Environmental Pollution 262 (2020) 114255

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Mechanistic insight to mycoremediation potential of a metal resistant fungal strain for removal of hazardous metals from multimetal pesticide matrix

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ARTICLE INFO

Article history:
Received 20 August 2019
Received in revised form
18 February 2020
Accepted 21 February 2020
Available online 6 March 2020

Keywords:
Bioaccumulation
Biosorption
Lindane
Multimetal
TEM-EDAX

ABSTRACT

Fungi have an exceptional capability to flourish in presence of heavy metals and pesticide. However, the mechanism of bioremediation of pesticide (lindane) and multimetal [mixture of cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb), zinc (Zn)] by a fungus is little understood. In the present study, *Aspergillus fumigatus*, a filamentous fungus was found to accumulate heavy metals in the order [Zn(98%)>Pb(95%)>Cd(63%)>Cr(62%)>Ni(46%)>Cu(37%)] from a cocktail of 30 mg L^{-1} multimetal and lindane (30 mg L^{-1}) in a composite media amended with 1% glucose. Particularly, Pb and Zn uptake was enhanced in presence of lindane. Remarkably, lindane was degraded to 1.92 ± 0.01 mg L^{-1} in 72 h which is below the permissible limit value (2.0 mg L^{-1}) for the discharge of lindane into the aquatic bodies as prescribed by European Community legislation. The utilization of lindane as a cometabolite from the complex environment was evident by the phenomenal growth of the fungal pellet biomass (5.89 ± 0.03 g L^{-1}) at 72 h with cube root growth constant of fungus (0.0211 $g^{1/3}$ $L^{-1/3}$ h^{-1}) compared to the biomasses obtained in case of the biotic control as well as in presence of multimetal complex without lindane. The different analytical techniques revealed the various stress coping strategies adopted by *A. fumigatus* for multimetal uptake in the simultaneous presence of multimetal and pesticide. From the Transmission electron microscope coupled energy dispersive X-ray analysis (TEM-EDAX) results, uptake of the metals Cd, Cu and Pb in the cytoplasmic membrane and the accumulation of the metals Cr, Ni and Zn in the cytoplasm of the fungus were deduced. Fourier-transform infrared spectroscopy (FTIR) revealed involvement of carboxyl/amide group of fungal cell wall in metal chelation. Thus *A. fumigatus* exhibited biosorption and bioaccumulation as the mechanisms involved in detoxification of multimetals.

1. Introduction

Eradication of various hazardous pollutants (heavy metals and pesticides) occurring in the natural resources such as rivers (Bhattacharya et al., 2015; Pandey et al., 2011) by bioremediation is being explored as an alternative to the conventional physical and chemical methods. Several studies depict the mechanism of metal uptake or degradation of organic compounds by plants (Müller et al., 2013; Yadav et al., 2010) as well as microorganisms (Amoozegar et al., 2007; Atkinson et al., 1998; Lee et al., 2019; Mishra and Malik, 2013; Zheng et al., 2018). Eventually, these techniques are also being explored for industrial wastewater treatment (Mishra and Malik, 2013; Shah, 2017). Fungus is particularly a lucrative option than unicellular bacteria in terms of harbouring enormous key remediating enzymes and possessing large surface to cell ratio (Deshmukh et al., 2016).

Microbial metal removal is an intricate process that depends on cell composition and physiology of the microorganism apart from

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https://doi.org/10.1016/j.envpol.2020.114255
0269-7491/

composition, concentration and chemistry of metal ions (Gadd, 2007). Also, the individual metals have a distinctive interaction among themselves when present in a complex multiple co-contaminant system which could be synergistic, antagonistic, or noninteractive and compete for metal binding sites in microorganisms (Pakshirajan and Swaminathan, 2009). Further, hydrophobic and lipophilic compounds, e.g. pesticides, interact with organic matrix on the biomass surface and membranes which results in adsorption of organic compounds (Gadd, 2009).

Limited studies have been conducted for bioremediation of single metal and single pesticide mixture (Thakur and Srivastava, 2011) or ternary metals (Kumar et al., 2016) or binary pesticide mixture (Liang et al., 2014). Additionally, in a system of organic compound and heavy metals, the effect of metals on degradation of the organic compound is complex which depends on physico-chemical and biological characteristics of the contaminated systems (Moreira et al., 2013). Aparicio et al. (2018) investigated the performances of single and mixed cultures of actinobacteria for remediation of lindane and chromium from a co-contaminated environment. Quadruple consortium showed enhanced (60%) chromium removal over single culture (50%) but almost similar lindane removal (55–60%). This reflected synergism amongst the microbial consortium for removal of chromium but not for lindane. Thus, a systematic investigation elucidating the mechanism for simultaneous remediation of organic and multiple inorganic contaminants is needed.

Our group evaluated the uptake of the individual metals from a cocktail of hexametals (Cd, Cr, Cu, Ni, Pb and Zn) mixture by *Beauveria bassiana* (Gola et al., 2016) and *Aspergillus fumigatus* (Dey et al., 2016). *A. fumigatus* demonstrated exceptional performance and could bring down the concentrations of Cd, Cu, Ni, Pb and Zn below the permissible limits for use in irrigation purposes as prescribed by Food and Agriculture Organization (FAO). Present study uniquely details the mechanistic insight of biosorption and bioaccumulation of hexametals into the *A. fumigatus* biomass and the degradation process of pesticide lindane from a simulated hexametals and pesticide (lindane) matrix which reflect the actual co-contaminated water systems. Hence this understanding would aid in the development of a better, evolved and realistic remediation technique which can be disseminated for *Ex-situ* bioremediation of river water for various agronomical purposes.

2. Materials and methods

2.1. Chemicals and reagents

Individual metal stock solutions of 10 g L^{-1} were prepared by dissolving their respective salts $Cd(NO_3)_2$, $K_2Cr_2O_7$, $Cu(NO_3)_2$, $Ni(NO_3)_2$, $Pb(CH_3COO)_2$ and $Zn(NO_3)_2$ in double distilled water. Lindane stock solution of 100 g L^{-1} was prepared by dissolving lindane powder in HPLC grade acetone. The reagents and calibration standards were prepared in deionized ultrapure water (RIONS Ultra 370 series) was used. All other chemicals used were of analytical grade and were obtained from Merck, Sigma and Qualigens.

2.2. Microorganism and culture media composition

The fungal strain was previously isolated from the soil sample collected from Okhla sampling site of the Yamuna river, New Delhi, India and characterized as *Aspergillus fumigatus* PD-18 with accession number KX365202 (Dey et al., 2016). Composite growth medium with following composition (g L^{-1}): (K_2HPO_4, 0.5; NH_4NO_3, 0.5; NaCl, 1.0; $MgSO_4.7H_2O$, 0.1; Yeast extract, 2.5 and Glucose, 10.0; pH 6.8 ± 0.2) was used. The media was sterilized at 121 °C for 15 min.

2.3. Multimetal bioaccumulation, lindane degradation and growth kinetics of the fungal strain in presence and absence of lindane

The approach for this study was to evaluate the potential of *A. fumigatus* for the parameters in terms of its growth, substrate (glucose) consumption, multimetal removal in presence of multimetal (MM) and then parallelly study these parameters under the exposures of multimetal and pesticide (lindane) stress (MML) in composite broth. The lindane degradation potential of *A. fumigatus* under multimetal pesticide mixture were also measured.

The multimetal mixture (MM) comprised of total 30 mg L^{-1} (5 mg L^{-1} of each of the individual Cd, Cr, Cu, Ni, Pb and Zn), respectively, supplemented with 1% of glucose in the composite media. This was carried out by keeping in view the occurrence of metals in the Yamuna river (Bhattacharya et al., 2015). The multimetal and pesticide mixture (MML) comprised of composite media with 30 mg L^{-1} multimetal, 30 mg L^{-1} lindane in addition to 1% of glucose. The biotic control comprised of composite media amended with only 1% of glucose. Two abiotic uninoculated controls comprised of media with only 30 mg L^{-1} multimetal and media with 30 mg L^{-1} multimetal + 30 mg L^{-1} lindane were also kept.

These studies were performed in series of Erlenmeyer flasks (250 mL) containing 100 mL of composite growth media. The flasks were inoculated with 1 mL of spore suspension (10^7 spores per mL) and incubated at 30 °C and 150 rpm for 72 h. At every 6 h interval, dry weight of the resultant biomass was measured gravimetrically and the supernatant was analysed for residual glucose, metal and lindane concentration. All the analytical measurements were done in triplicate. The amount of metals accumulated by the fungal strain was calculated from the difference between initial concentration and the concentration remaining in supernatant. The experimental data obtained from the batch multimetal accumulation, lindane degradation and fungal growth experiments were subjected to cube root equation Papagianni, 2004.

$$M^{1/3} = kt + M_0^{1/3} \text{ (Papagianni, 2004)}$$

where M = dry cell weight (g L^{-1}) of fungal cells at time t(h), M_0 = initial dry cell weight (g L^{-1}), k = cube root kinetic constant ($g^{1/3}$ $L^{-1/3}$ h^{-1}).

Specific uptake capacity, qm (mg g^{-1}) of fungal biomass was determined using following equation

$$(q_m) = \text{total metal removed (mg L}^{-1}\text{)/total dried biomass (g L}^{-1}\text{)}$$

2.4. Analysis of residual heavy metals and estimation of residual sugar content

Residual metal ions (cadmium, chromium, copper, nickel, lead and zinc) concentration in water sample were determined after digestion with concentrated nitric acid in microwave digester (Anton Paar Multiwave Pro) using standard method (Eaton et al., 2017). The digested solution was quantified through Atomic Absorption Spectroscopy (ECIL AAS4141). Residual sugar content in the samples were analysed by the reagent dinitrosalicyclic acid method (DNS) for reducing sugar (Miller, 1959) at 540 nm using spectrophometer (PerkinElmer Lamda 35 UV/Vis systems).

2.5. Estimation of residual lindane concentration

Residual lindane in the samples was estimated using standard method (Singh and Singh, 2019). Briefly, the sample solutions were

added to the HPLC grade ethyl acetate and vortexed that led to formation of two layers of organic and aqueous phase. The organic phase (ethyl acetate) was transferred to another falcon tube which was evaporated by vacuum evaporator. The residual lindane in the falcon tube was diluted by HPLC grade ethyl acetate and estimated by Gas Chromatography (Agilent 7890A).

The oven temperature was programmed to 180 °C while the injector and interface temperatures were kept at 220 °C and 250 °C, respectively. The carrier gas used was nitrogen. Lindane degradation rate constant (k) was determined by plotting lindane concentrations at different time intervals versus time on semi logarithmic graph.

$$\ln C = -Kt + \ln C_0$$

where C is the lindane concentration at 72 h and C_0 is the initial lindane concentration

2.6. Study of mechanism of metal bioaccumulation and biosorption by analytical instruments at physiological level

To understand the metal uptake mechanism under multimetal as well as multimetal plus pesticide environments, *A. fumigatus* was grown in composite media till the fungus growth reached the late log phase (120 h) and the pellets were harvested thereafter. The morphological changes due to these stresses as well as the localization of multimetals inside the fungal biomass were assessed utilising tools such as Scanning Electron Microscope (SEM); Fourier Transform Infrared Spectroscopy (FTIR); Transmission Electron Microscopy coupled with EDX.

2.6.1. Scanning electron microscopy (SEM)

The harvested fungal pellets were fixed in 2.5% glutaraldehyde for 12–18 h at 4 °C according to the standard protocol as described by (Gola et al., 2016). Afterwards fungal specimens were gold-coated by cathodic spraying (Polaron gold) and examined under scanning electron microscope (ZEISS EVO 50) at acceleration voltage of 20 kV with magnification: = 5000 X.

2.6.2. Fourier transform infrared spectroscopy (FTIR)

The harvested fungal pellets were first washed with phosphate buffer (pH 7.2) and then lyophilised. The FTIR spectra for the dried fungal pellets were recorded for the wave number range of 520–4000 cm^{-1} using Spectrum One (PerkinElmer) spectrometer equipped with DTGS detector. The FTIR spectra was interpreted by comparing it with standard spectra of various organic compounds (Silverstein et al., 2014).

2.6.3. Transmission electron microscopy coupled with energy dispersive X-Ray spectroscopy (TEM-EDX)

The harvested fungal pellets were washed with phosphate buffer (pH 7.2) and subsequently treated with primary fixative 1% glutaraldehyde and 2% paraformaldehyde for 12–18 h at 4 °C, followed by treatment with secondary fixative for 2 h in osmium tetra oxide (1%) in phosphate buffer at 4 °C. The fungal specimens were cut into ultrathin section of 60–80 nm thickness using an ultramicrotome (Leica EM UC 6) and stained in alcoholic uranyl acetate for 10 min followed by staining in lead citrate for 10 min. The grids were examined in transmission electron microscopy (JEOL JEM-2100F) operated at 120 kV.

2.7. Statistical analysis of the data

All the experiments were conducted in triplicates. Statistical calculations were performed with SPSS 21. Significant effect of lindane concentration on metal uptake was calculated by means of one-way multivariate analysis of variance (MANOVA) using Wilk's lambda test statistic. The dependent variables (metals) were tested for homogeneity of variance using Levene's test.

3. Results

The results obtained in this study depicted the notable growth of *A. fumigatus* PD-18 in presence of multimetal and pesticide lindane. Furthermore, the tools such as SEM, TEM-EDAX mapping and FTIR revealed biosorption and bioaccumulation of metals in the fungal biomass.

3.1. Growth, glucose utilization, metal accumulation and pesticide degradation potential of A. fumigatus

Under multimetal + pesticide exposures (MML), glucose was completely consumed in ~72 h (Fig. 1) and (Supplementary Figs. S1–S3) as compared to 60 h under multimetal stress (MM), and 30 h for biotic control (BC).

This strain also aided in effective degradation of lindane. Maximum biomass production by ***A. fumigatus*** in the three types of exposures were as follows, MML (5.89 ± 0.03 g L^{-1}) at 72 h > BC (5.42 ± 0.01 g L^{-1}) at 36 h > MM (4.59 ± 0.01 g L^{-1}) at 66 h. While the comparable cube root growth kinetic constant k of the fungal strain were as follows, BC (0.0562 ($g^{1/3}$ $L^{-1/3}$ h^{-1}) > MM (0.0226 $g^{1/3}$ $L^{-1/3}$ h^{-1})>MML (0.0211 $g^{1/3}$ $L^{-1/3}$ h^{-1}) for the strain *A. fumigatus* (Supplementary Table 1). However, the metal accumulation potential of the strain was affected in presence of lindane (Fig. 1). After 72 h of cultivation, the final concentration of residual multimetal concentration in the medium in case of both the exposures were (MML) 9.92 ± 0.001 mg L^{-1} and (MM) 2.41 ± 0.001 mg L^{-1}, respectively. Correspondingly, the total multimetal accumulation by *A. fumigatus* were calculated as (MML) 20.04 ± 0.02 mg L^{-1} and (MM) 27.59 ± 0.09 mg L^{-1}, respectively (Supplementary Table 1). The multimetal concentration in both the abiotic controls (in absence and presence of lindane) remained same.

About 5% of multimetal had been removed from medium in the initial 6 h in the presence of lindane. After 6 h, multimetal removal almost gradually paralleled lindane degradation. It is apparent that rapid removal of lindane (62% of the initial concentration) was observed in first 6 h. The residual lindane concentration decrement was in parallel to the fungal growth, achieving 96% lindane removal at the end of 72 h (Supplementary Fig. S4).

The result of gas chromatograph (GC) is also shown in Supplementary Fig.S5 (a-b). The chromatogram shows the residual concentration of lindane in the medium taken at 0 h and after 72 h growth period. The GC analysis revealed a single peak of lindane with retention time ~8.7 min. Lindane concentration in the abiotic non-inoculated control setup remained constant implying no abiotic loss. A first-order kinetics model was used to determine the rate constants (k) for lindane degradation in the presence of co-substrate glucose. The degradation rate constant (k) was found to be 0.0289 h^{-1}.

Based on metal accumulated from the medium in 72 h, each metal declined in presence of lindane in the order Zn (98%)>Pb (95%)>Cd (63%)>Total Cr (62%)>Ni (46%)>Cu (37%) (Fig. 2). Overall there was significant accumulation of metals in absence of lindane. The metal removal trend was Cd (100%)>Ni (99%)>Cu (98%)>Pb (91%)>Zn (84%)>Cr (81%) (Fig. 2). The addition of lindane had significant effect on the accumulation of all the metals viz. Cd, Cr, Cu, Ni, Pb as well as Zn. (Here $p < 0.05$ according to one-way MANOVA) as shown in Supplementary Table 2. The Wilks' Lambda has a p-value <0.001.

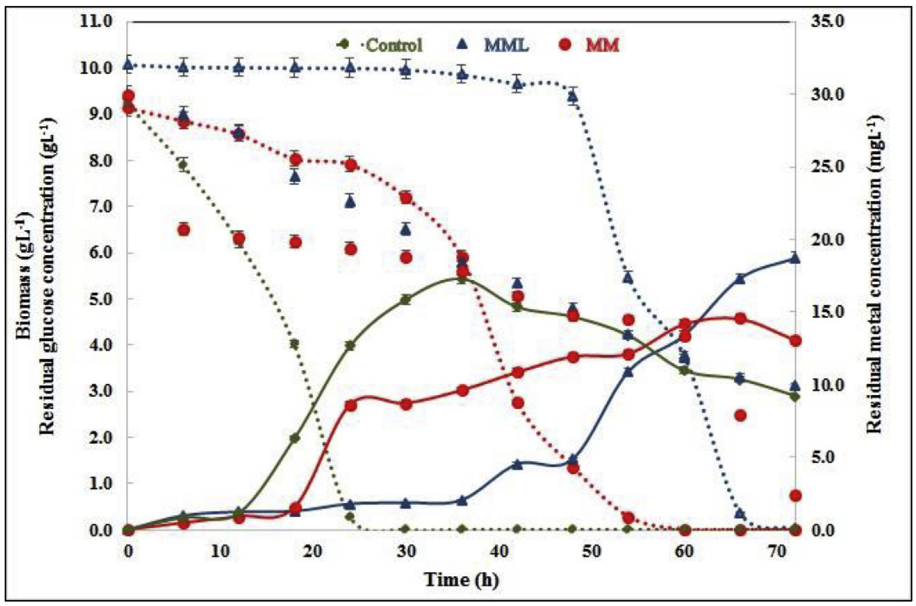

Fig. 1. Residual glucose concentration (dotted lines), residual metal concentration (symbols) in broth and biomass production (solid lines) by *A. fumigatus* in presence of 1% glucose in BC; 30 mg L^{-1} MM; 30 mg L^{-1} MM & 30 mg L^{-1} lindane.

3.2. Physiological analysis of metal accumulation by A. fumigatus

3.2.1. SEM analysis

The SEM micrographs showed a clear distinction between the control (Supplementary Fig.S6 a) and the mycelia stressed with MM and MML (Supplementary Fig. S6 (a-c)). The fungal hyphae in control is loosely packed and ribbon like while MM brought slight shrinkage and MML exposure exhibited rough surface.

3.2.2. TEM-EDX analysis

The TEM-EDX micrographs as shown in Fig. 3 (a) (I-IX), revealed that fungal isolate accumulated Ni and Zn in its cytoplasm while Cd, Cr, Cu and Pb in cell membrane/cell wall in case of MM exposure. Under MML exposure, this strain had tendency to accumulate more of Cr in cytoplasm besides the metals Ni and Zn. While in the cell membrane/cell wall there was accumulation of metals Cd, Cu, and Pb as depicted in Fig. 3 (b) (I-IX).

3.2.3. FTIR analysis

FTIR spectra for fungal biomass grown under three conditions (Fig. 4) exhibited various functional groups involved in the multi-metal binding. The dominant functional groups present in biotic control are O–H and N–H stretching (3000-3500 cm^{-1}), C–H stretching (2800-3000 cm^{-1}) C=O stretching in carboxyl or amide I band groups (1600-1700 cm^{-1}), N–H bending in amide III and C–N stretching in –CO–NH– groups (1500-1600 cm^{-1}), C–OH and C–O–P stretching (1000-1200 cm^{-1}), nitro and disulphide groups (800-850 cm^{-1}). In case of MM laden biomass there were disappearance of peaks denoting masking of C–H stretching when compared to control biomass. There was masking of OH and NH stretching groups which were evidently involved in MM as well as in MML treated samples. Also, there was disappearance of carboxyl/amide group stretching and thiol group stretching as observed in case of MML laden biomass.

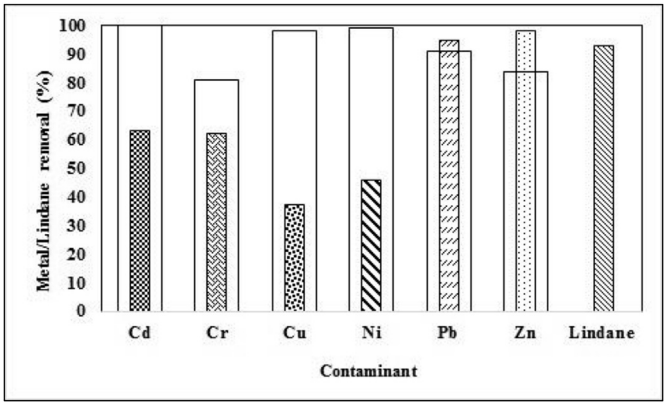

Fig. 2. Removal of metals in presence of 1% glucose, 30 mg L^{-1} multimetal and absence (blank bars) or presence (patterned bar) of 30 mg L^{-1} lindane.

4. Discussion

4.1. Growth, glucose utilization, metal accumulation and pesticide degradation potential of A. fumigatus

The findings of this study clearly show that *A. fumigatus* could tolerate and degrade lindane by cometabolism with amendment of 1% glucose which served as an energy source and aided in rapid lindane degradation and consequently in higher biomass yield of *A. fumigatus* (Supplementary Fig. S4). It is important to note that *A. fumigatus* could not grow with only lindane as a sole carbon source in composite medium in 72 h (data not shown).

It is known that the breakdown of organic compounds is associated to an inducible system. Where occurrence of heavy metals in a co-contaminated system may impede the degradation of organic pollutants by competing with essential metal cofactors and enzymes eg. oxygenases and consequently reduce the enzymatic activity in microorganisms (Sandrin and Maier, 2003). On the contrary, *A. fumigatus* on addition of 1% glucose was able to degrade 30 mg L^{-1} lindane to 1.92 ± 0.01 mg L^{-1} in 72 h which is below the permissible limit value (2.0 mg L^{-1}) for discharge of lindane into the aquatic environment according to European Community legislation (Supplementary Fig. S4).

Organic compounds contamination may derail the primary

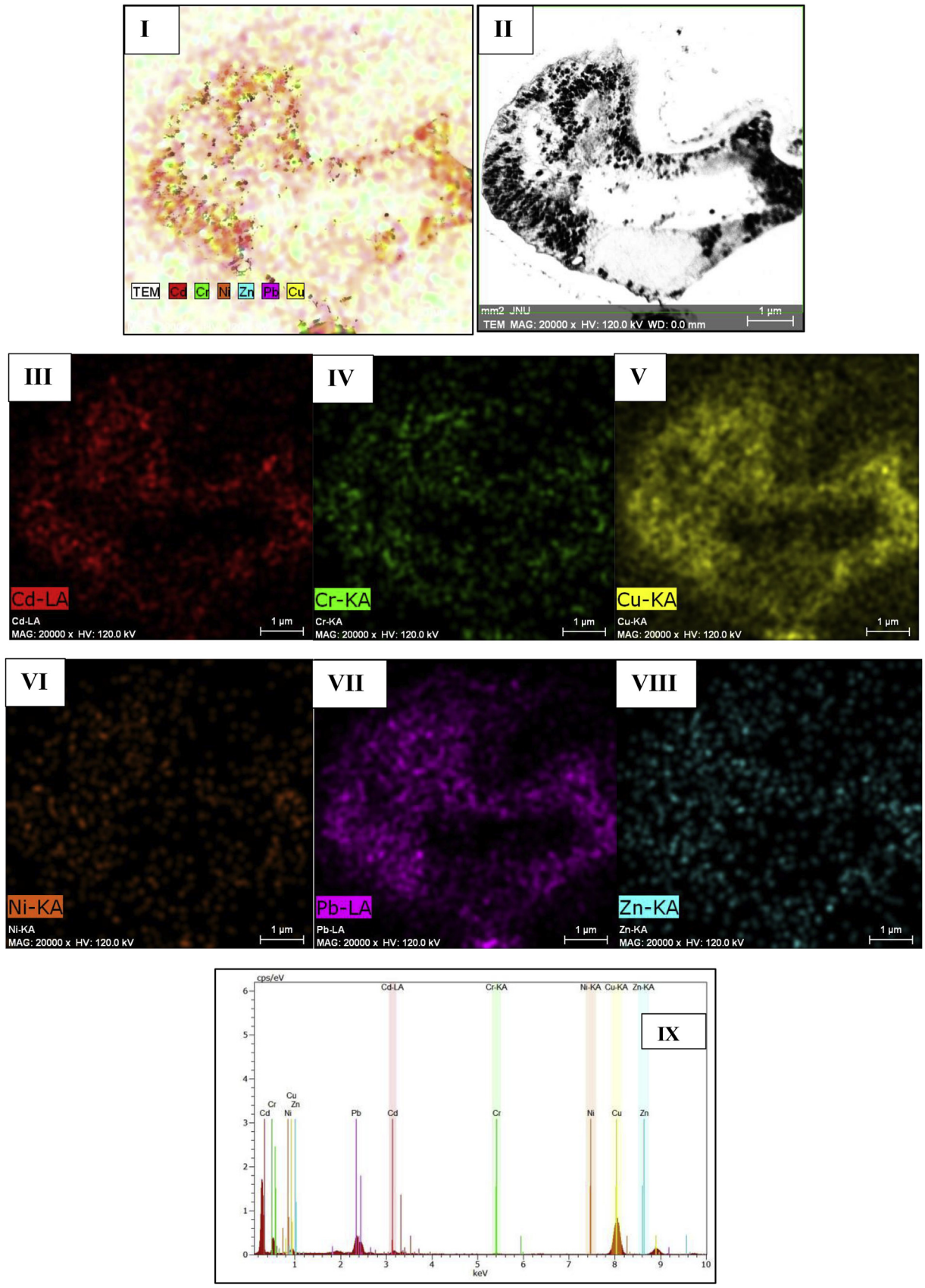

Fig. 3. (a): TEM-EDAX mapping of pellets of *A. fumigatus* in 30 mg L^{-1} multimetal. (II) TEM micrograph in presence of multimetal (Cd, Cr, Cu, Ni, Pb, Zn); (I-IX) EDAX depicting the exact location of accumulation of individual metal onto the different organelles of the fungal cell; (IX) EDAX graph confirm the presence of individual metals inside the fungal cell, (b): TEM-EDAX mapping of pellets of *A. fumigatus* in 30 mg L^{-1} multimetal and 30 mg L^{-1} lindane. (II) TEM micrograph in presence of multimetal (Cd, Cr, Cu, Ni, Pb, Zn); (I-IX) EDAX

depicting the exact location of accumulation of individual metal onto the different organelles of the fungal cell; (IX) EDAX graph confirm the presence of individual metals inside the fungal cell.

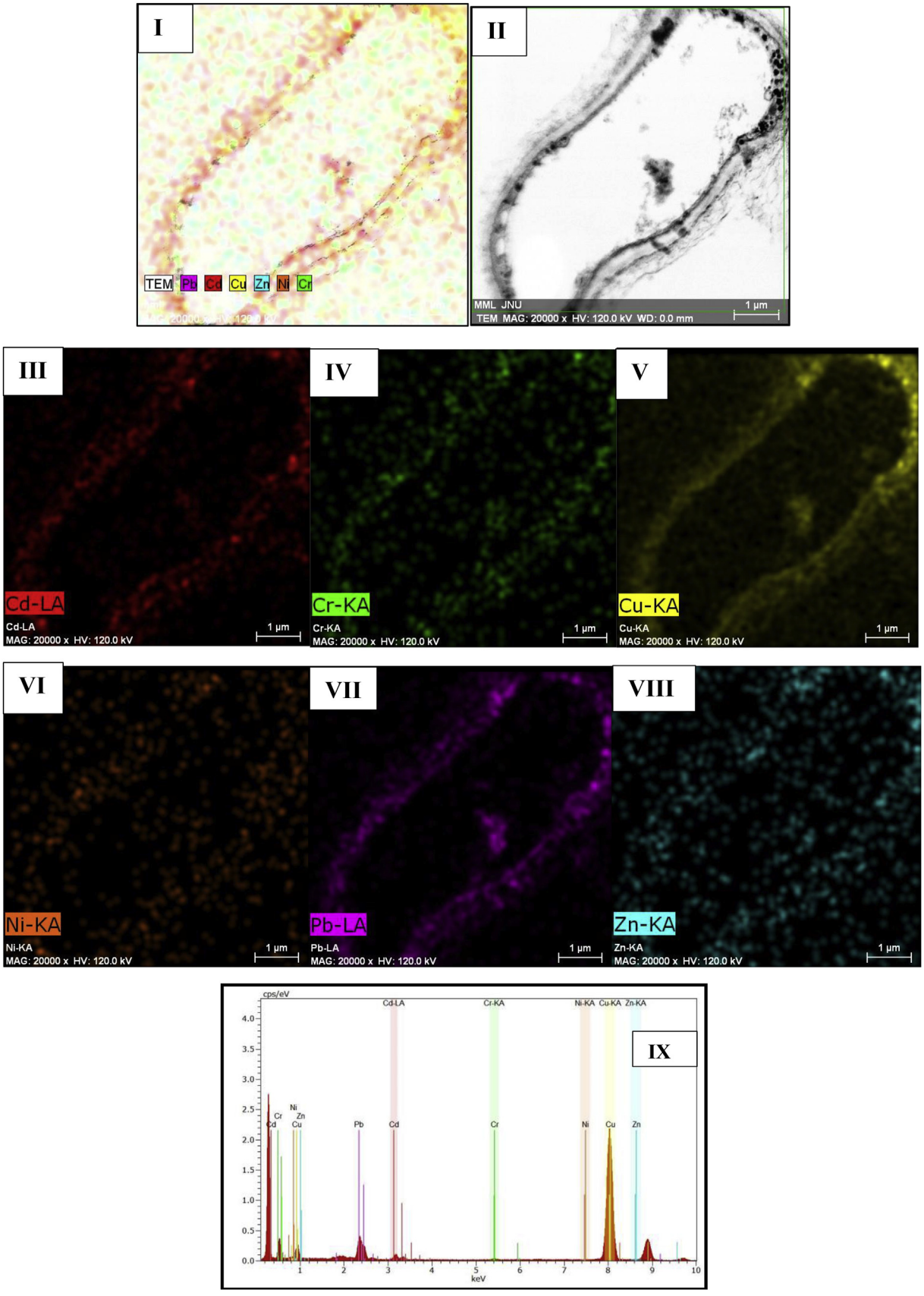

Fig. 3. (*continued*).

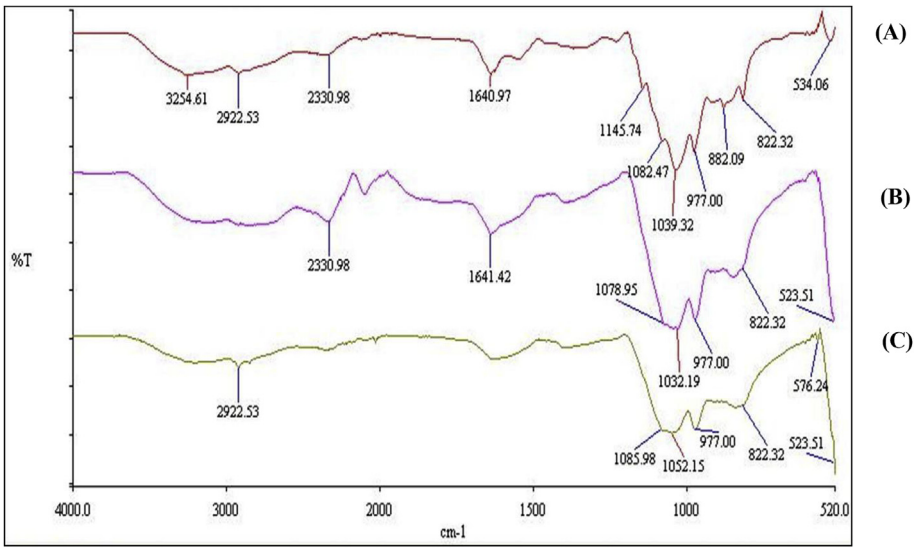

Fig. 4. FTIR spectra of pellets of *A. fumigatus* (A) In absence of multimetal and lindane (Biotic control); (B) 30 mg L^{-1} multimetal (MM) (C) 30 mg L^{-1} multimetal + 30 mg L^{-1} lindane (MML).

substrate (glucose) utilization by microorganisms. Similarly, in this study glucose consumption was delayed with the introduction of pesticide to the multimetal mixture. It was observed that *A. fumigatus* could use glucose only after the first 48 h of incubation, resulting in sequential 15% glucose removal and 83% lindane degradation (Fig. 1).

Other researchers too have enumerated the direct correlation between the microbial growth and its degradation ability along with cometabolism. Schrijver and Mot (1999) studied cometabolism in presence of a carbon source (glucose) in actinomycetes. They concluded that pesticides serve as carbon, nitrogen or phosphorous source through partial transformation reactions. The simple growth substrates (carbon sources viz. fructose, glucose or small chain fatty acids viz. sodium succinate or sodium acetate) promote cell growth, enhance the degradation of heavy molecular weight organic substrate (pesticide) and also act as a cosubstrate and aid cometabolism by inducing production of catabolic enzymes such as laccase, peroxidase (Xie et al., 2009) or heme thiolate monooxygenases (Mougin et al., 1997).

The biodegradation of lindane and complete utilization of glucose is evident from the growth of the *A. fumigatus*. Though lindane derailed the growth of fungal biomass, which required an acclimatization phase for growth to occur (Fig. 1). The lag phase extended to ~18 h multimetal (MM) and ~36 h multimetal + lindane (MML), respectively, as the complexity of contamination increased. Thus, the current growth profile clearly indicates the adaption of *A. fumigatus* to MM and MML exposure during extended lag phase.

Remarkably, in this study, while there was slow growth under MM exposure, MML exposure resulted in higher growth beyond 48 h and the biomass obtained was more than the biomass obtained in the biotic control (Fig. 1). This observation evidently indicates the degradation of organic contaminant lindane as well as the utilization of its breakdown products as carbon source for the growth of the fungus. Additionally, with the introduction of organic contaminant (pesticide), the overall growth rate of fungus decreased. The higher biomass obtained in case of MML may be due to the delayed lag phase resulting in the slow utilization of the lindane degradation products (Supplementary Table 1).

Also, the extent of inhibition of metal uptake by the fungal strain *A. fumigatus* can be postulated with the metabolism of lindane due to the formation of metabolites as evident from the appearance of new peaks in chromatogram beyond 6 h (Supplementary Fig. S5b). Other researchers too noted the formation of metabolites (Guillén-Jiménez et al., 2012; Mougin et al., 1996). They reported the formation of metabolites like benzoic acid, pentachlorocyclohexane or tetrachlorocyclohexene, tetrachlorocyclohexene epoxide, tetrachlorocyclohexenol on degradation of lindane by fungi. Thus, fungus breaks down the recalcitrant lindane into its metabolites that alleviates the suppression of lindane biodegradation which could be brought about by the presence of multimetals (Cd, Cr, Cu, Pb and Zn).

The multimetal uptake by *A. fumigatus* was initiated after few hours of incubation in case of lindane amended medium, (Fig. 1). It is evident that the multimetal uptake was less and disrupted in the presence of lindane. This indicated an extended lag phase, followed by slow utilization of the primary growth substrate glucose which led to less biomass formation and consequently slow and less metal uptake.

The metal removal trend in the absence of lindane was Cd (100%)>Ni (99%)>Cu (98%)>Pb (91%)>Zn (84%)>Cr (81%) (Fig. 2). Cd was completely removed while the metals Ni, Cu and Pb had almost similar percentage of removal.This selective metal removal efficacy in a multiple metal system depends on metal ion characteristics such as electronegativity, atomic radii, ionic radii of the individual ions (Açıkel and Alp, 2009; Gola et al., 2017).The fungal biomass surface comprises of chitin and chitosan having inorganic and organic groups (In et al., 2019; Sağ, 2001).Thus, the more electropositive metal ion will be more strongly attracted to the surface of the biomass.The Sanderson electronegativity of the metal ions follows the order Pb(1.92)<Ni(1.94)<Cd(1.98)≤Cu(1.98)<Zn(2.22)<Cr(3.37).Therefore,in this multimetal mixture environment, bioaccumulation of Zn and Cr decreased consistently as they are more electronegative and would have less affinity towards this fungal biomass. Nonetheless, the metals Cd, Cu, Ni, Pb and Zn were brought down below the permissible mandates of FAO.

The percentage of metal accumulated from the multimetal + lindane amended medium in 72 h (Fig. 2) was in the order of Zn (98%)> Pb (95%)> Cd (63%)> Total Cr (62%)> Ni (46%)> Cu (37%). This decreased uptake of metals by the fungus in presence of a co-contaminant could be attributed to the phenomenon of

screening effect by pesticide lindane. Presence of lindane that is adsorbed onto the fungal biomass as shown in the SEM micrograph (Supplementary Fig. 6c) can mask the sorption sites on cell surface and act as a blanket. This was further established by the FTIR results (Fig. 4) that depicted low molecular weight organic acids viz. carboxylic and amino groups of the fungal cell membrane (Liu et al., 2017) which were found to be binding with pesticide lindane. Thus, these factors resulted in reduced binding of the metals.

On the basis of metabolism of fungus, it can be hypothesized that in presence of lindane, slow uptake of metals could be attributed to the energy provided by the electron acceptors NAD^+. The degradation of lindane and the accumulation of metals may not take place simultaneously. At first instance energy in the fungus maybe channelized for the NADH dependent lindane breakdown. On the second instance, the residual energy after lindane degradation may be utilised for uptake of more soluble and bioavailable metals viz. Cd, Cu, Ni and Zn. Metals like Cr and Pb are less soluble (Igiri et al., 2018). However, it can be postulated that Pb having higher atomic number and a soft acid has the affinity to bind with thiol group (ligand) in the metallothionein occurring in cell membrane of the fungus (Gutiérrez et al., 2019; Nies, 1999) as depicted in the TEM-EDAX micrograph (Fig. 3 (b) VII).

Interestingly, *A. fumigatus* exhibited higher removal of Pb (95%) and Zn (98%) in the presence of lindane than in the absence of lindane (Fig. 2). This phenomenon of elevated Pb and Zn sequestration in the fungal cell could be attributed to enhanced permeability of the cell wall due to change in the cell structure dynamics by the extreme toxicity of the pesticide (Słaba et al., 2009). The researchers observed that increase in zinc binding by the growing mycelium of *P. marquandii* in the presence of the pesticide was caused by the changes in the wall and membrane composition induced by simultaneous toxic interaction of zinc and alachlor. Another study revealed that polyaromatic hydrocarbons may damage microbes and network with lipophilic components of biomembranes, leading to change in permeability of biomembranes and permitting heavy metals to enter easily into microbial cells (Shen et al., 2006). Furthermore, this rupture in the cell membrane/wall of the fungus is correlated with TEM-EDAX result (Fig. 3 (b)II) where interrupted dark electron region could be observed throughout the cell membrane of the fungal cell.

The total bioaccumulated multimetal quantities per unit mass of biomass on dry weight basis (specific uptake capacity) represented by q_m reduced in case of MML (3.40 mg g^{-1}) when compared to MM (6.70 mg g^{-1}) as shown in Supplementary Table 1. In our previous study, heavy metal bioaccumulation by a fungus was found to depend upon the type of fungal species, concentration of heavy metals as well as the dynamics of the cocktail of heavy metals (Dey et al., 2016). Thus in this study the uptake of heavy metals by *A. fumigatus* was affected with the introduction of the co-contaminant lindane.

Furthermore, it is noteworthy that biodegradation of lindane by microorganisms have been studied by several researchers (Ceci et al., 2015; Garg et al., 2012; Lodha et al., 2007; Mougin et al., 1996; Sagar and Singh, 2011). Yet, the degradation of the pesticide lindane in the presence of hexametal by a fungus has not been studied so far.

4.2. Physiological analysis of metal accumulation by A. fumigatus

4.2.1. Scanning electron microscope (SEM) analysis

Scanning Electron Microscope (SEM) analysis provided mapping of surface details. The metal resistant microorganisms exhibit various structural and morphological adaptations under multimetal and organic contaminant stress. The SEM micrographs showed a clear difference between the control (Supplementary Fig. S6a) and mycelia stressed with 30 mg L^{-1} multimetal and 30 mg L^{-1} multimetal +30 mg L^{-1} lindane (Supplementary Figs. S6(b–c)). The fungal hyphae in control is loosely packed and ribbon like (Supplementary Fig. S6a). The mycelia in the presence of 30 mg L^{-1} multimetal brought slight shrinkage (Supplementary Fig. S6b). Lundy et al. (2001) observed decrease in mycelial length of the fungus *Achlyabi sexualis* in presence of metals Cu, Co, Hg, Zn and Cd. Toxic metals bind and chelate with cell membrane and consequently result in distortion of the cell structure as well as loss of cell function (de Silóniz et al., 2002; Yilmazer and Saracoglu, 2009).

Thus in a stressful environment of individual metals, the mycelia aggregates and reduces the exposed surface area in order to facilitate the concentration of intracellular polysaccharides, metal chelating pigments and agents like melanin and siderophores in the fungal mycelia which accounts for the chelation of metals (Gadd, 2007).

Interestingly, the impact of 30 mg L^{-1} multimetal+ 30 mg L^{-1} lindane on the mycelial structure had pronounced rough surface due to the adsorption of lindane onto the fungal biomass when compared to the 30 mg L^{-1} multimetal. Furthermore, from Supplementary Fig. S6 (c), granular deposition of pesticide lindane on the mycelia can be observed, which indicates the adsorption of lindane on surface of the fungal strain. This observation is substantiated with the previous observation of Ghosh et al. (2009), who postulated the involvement of hydrophobic interaction of lipid component in fungal biomass for the adsorption of lindane onto its biomass. Furthermore, the adsorption of lindane onto the fungal biomass surface corroborates the reduced chelation of the metals Cd, Cr, Cu and Ni in this study.

4.2.2. Transmission electron microscopy coupled with EDX analysis

Transmission Electron Microscopy coupled with EDX technique detailed the intracellular structures in the fungal cell. Thus, it demonstrates the mechanisms of living processes at physiological level. The metal resistant microorganisms exhibit preferences for metal uptake in a multimetal and organic contaminant stress. The TEM-EDX micrographs depicted, the fungal isolate accumulated Ni and Zn throughout the cell membrane and cytoplasm while Cd, Cr, Cu and Pb in the cell membrane/cell wall in case of 30 mg L^{-1} initial multimetal (Fig. 3 a (I-IX)). There is an observance of dark electron region bordering throughout the fungal cell (Fig. 3 (a)II).

When the fungal biomass was exposed to the 30 mg L^{-1} multimetal +30 mg L^{-1} lindane (Fig. 3 (b) (I-IX)) this strain had the tendency to accumulate the metals Cd, Cu and Pb in its cell wall/membrane while the metals Cr, Ni and Zn in its cell membrane and cytoplasm as depicted in the figures. So, the metals Cr, Ni and Zn had the tendency to be bioaccumulated onto the fungal cell cytoplasm including the intracellular vacuoles in both the exposures. These intracellular vacuoles hoard the thiol containing compounds such as GSH and metallothioneins which are responsible in channelizing the metals into the vacuoles to reduce the metal load in the cytoplasm (Ge et al., 2011). Furthermore, from the micrograph (Fig. 3 (b) VII) it can be inferred that a distinct dense layer of the Pb being formed onto the cell membrane which denotes the higher accumulation of this metal due to the rupture of the cell wall in the presence of the pesticide lindane.

Srivastava and Thakur (2006) studied the metal rich area of the cell wall by TEM-EDX, confirming the role of cell wall of the fungal strain *Aspergillus niger* as the primary accumulator of chromate from the solution. Thus chitin and chitosan components of the fungal cell wall are instrumental in sequestration of metal ions (de Lima et al., 2013; Purchase et al., 2009).

4.2.3. Fourier Transform Infrared Spectroscopy (FTIR) analysis

As biosorption have significant roles in metal remediation, it is pertinent to know the various functional groups that aid in binding of metals in the fungal biomass. The FTIR spectra of the lyophilised fungal biomass of *A. fumigatus* after metal uptake were examined for masking and presence of functional groups involved in metal chelation.

The dominant functional groups present in the biotic control are O–H and N–H stretching, C–H stretching, C=O stretching in carboxyl or amide I band groups, N–H bending in amide III and C–N stretching in –CO–NH– groups, C–OH and C–O–P stretching, nitro and disulphide groups. So, when compared with the biotic control (Fig. 4), in case of the 30 mg L^{-1} multimetal laden biomass, there was masking of the C–H stretching group. Guibal et al. (1995) studied the role of amides in metal chelating in *Aspergillus, Penicillum, Mucor*. The NH_2 group is a chemical active site in chitin of the fungal cell wall responsible for metal binding (Niu and Volesky, 2006). Further, chitin, glucan, mannan and proteins are the components of fungal cell walls. Apart from these it also contains other polysaccharides, lipids and pigments (melanin) which facilitate binding of many metal ions. This metabolism independent process comprising of the negatively charged groups of carboxyl, phenolic, hydroxyl, carbonyl and methoxyl are important as they bind to the oxygen binding sites which are present in phenolic polymers and melanins of the fungal cell wall (Gadd, 2004). These functional groups of the cell wall moderate the accumulation of the toxic metals inside the cytoplasm that houses most of the enzymes which are required for cell metabolism (Ge et al., 2011).

In case of 30 mg L^{-1} multimetal+ 30 mg L^{-1} lindane laden biomass there was masking of carboxyl/amide group stretching. Thus, confirming the adsorption of the pesticide lindane by the low molecular weight organic acids viz. carboxylic and amino groups present in the cell wall of the fungus. This in turn may lead to competition with the binding of inorganic heavy metals onto the fungal cell. Thus, enhancing the bioavailability and mobility of the pesticide for degradation by the microbe (Kreeke et al., 2010). This hypothesis also validates with SEM micrograph (Supplementary Fig. S6c) which depicts adsorption of lindane on the fungal biomass resulting in interference with metal uptake.

Thus, overall this fungus adopts stratagems to alleviate multimetal and pesticide stress, which include adsorption of lindane pesticide on the fungal biomass, intracellular metal import to the cytoplasm and chelation of metals to the fungal cell wall components. These mechanisms work in a coordinated manner to maintain homoeostasis in the fungus.

5. Conclusions

This study investigated the remediation of pesticide lindane and biosorption and bioaccumulation of multimetal (mixture of cadmium, chromium, copper, nickel, lead, zinc) by previously isolated *A. fumigatus* PD-18 from composite medium. The results deduced the enhanced uptake of metals Pb and Zn by fungus in presence of lindane. Remarkably, lindane was degraded below permissible limit value as prescribed by European Community legislation. However, the bioaccumulation of metals Cd, total Cr, Cu, Ni ions was impeded on addition of lindane.

Lindane adsorption on the fungal biomass is clearly depicted by SEM analysis. The localization of the metals Cr, Ni and Zn in the cytoplasm was revealed by TEM-EDX while FTIR analysis shed light on the competition between metal sequestration and the adsorption of pesticide lindane on the functional groups of carboxyl/amide.

Overall this study provides the invaluable detailed information on the strategy that *A. fumigatus* adopts for removal of metals from different complex contaminant conditions. Essentially the extracellular metal chelation onto fungal cell surface and the intracellular sequestration of metals into the organelles in the cytoplasm was demonstrated. Thus, this knowledge may lead to the optimization of recovery process of the metals from the biomass and development of a field-worthy remediation systems for complex mixtures.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Priyadarshini Dey: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft. **Anushree Malik:** Supervision, Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Funding acquisition, Writing - original draft. **Abhishek Mishra:** Writing - review & editing. **Dileep Kumar Singh:** Writing - review & editing. **Martin von Bergen:** Writing - review & editing. **Nico Jehmlich:** Writing - review & editing.

Acknowledgement

Priyadarshini Dey gratefully acknowledges the scholarship from the Ministry of Human Resource and Development (Government of India) and German Academic Exchange Service (DAAD). This work received financial support from the project grant by National Fund for Basic, Strategic and Frontier Application Research in Agriculture (NFBSFARA) [Grant No. NFBSAFARA/WQ-2023/2012-13], Indian Council of Agricultural Research, New Delhi, India.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.114255.

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