasing metal complexity removal of Cr decreased significantly ($p < 0.05$) with the addition of different metals whereas the removal of Pb, Zn and Ni remained unaffected. However, there was a variation in the removal pattern of the above elements with different metal combinations. There was competition between different metal ions that caused different removal patterns. SEM showed that hexa-metal stress caused fungal hyphae breakage. The TEM–HAADF revealed the precise location of each metal accumulation site inside *A. fumigatus* cell. The toxicity effect due to increasing metal complexity did not follow a linear trend. The above study showed that in a multi-metal system, the toxicity effect on the fungus varies with different metal combinations rather than the number of metals present indicating its usefulness towards the treatment of metal-contaminated wastewater.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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