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Heavy metal tolerance of filamentous fungal strains isolated from agricultural field near waste dump site of Dhapa, Kolkata

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Indigenous filamentous fungal strains from maize field near garbage dump site of Dhapa, Kolkata exhibited remarkable tolerance in heavy metal rich media. In the present study about eleven fungal strains were isolated from the soil (15-30cm depth) of maize field in Dhapa. The isolates were identified and assessed for their tolerance to heavy metal concentrations of cadmium (Cd^{+2}), chromium (Cr^{+4}), lead (Pb^{+2}), copper (Cu^{+2}) and cobalt (Co^{+2}). Out of the isolates, 2 fungal species of *Aspergillus* and *Chaetomium* were further selected for their higher potential for metal tolerance. Both the isolates showed highest tolerance to Pb with high tolerance index (TI =/>1). Very high tolerance was also exhibited by *Aspergillus* against Co^{+2} (TI=1). Tolerance of *Aspergillus* and *Chaetomium* to Cd was found to be least with very low tolerance index of 0.27 and 0.38, respectively. Moderate tolerance was exhibited by *Aspergillus* against Cu and Cr . On the other hand *Chaetomium* showed moderate tolerance against Co and Cr and low tolerance against Cu. The fungal strains were subjected to different concentrations of Pb and Cd for determination of Minimum inhibitory concentrations (MIC). The MIC values of Pb and Cd were found to be 2000-2200 ppm and 200-400 ppm in both the fungal isolates. Growth pattern in metal rich media revealed that *Aspergillus* was more tolerant than *Chaetomium* The concentrations of the most adapted and toxic heavy metals (Pb and Cd) in the soil were evaluated by Atomic absorption spectrophotometer . The Pb and Cd contents were found to be 218 mg/kg and 2.15 mg/kg which are significantly higher than the control non contaminated soil as per Kabata-Pendias, 2011. The exceptional trait of heavy metal tolerance displayed by the fungal species indicated their potential as an effective agent for metal removal.

Key words: Dhapa, fungi, heavy metal, tolerance index, minimum inhibitory concentration

INTRODUCTION

Bioremediation is defined as the utilization of microorganisms to reduce or eliminate environmental pollutants by metabolically mediating desired chemical reactions or physical processes.Bioremediation is an eco friendly and cost effective method as compared to conventional chemical and physical environmental pollutants by metabolically mediating desired chemical reactions or physical processes. Bioremediation is an eco friendly and cost effective method as compared to conventional chemical and physica techniques. Heavy metal contamination of the ecosystem is a major problem nowadays (Gavrilesca, 2004; Malik, 2004; Srivastava and Thakur, 2006). Due to their non biodegradable nature and high concentration as contaminants, heavy metals are very toxic to biological systems in their soluble forms and several of them have carcinogenic and mutagenic effects (Diels *et al*., 2002). In recent years excessive deposition of toxic heavy metals in the soil and water resources is caused by expansion of industrial activities and technological development. Microorganisms are reported to play an important role in bioremediation of heavy metals in contaminated soil and water ecosystem (Parameswari *et al*., 2010). Microorganisms can uptake metals either actively by bioaccumulation or passively by biosorption (Hussain *et al.,* 2003). Generally, the contaminated sites are the sources of metal tolerant micro-organisms. In naturally polluted environments, the microbes' response to heavy metals toxicity depends on certain factors such as the concentration, availability, type of metals, the nature of medium and microbial species

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Fungi and yeast biomasses are known to tolerate heavy metals (Gavrilesca, 2004). Fungi can adapt and grow under various extreme conditions of pH, temperature and nutrient availability, as well as high metal concentrations (Anand *et al.*, 2006). Fungi are reported to tolerate and detoxify metals by several mechanisms including valence transformation, extra and intracellular precipitation and active uptake. Several metal-tolerant filamentous fungi viz. *Rhizopus, Trichoderma, Aspergillus*, *Chaetomium*, *Penicillium*, *Fusarium*, and *Curvularia* have been isolated from multiple heavy metal contaminated soils (Iram *et al.*, 2009; Fazil *et al.*, 2015; Adegbite *et al.,* 2018).

The use of dumpsites as farm land is a common practice in urban and sub-urban locations (Ogunyemi *et al.*, 2003). These wastes often contain heavy metals such as As, Cd, Cu, Fe, Hg, Mn, Pb, Ni and Zn in various forms and at different contamination levels. Some of the heavy metals like As, Cd, Hg and Pb are particularly hazardous to plants, animals and humans. Plants grown in these contaminated agricultural field can absorb heavy metals in form of mobile ions present in the soil through their roots or through foliar absorption. These absorbed metals get bioacumulated in the roots, stems, fruits, grains and leaves of plants and thus can cause health hazards when consumed as vegetables.

In the present investigation soil was collected from the agricultural field adjacent to the waste disposal ground of Dhapa near Eastern bypass in Kolkata. This site was chosen for study as the soil in this field is infested with high concentration of heavy metals (Bhattacharyya *et al.*, 2017). Different kinds of crops and vegetables grown here are sold in urban vegetable markets of Kolkata. The objective of this study is to isolate fungal species from the soil of agricultural field of Dhapa.These fungi were further screened to study their ability to tolerate five heavy metals viz. Cr, Cd, Pb, Cu and Co, so that they can be used as potential organisms in mycoremediation process.

MATERIALS AND METHODS

Sampling of soil and sampling site

Agricultural field of maize near Dhapa dumping area have been chosen as sampling site (Fig.1). Dhapa dumping area is located on the fringes of the East Kolkata Wetlands. This area is owned by Kolkata Municipal Corporation (KMC) and lies within Ward Nos. 57 and 58 of the KMC administrative boundaries. Soil was collected in ziplock pouches from 15-30cm depth of the rhizosphere region and stored at 4°C for further study.

Fig 1. Sampling site: Maize field adjoining to Dhapa dumping site.

Determination of physical and chemical property of soil

The pH and moisture content of the soil sample was measured. The pH of soil and water (1:5) mixture was determined using a pH meter. The soil sample was dried at 80°C in hot air oven and moisture content was determined. The total concentration of heavy metals Pb and Cd were estimated by digestion of soil sample. About 10g of soil was taken in a conical flask and to it 15 ml conc. HNO_3 and 35ml distilled water were added. The mixture was boiled for 30 mins. Then again conc. HNO_3 and distilled water were added and the mixture was boiled till clear transparent solution was obtained. After this, the mixture was cooled and filtered in a volumetric flask by Whatman no.1 filter paper and the volume was made up to 100 ml by addition of distilled water. The metal concentrations were determined by Atomic Absorption Spectrophotometric analysis (AAS - Thermo SCIENTIFIC iCE 3000 Series).

Isolation of fungal strains

Serially diluted soil samples were added on potato dextrose agar or PDA (potato : 400g, dextrose : 20g, distilled water : 1l, agar powder : 20g) plates, amended with streptomycin (Himedia) and incubated for 4 to 6 days at 30 ± 2°C. Morphologically distinct fungal colonies appeared on the plates and were isolated in pure form. The pure cultures were maintained on PDA slants and subcultured at regular intervals.

Identification of fungi

The fungi were cultured in Czapek dox's medium (sucrose : 30g, sodium nitrate : 2g , Dipotassium phosphate : 1g, potassium chloride : 0.5g, magnesium sulphate : 0.5g, ferrous sulphate : .01g, distillted water : 1l, agar powder : 15g) for the purpose of identification. Fungal cultures were identified from their pure culture on the basis of both macroscopic (characteristics visible by naked eyes i.e. colony morphology, colour and appearance of colony, shape) and microscopic (septation of mycelium, shape, diameter and texture of conidia, presence of specific reproductive structure or sterile mycelia) characteristics. Identification was done by using the manuals of Gilman (1967) , Domsch *et al.* (1980) and Watanabe (2010).

Determination of tolerance index (TI) of fungi against heavy metals

Fungal isolates were screened on the basis of their tolerance to chromium (Cr^{6+}), lead (Pb^{2+}), Cobalt (Co^{2+}), cadmium (Cd^{2+}) and copper (Cu^{2+}). The most tolerant strains were tested for estimation of Tolerance index (TI). A mycelial disc (5mm) from young fungal colonies was inoculated aseptically on PDA plates supplemented individually with 1mM of heavy metal salts. The metal salts used were potassium chromate [K_2CrO_4], lead nitrate [$Pb(NO_3)_2$], cadmium chloride [$CdCl_2$], cobalt chloride [$CoCl_2$] and copper sulphate [$CuSO_4$]. Control plates without heavy metals were also inoculated with mycelial discs. The inoculated plates were incubated at 30 ± 2°C for at least 12 days. The effect of the heavy metal on the growth of the isolates and tolerance index was estimated by measuring the radial mycelial growth (mm) against the control. The tolerance index was calculated as the ratio of the radial mycelia growths of the treated sets to that of the untreated control sets (Joo and Hussein, 2012). The tolerance index value was rated as: 0.00 – 0.39 (very low tolerance), 0.40 – 0.59 (low tolerance), 0.60 – 0.79 (moderate tolerance), 0.80 – 0.99 (high tolerance) and 1.00 – >1.00 (very high tolerance) as per Oladipo *et al* (2018).

Determination of Minimum Inhibitory Concentrations (MICs)

Minimum Inhibitory Concentration (MIC) was determined by observing the tolerance of fungal isolates to different concentrations of heavy metals (Joo and Hussein, 2012). Potato Dextrose Agar media supplemented with different concentrations of heavy metal was used separately for inoculation of agar mycelial discs. Three replicates were used for each concentration of metal, as well as for control (without metal salt). All the treated and control Petri plates were incubated for 7 days at 30 ± 2°C. After the incubation period the radial mycelia growth was determined and the minimum inhibitory concentration (MIC) was determined. MIC is defined as the lowest concentration of metal that inhibits visible growth of the isolate.

Statistical analysis

Statistical analysis of data was done by student's t test at 5 % level of significance using SPSS -20 statistical software.

RESULTS AND DISCUSSION

The pH and moisture content of soil sample were measured as 7.2 and 25 % respectively. Atomic absorption spectrophotometric analysis was carried out to measure the concentrations of Pb and Cd in the soil. The concentrations of Pb and Cd in the acidic soil solution of the Dhapa agricultural field were estimated as 217.85 mg kg^{-1} and 2.15 mg kg^{-1} respectively. The Pb and Cd concentrations in the soil were found to be within the range of maximum allowable concentration (MAC) i.e. 50-300 mg kg^{-1} and 1-5 mg kg^{-1} as per Kabata-Pendias (2011). However the values are significantly higher than the reported standard concentrations of Pb and Cd in control non contaminated soil (27 mg kg^{-1} and 0.4 mg kg^{-1} as per Kabata-Pendias, 2011).

Isolation and Identification of fungal strains

Eleven fungal isolates were isolated from the soil sample and out of them nine fungal isolates were identified. Five out of nine isolated fungi belonged to the genera *Aspergillus* sp. and two belonged to genera *Penicillium* sp. and *Chaetomium* sp. each. All the isolates were screened for metal tolerance. Out of the isolates two most tolerant species of *Aspergillus* and *Chaetomium* (Fig. 2) were chosen for performing further study.

It has been reported earlier that heavy metal contamination resulted in decrease in microbial diversity in the soil and water . This is due to decrease in number of metal sensitive species and enhanced growth of metal tolerant/ resistant species (Almås *et al.*, 2004).

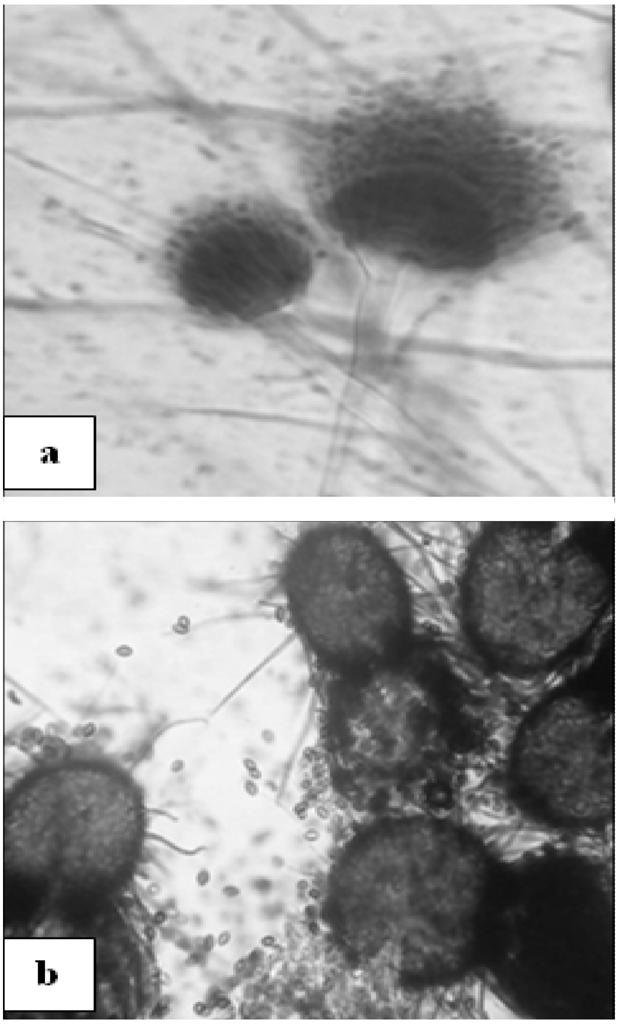

Fig 2. Microscopic photographs of fungal isolates. a. *Aspergillus* sp.; b. *Chaetomium* sp.

Tolerance index of fungi against heavy metals
Tolerance of the tested fungal strains against the heavy metal concentration (1mM) was evaluated by calculating their tolerance index relative to the control (Table 1). The fungal strains exhibited retarded (T<1), similar (T=1) or enhanced (T>1) growth in presence of heavy metal relative to control. Both *Aspergillus* sp. and *Chaetomium* sp. showed highest tolerance index against Pb (TI >/ =1). Very high tolerance was also exhibited by *Aspergillus* sp. against Co^{+2} (TI=1). Tolerance of *Aspergillus* and *Chaetomium* to 1mM Cd^{+2} was found to be least with very low tolerance index of 0.27 and 0.38, respectively. Moderate tolerance was exhibited by *Aspergillus* against Cu (TI=0.75) and Cr (TI=0.70

Table 1: Tolerance index of *Aspergillus* and *Chaetomium* isolates in heavy metal rich media

Name of fungal species	Tolerance index of heavy metals +				
	Cr	Cd	Pb	Cu	Co
Aspergillus	0.7 ± 0.01*	0.27 ± 0.05	1.0 ± 0.03*	0.72 ± 0.05*	0.95 ± 0.03*
Chaetomium	0.6 ± 0.03*	0.38 ± 0.03	1.1 ± 0.02*	0.42 ± 0.04*	0.75 ± 0.04*

+Average of three replicates ; *P < 0.05

On the other hand *Chaetomium* showed moderate tolerance against Co (TI=0.72) and Cr (TI=0.61) and low tolerance against Cu (TI=0.42). The tolerance of *Aspergillus* in different heavy metals is represented in the following order Pb>Co>Cu>Cr>Cd. On the other hand, the order of different heavy metal tolerance of *Chaetomium* sp. is as follows Pb>Co>Cr>Cu>Cd (Fig. 3 & 4). Both the species showed highest adaptation to Pb, where as Cd was observed to be the most toxic metal as it showed strongest inhibition effect on the growth rate of the fungal strains. Results also suggested that tolerance index of *Aspergillus* for Pb, Co, Cu and Cr was significantly higher ($p < 0.05$) than *Chaetomium.* Similar observations on isolation of resistant/tolerant fungal strains from heavy metal contaminated soil has been reported earlier. Ezzouhri *et al.* (2009) isolated thirty-six micro-organisms, represented by fungi and yeasts strains from heavy metal-contaminated sites in Tangier, Morocco. Filamentous fungi isolated belonged to the genera *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Geotrichum*. They were screened for their resistance to heavy metals. Results revealed that the majority of the isolates were resistant to Pb, Cr, Cu and Zn, whereas to Cd only the fungus *Penicillium* sp. was tolerant. The level of resistance depended on the isolate tested, as well as the site of its isolation. Filamentous fungal species were isolated from gold and gemstone mine site soils in south western Nigeria and were assessed for their tolerance to varied heavy metal concentrations by Oladipo *et al.* (2018).

Fungal strains were identified as *Fomitopsis meliae*, *Trichoderma ghanense* and *Rhizopus microsporus*. All test fungal species exhibited tolerance to Cu, Pb, and Fe at all test concentrations (400–1000 mgkg^{-1}) with tolerance index >1. Akhtar *et al*. (2013) isolated nineteen fungal strains from soils irrigated with untreated municipal/industrial effluent in peri-urban areas of Gujranwala and Sialkot in Pakistan using dilution technique. The isolated fungal strains were screened for metal tolerance index at 1 mM cadmium (Cd), nickel (Ni) and copper (Cu) concentrations. The isolated fungi belonged to the genera *Aspergillus* , *Curvularia*, *Acrimonium* and *Pythium*. The results revealed that the order of tolerance of isolates for metals was Cd> Cu > Ni and *Aspergillus* sp. isolates were more tolerant than other fungi.

400 respectively. The MIC values also indicated that Cd exerted significantly more toxic effect on growth of the fungal species than Pb. The growth patterns in heavy metal rich media revealed that *Aspergillus* was significantly more tolerant to the heavy metals than *Chaetomium* at higher concentrations (Fig. 5 a & b). The high adaptability of the species towards Pb may be due to its role in fungal metabolism (Joo and Hussein, 2012).

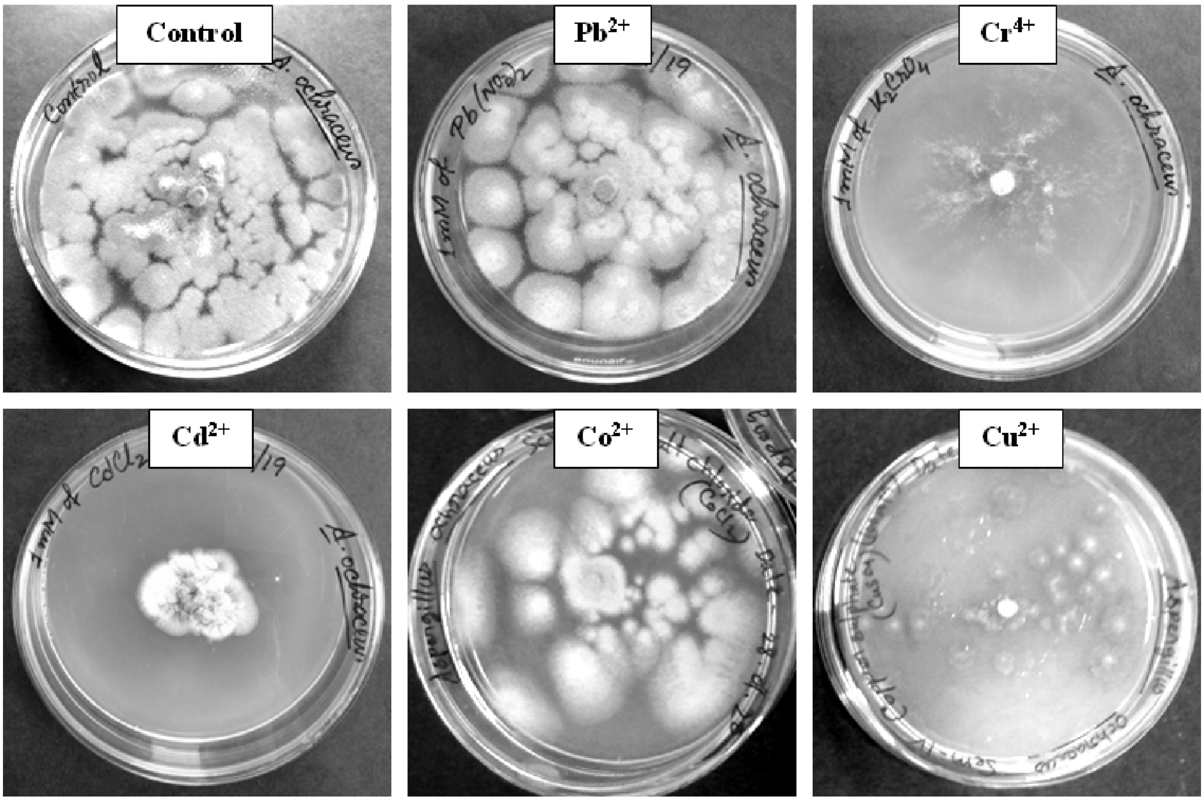

Fig 3. Tolerance index of *Aspergillus* sp. in heavy metal rich media.

Determination of MIC

The MIC values of the most adapted and toxic metals Pb and Cd against the fungal isolates were determined. Variations in the growth pattern of fungi were observed in different concentrations of heavy metals. At lower metal ions concentrations, the tested fungal isolates were very tolerant and exhibited strong growth. Where as, at higher metal ion concentration significant reduction in growth was observed. The MIC values of both *Aspergillus* and *Chaetomium* were found to be same for both the metal ions. The MIC values of Pb and Cd were found to be in the range of 2000-2200 and 200-

Soil in the agricultural field adjoining to waste disposal site is contaminated with toxic non biodegradable heavy metals mainly by waste water irrigation. Plants grown in these contaminated soils absorb heavy metals which accumulate in the roots, stems, leaves, flowers and grains and thus get translocated and biomagnified through the food chain. So consumption of vegetables and fruits grown in these contaminated soils may cause serious health hazards. In the present investigation Dhapa was chosen as the sampling site. The soil of the agricultural field adjoining to Dhapa dumping site has been reported earlier to be rich in heavy metals due to continuous disposal of waste water.

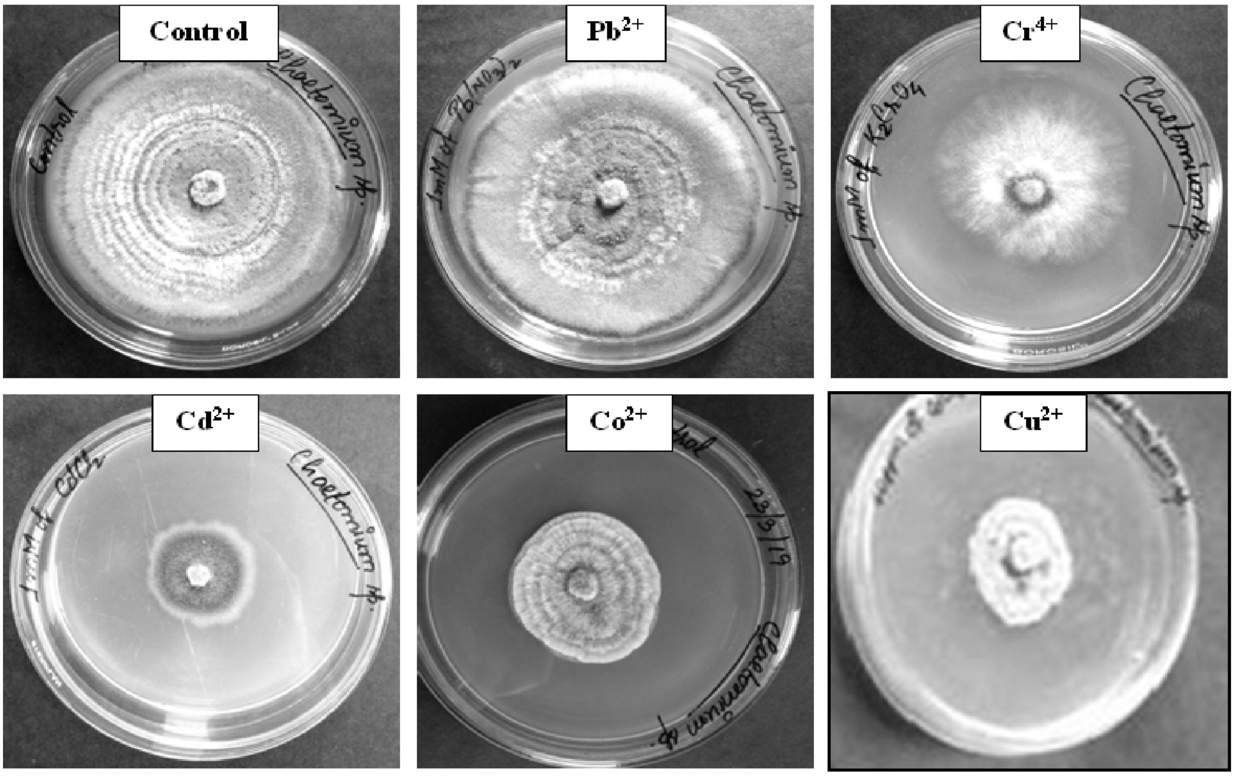

Fig 4. Tolerance index of *Chaetomium* sp. in metal rich medium

A survey based study was conducted at Dhapa by Bhattacharyya *et al.* (2017).

Random GPS based soil sampling was carried out to collect soil, water and plant samples from the whole Dhapa area. It was observed that among all the heavy metals (Zn, Cu, Fe, Mn, Pb, Cd, Ni) estimated, the highest mean concentration in the soil was found in case of Pb, followed by Fe > Cu > Mn and least concentration was found in case of Ni in three depths respectively. The heavy metal concentrations (Pb, Zn, Ni and Cd) in the vegetables grown in Dhapa were found to be above the permissible level. In the present investigation both the isolates of *Aspergillus* and *Chaetomium* showed highest tolerance to Pb and moderate tolerance to Cr. *Aspergillus* was more tolerant to Cu than *Chaetomium* and Cd was the most toxic metal for both the species. The high tolerance index and MIC values of Pb of both the fungal isolates showed that these fungi are resistant to Pb and thus can be used for metal removal. The high concentration of Pb in the soil may be responsible for better adaptatibility of these species in the contaminated soil. These results are in conformity with earlier findings. Bala *et al.* (2020) isolated metal tolerant fungi from refuse dumpsite soil in Nigeria using pour plate method. These fungi were identified as *Aspergillus niger*, *Penicillium chrysogenum* and *Rhizomucor* sp. The fungal isolates were screened for Cd, Pb and Zn. *Penicillium chrysogenum* showed higher Pb removal or biosorption potential than *Aspergillus niger*. Opaluwa *et al.* (2012) assessed the pollution status of the farm lands around the dumpsites in Lafia metropolis of Nasarawa state in Nigeria. The level of heavy metals (As, Cd, Co, Cu, Fe, Ni, Pb and Zn) in soils, plant leaves and crops from farmlands around dumpsites were determined using digestion and Atomic Absorption Spectrophotometer methods. The values of all the metals analyzed for samples from dumpsites were higher than those from the control site suggesting possible mobility of metals from dumpsites to farmlands through leaching and runoffs. Tolerance level of different fungi isolated from contaminated peri-urban agricultural soils of Faisalabad towards heavy metals was studied by Iram *et al.* (2012). Among all the tested fungal strains, few isolates of *Aspergillus flavus*, *Aspergillus niger* and *Fusarium* were tolerant to Cr and Pb. Lin *et al.* (2020) analyzed the fungal isolates from heavy metal

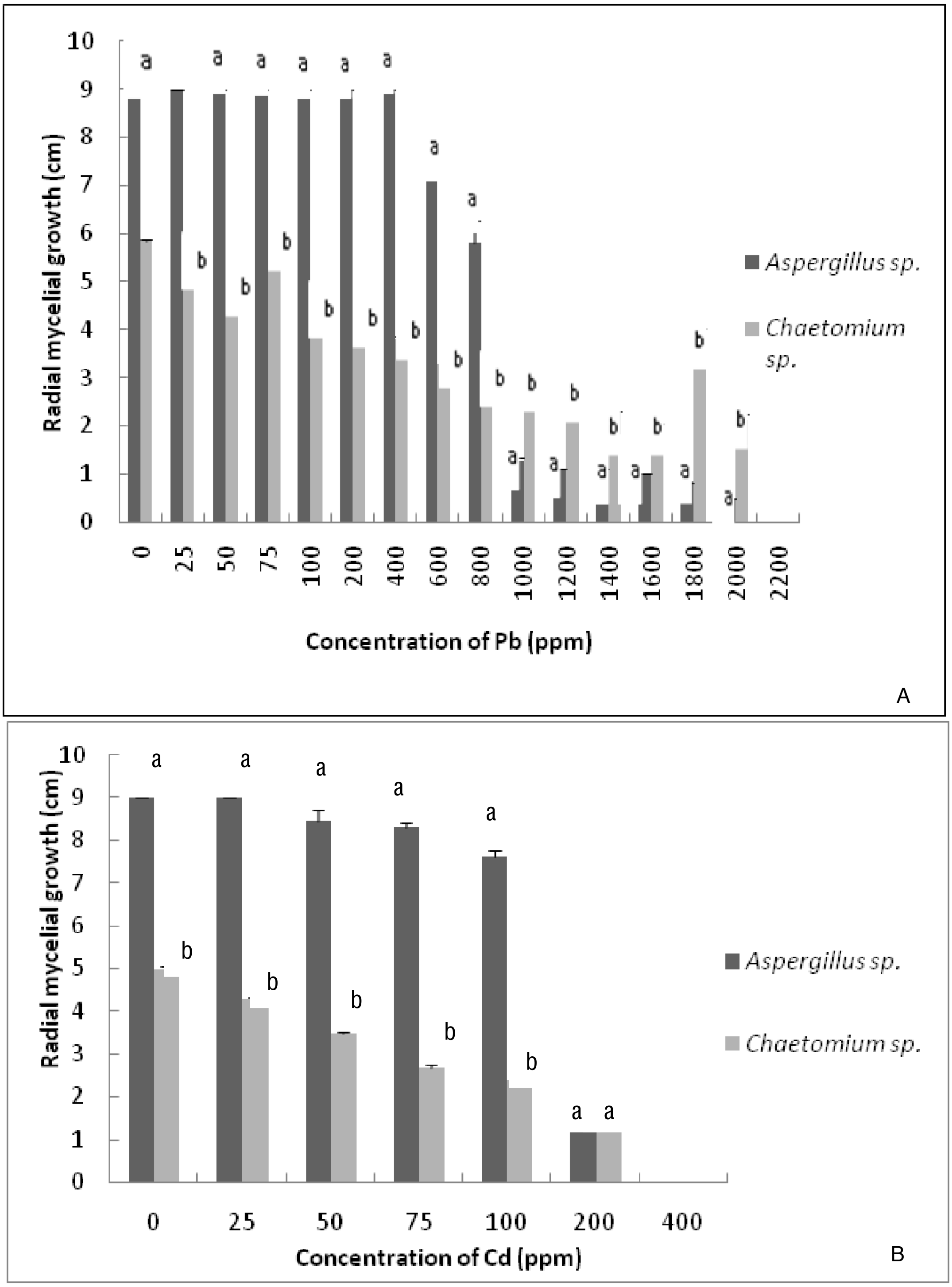

Fig 5. (a & b). Effect of varied concentrations of heavy metals Pb (a) and Cd (b) on radial growth of fungi. The bars showing growth of different fungal species in each concentration with different letters are significantly different ($p<0.05$).

contamination sites in paddy field of eastern China to identify species that have strong adaptability to heavy metals. The most dominant genera of tolerant fungi were observed as *Aspergillus*, *Penicillium*, and *Fusarium*. These isolates had strong biodegradability on heavy metals and as a result they can reduce metal toxicity to create a proper soil environment to grow food crops.

CONCLUSION

In the present investigation two Indigenous filamentous fungal isolates from agricultural field near garbage dump site of Dhapa, exhibited remarkable growth and tolerance in heavy metal-rich media. Among the five heavy metals studied, the highest level of tolerance was displayed by

Aspergillus sp. and *Chaetomium* sp. to elevated concentration of Pb. This exceptional trait of metal tolerance may indicate their bioremediative potentials. Biological treatment based on the use of these fungal isolates is not only cost effective, but also a more eco friendly alternative technique to existing conventional chemical methods for metal removal.

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