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A HIGHLY HEAVY METAL TOLERANT *Fusarium solani* WITH EFFICIENT BIOACCUMULATION POTENTIALITY FROM CONTAMINATED SOIL

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ABSTRACT

Bioremediation of toxic metal contaminated soil and wastewater by filamentous soil fungi is a promising approach. In the present study, tolerance and bioaccumulation ability of *Fusarium solani* were tested for four heavy metals viz. cadmium (Cd), lead (Pb), nickel (Ni) and chromium (Cr). The fungus, isolated from textile-dye contaminated soil, was cultured on potato dextrose agar (PDA) and potato dextrose broth (PDB) supplemented with different metals to investigate metal tolerance and removal efficiency respectively. After 10 days of fungal growth in metal containing PDB, the remaining metal amount was evaluated by atomic absorption spectrophotometer (AAS). Minimum inhibitory concentrations of heavy metals up to which the isolate could grow were 2100, 3600, 2700 and 3900 ppm for Cd, Pb, Ni and Cr respectively. The highest tolerance index was recorded against Cr (1.07), followed by Pb (1.02), Ni (0.77) and Cd (0.66). Up to certain concentration, both Cr and Pb showed stimulatory effect on the growth of the isolate. Maximum bioaccumulation was observed for Cr (86.5%) followed by Pb (85.5%) > Ni (75.3%) > Cd (68.6%). The fungal isolate showed maximum growth, tolerance and bioaccumulation at pH 6 and 28°C temperature. FTIR spectra of dried biomass grown with or without heavy metals showed stretching vibration at 4000 – 400 cm⁻¹ which revealed the interaction between heavy metals and functional groups of biomass. Thus, it can be concluded that this fungal isolate has a potential of being a suitable for efficient heavy metal bioaccumulation and bioremediation of polluted soil and waste water.

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1 Introduction

Environmental pollution is a global challenge and among various types of pollution, heavy metals are one of the most hazardous pollutants (Xiezhi et al., 2005). Discharge of these heavy metals into soil and water is a foremost health concern worldwide, which has long-term harmful effects on the ecosystem, since they cannot be broken down to non-toxic forms (Singh et al., 2011). Even at very low concentrations many of them show toxic effects. Cadmium, arsenic, lead, chromium, nickel, copper, mercury, selenium, zinc, silver etc. are cytotoxic, mutagenic and carcinogenic in nature when they enter in the food chain (Salem et al., 2000). Presence of these toxic materials in soil and water affects both flora and fauna, in addition to pollution of groundwater through leaching. Further, contaminated soil can lead to reduction of plant growth, performance and product quality in agriculture. It affects the microbial activity in the ecosystem. It is also harmful to public health and other organisms due to environmental stress (Khosravi et al., 2009; Mohsenzade et al., 2012). Heavy metals are discharged into the environment by sewage and waste materials of paint industry, metal plating, batteries, textile dyeing industry, metallurgy, pesticides, combustion of fossil fuels, released oil ingredients in the soil, ores washing, mining, natural erosion of rocks, coloured material etc. (Vadkertiova & Slavikova, 2006). Bioremediation is an eco-friendly and encouraging technology which helps in heavy metal removal from polluted water and soil. For survival in heavy metal polluted soil, microorganisms improve and adopt various decontaminating mechanisms such as bioaccumulation, biosorption and biotransformation. These organisms can be exploited either *ex situ* or *in situ* for bioremediation (Gadd, 2000; Lim et al., 2003; Malik, 2004; Lin & Lin, 2005). Worldwide bioremediation technologies were surveyed by Elekwachi et al. (2014) for addressing the environmental problems.

Generally, for heavy metal tolerance and bioremediation in filamentous fungi two mechanisms i.e. extracellular and intracellular sequestration have been proposed. In extracellular mechanism (biosorption), heavy metals bind to the fungal cell wall without entry into the cell. The fungal cell wall is made up of glucan and chitin, which are negatively charged containing various anionic structures (Maghsoodi et al., 2007). Thus fungi cell wall could bind with heavy cations metals. In the intracellular mechanism, toxic metal ions are either extruded from the cytosol, out of the cell or by metal sequestration into vacuolar compartment by transport proteins (Tawab Abdul & Maqsood, 2007). In this study, the tolerance of fungal strain *Fusarium solani* for heavy metals Cd, Pb, Ni and Cr was studied. The main objective of the work was to investigate the effect of metal concentration, pH and temperature, at which optimum metal tolerance was found. Further, tolerance index, minimum inhibitory concentration (MIC) and removal percentage of heavy metals were also investigated.

2 Materials and Methods

2.1 Heavy metal stock solutions preparation

Stock solutions of heavy metals with 10000 ppm concentration Cd(II), Pb(II), Ni(II) and Cr(III) were prepared by dissolving analytical reagent grade salts of CdCl₂ · H₂O, Pb(NO₃)₂, NiCl₂·6H₂O, Cr(NO₃)₃ in milli-Q water. From the metal stock solution, further concentrations of each metal were prepared.

2.2 Soil sample collection

Soil samples were collected from polluted soil to a depth of 15 cm from dumping areas contaminated with effluent of textile dyeing factory at Santipur, Nadia District, West Bengal. Soil was collected in sterilized plastic bag, transported to the laboratory in icebox and stored in refrigerator in 4°C for further work within 12 h.

2.3 Isolation of the Fungal isolate

Soil sample (1g) was suspended in 10ml of sterile distilled water and further dilutions were made. To isolate *F. solani*, soil dilutions of 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ were used to evade over-crowded fungal colonies. Soil suspension of 100µl of each concentration was added to sterile petri plates containing 30 ppm streptomycin, 20ml of sterile Potato Dextrose Agar (PDA) medium. Three replications were maintained for each treatment. The culture plates were incubated at 28±1°C in the dark and monitored everyday up to 7 days. Each developed fungal colony was sub-cultured to obtain pure cultures (Ezzouhri et al., 2009; Kumar et al., 2015).

2.4 Identification of the Fungal isolate

Isolated *F. solani* species identification was carried out by the method of Booth (1977) and Smith (2007) based on colony characteristic on PDA (colonial morphology, color, appearance) and microscopic characteristics (septation of mycelium, shape diameter and texture of reproductive structure i.e. conidia).

Molecular identification of the fungal isolate was done by sequencing of 18S rDNA. Genomic DNA was isolated by CTAB method (Datta & Chaudhuri, 2017). For rDNA amplification ITS (ITS4, ITS5)) Forward: 5'-TCCTCCGCTTATTGATATGC-3' and Reverse 5'-GAAGTAAAAGTCGTAACAAGG-3' primers (Schoch et al., 2012) were used. To perform PCR, a total reaction volume of 50µl was used which contained 0.25 mM of each dNTP, 1.5 mM MgCl₂, 1x Taq buffer, 1.5 U of Taq DNA Polymerase, 0.2 µM of each primer, and 200 ng of template DNA. Condition for amplification of the PCR cycling was 95°C for 10 minute; 35 cycles at 95°C for 15 second, 52°C for 30 second, and at 72°C for 1.5 second; 72°C for 7 minute and hold at 4°C (Schoch et al., 2012).

2.5 Minimum inhibitory concentration (MIC)

Potato dextrose agar (PDA) culture medium was supplemented with increasing heavy metal concentrations viz. 200, 400, 600, 800, 1000, 1500, 2500, 3000, 3500 and 4000 ppm. The culture plates were inoculated with 5mm inoculum disks from pure young cultures. Fungal growth on metal containing culture plates were observed after 10 days of inoculation. The lowest concentration of heavy metal which prevented visible fungal growth was considered as minimum inhibitory concentration (Fazli et al., 2015).

2.6 Tolerance Index of Fungi

The tolerance index (TI) was defined as the division of fungal growth in heavy metal containing plates with fungal growth in without metal plates i.e. control plates. The higher TI value indicated greater resistance. Five mm disks of fungal isolate from 10 day old pure cultures were inoculated into petri plates containing PDA supplemented with 200 ppm of Cd, Pb, Ni and Cr, separately, a combination of two metals of 100 ppm each as well as in mixture of all four metals (4x50 ppm). Parallel cultures, without metal were maintained as a control. Three replications were maintained for each treatment. The inoculated plates were then incubated at 28±1°C for 7 days. The radial growth of the fungal mycelia on culture plate from four measurements (in millimetres) that passed through the centre was evaluated. The mean of evaluated diameter measurements for each plate on the 7th day was recorded.

2.7 Bioaccumulation of heavy metals

For the determination of bioaccumulation ability of the fungal isolate, three 5 mm inoculums disks from 10day old mycelia culture, was inoculated into 150 ml conical flask containing 50 ml potato dextrose broth (PDB) and 20, 30, 40 and 50 ppm of each heavy metal, in separate conical flasks. Initial concentration of heavy metals in each conical flask was tested by Atomic Absorption Spectrophotometer (AAS) before fungal inoculation. The pH was adjusted to 6 at which the optimum fungal growth was found. In controls PDB medium with 20 ppm of each metal in conical flasks, without fungal inoculum were maintained. All the flasks were incubated on a rotary shaker at 28±1°C in dark. After 10 days of incubation, the flasks containing fungal mycelia were filtered through filter paper (Whatman No.42). For determination of total metal concentration, the filtrate was subjected to acid digestion with HNO₃ and HCl in 1:3 ratio, on a hot plate, until the red nitrous fumes production stopped and the liquid becomes colorless. The cooled liquid was diluted with milli-Q water and filtered through Whatman's No.1 filter paper (Uddin et al., 2016). Heavy metal content was analyzed using AAS (Juwarkar, 1988). The residual fungal mycelia were rinsed three times with milli-Q water and dried in hot air oven at 70°C until the weight became

constant. The dried fungal biomass was defined as dry biomass and weighed (g). The percentage of heavy metal uptake by the fungal mycelia was calculated by using the following equation (Mohsenzadeh & Shahrokhi, 2014; de Lima et al., 2013)

$$q = \frac{(C_i - C_f)}{C_i} \times 100$$

where, q is the metal uptake percentage (%); C_i (ppm) is the initial metal concentration; C_f (ppm) is the final metal concentration.

The effect of bioaccumulation of heavy metals at 20ppm of each metal by *F.solani* at different pH and temperature was also studied.

2.8 FTIR analysis

To analyse the functional groups on the dried fungal biomass surface before and after metal biosorption, Fourier transform infrared spectra (FTIR) method was used. The dried biomass was treated with KBr pellets; spectra were recorded at the range of 4,000–400 cm⁻¹ using a KBr window with a spectrometer. In order to find out the differences between the biomass treated with metals and biomass without metal were also analysed with FTIR.

3 Results and Discussion

Based on colonial morphology, appearance and color of fungal mycelia on culture plate and microscopic characteristics viz. mycelia septation, size, shape, diameter, texture and septation of reproductive structure i.e. conidia and also on 18S rDNA sequencing, the fungal isolate was identified as *F.solani*. The 18S rDNA fragment was submitted to the NCBI gene bank and the accession number was MK829000.

3.1 Minimum inhibitory concentration (MIC)

It was found that the resistance level of *F.solani* was different for the different tested metals (**Figure 1**). The order of concentrations of heavy metals up to which mycelia of *F. solani* grown was Cr (3900 ppm) > Pb (3600 ppm) > Ni (2700 ppm). These results revealed that *F. solani* showed maximum resistant to Chromium; this higher MIC value indicated more resistance to that metal. Cadmium showed highest toxic effect among all four metals on cellular metabolic activity which directly affected mycelial growth as a result it showed lowest MIC value. The MIC values of the fungus evaluated in the present study are higher than those reported by Parameswari et al. (2010) and Zafar et al. (2007). However, MIC values reported by Ahmad et al. (2005) are in agreement with the findings of present study.

3.2 Tolerance Index of Fungi

Heavy metal tolerance index of *F.solani* represented by **Figure 2**. Tolerance index of *F.solani* in heavy metals (both in single and

multi-metal culture) ranged from 0.57 to 1.07 at 200 ppm concentration. Higher TI value indicates higher resistance to the particular metal ion. In single metal culture, Cr containing plates showed maximum tolerance (1.07), followed by Pb (1.02), Ni (0.77) and Cd (0.66). In multi-metal cultures, it showed greater growth than on control plate and the T.I. value was 1.07. Akhtar et al. (2013) also reported that *Aspergillus flavus* (SF-4) showed ≥ 1 T.I value for Ni and Cu. In bi-metal culture Cd + Cr (100ppm +100ppm) containing culture plate showed highest growth i.e. maximum tolerance (0.82) followed by Cd + Pb (0.76) and Cd + Ni (0.57). *F.solani* showed not only higher tolerance to Pb and Cr but its growth was stimulated in presence of these two (Figure 3). According to Roane & Pepper (2000) the distinction in degree of resistance to different metals was undoubtedly due to the potential variation in the resistance mechanism. It was reported by Perfus-Barbeoch et al. (2002) that Cd inhibition to physiological processes such as growth and photosynthesis at less than 2 ppm concentration had also been observed in microorganisms.

3.3 Bioaccumulation of Heavy Metals

In the present study *F.solani* showed a wide range of diversity in heavy metals bioaccumulation at different concentrations (Figure 4). It was observed that with increasing of heavy metals concentration, the bioaccumulation percentage decreased. At 20 ppm of heavy metals, the isolate showed highest accumulation of Cr (86.5%) followed by Pb (85.5%), Ni (75.3%) and Cd (68.6%). Bioaccumulation percentages of heavy metals were significantly decreased with increase of metal concentrations (Table 1). In Cr treatment, weight of fungal biomass was greater than the biomass in untreated PDB after 10 days of incubation period. While lower concentrations of Cr stimulated the fungal growth, its increased concentration resulted in increased toxicity.

Khurshid et al. (2016) reported that *F. oxysporum* showed 80- 90 % bioaccumulation of Cr (IV) at the concentration of 5 – 350ppm. At higher metal concentrations bioaccumulation efficiency decreased due to saturation of ligands on the fungal cell wall (Rao et al., 2005). Functional groups in fungal cell wall involved in the binding of heavy metals are carboxyl, hydroxyl and phosphate which have high covalent affinity towards metal ions.

Figure 1 Minimum inhibitory concentrations (MIC) of cadmium, lead, nickel and chromium. Each data is mean of 3 replicates. The line on each bar represent standard deviation. MIC of different metals differ significantly ($P < 0.05$).

Figure 2 Tolerance Index of *Fusarium solani* at 200 ppm (single metal), 2×100ppm (bi- metals) and 4×50ppm (multi-metals). Data of each column is mean of $n = 3$. Bar line of each column indicates standard deviation. T.I of different metals or combination of metals were significantly different ($P < 0.05$) except Cd+Ni containing culture.

Figure 3 Effect of heavy metals on fungal growth and morphology. A. is control plate (without any metal); B, C, D and E (with Cd, Pb, Ni and Cr respectively); F, G and H (with Cd+Pb, Cd+Ni and Cd+Cr respectively); I (with Cd+Pb+Ni+Cr)

Table 1 Two way ANOVA test for calculation of significance differences in bioaccumulation potentiality by fungal mycelia.

Factors effecting bioaccumulation	Cadmium	Lead	Nickel	Chromium
	S	S	S	S
Metal concentrations				
20	-	-	-	-
30	ns	ns	ns	ns
40	ns	**	ns	*
50	*	**	*	**
pH value				
4	-	-	-	-
5	**	**	***	*
6	***	***	***	***
7	ns	*	***	ns
8	ns	ns	ns	ns
Temperature				
18	-	-	-	-
28	***	***	***	***
38	**	***	**	**

S = significance level; ns = non significant; *** = very high significant ($P < 0.001$); ** = high significant ($P < 0.01$); * = significant ($P < 0.05$).

Figure 4 Heavy metal bioaccumulation percentage of *Fusarium solani* from batch culture of different concentrations of metals at pH 6. Each data is mean of 3 replicates and the lines of the bars are standard deviation (significance level detail based on Table 1).

Figure 5 Bioaccumulation percentage of heavy metals at different pH by *Fusarium solani* from batch cultures containing 20 ppm of metal. The data are mean of n = 3 and the bar lines represent standard deviation (significance level detail based on Table 1).

Figure 6 Heavy metal uptake percentages at different temperature by *Fusarium solani* at pH 6 and 20 ppm metal concentrations from batch culture. Bar lines represent standard deviation which was calculated based on 3 replicates (significance level detail based on Table 1).

3.4 Effect of pH on Heavy Metals bioaccumulation

Fungal growth is dependent on the pH of the culture medium. Bioaccumulation of heavy metals by *F. solani* was maximum (Cd = 68.6 %, Pb = 85.5 %, Ni = 73.3 % and Cr = 86.5 %) at pH 6.0 while lower degree of heavy metal accumulation was observed at pH of 4.0 and 7.0-8.0 (**Figure 5**). Bioaccumulation at pH values lower and greater than 6 decreased significantly (**Table 1**).

At low pH, the surface ligands of fungal cell wall are closely bound with the H⁺ ions and thus prevent the metal cations to bind. The carboxylic groups of fungal cell wall do not dissociate at low pH and therefore the metal ions cannot bind (Choudhary & Sar, 2009). With increase in the pH, the negative charges increase the ligands which favour electrochemical attraction and as a result binding of heavy metal cations increases (Johncy et al., 2010). At high pH value heavy metals get precipitated causing very low biosorption (Pinoa et al., 2006).

3.5 Effect of Temperature on Heavy Metals bioaccumulation

Bioaccumulation of heavy metals observed in *F. solani* was optimum at 28°C for all four metals followed by 38°C and 18°C (**Figure 6**). At 28°C bioaccumulation of all four heavy metals were Cr (86.5%)>Pb (85.5 %) > Ni (75.3%) > Cd (68.6 %). Bioaccumulation of metal ions were increased significantly at 18°C to 28°C and decreased significantly at 28°C to 38°C temperature

(**Table 1**). Biosorption of heavy metals at the temperature within the range from 25 to 35°C affects only to a lesser extent (Veglio & Beolchini, 1997). At these temperatures, kinetic energy of the solute and surface activity increases as a result of which biosorption of heavy metals also increases (Sag & Kutsal, 2000). Higher temperature causes physical damage to the biosorbent and reduces biosorption capacity (Srivastava & Thakur, 2006). Room temperature provides optimum biosorption by a fungus (Vijayaraghavan & Yeoung, 2008).

3.6 FTIR spectra analysis of fungal biomass with and without heavy metal biosorption

FTIR spectroscopy was used to find out the characteristic functional groups responsible for biosorption of metal ions. The comparative study of the spectra was shown in the **figure 7**. FTIR spectra showed distinct peaks at the range of 4000 to 400 cm⁻¹. Stretching of the broad and strong bands of -OH and -NH were attributed at 3500 to 3000cm⁻¹. Stretching vibrations at 2900 attributed the C-H, at 1620 to 1590cm⁻¹ for C=O, and amide groups, at 1400 cm⁻¹ due to N-H of amine groups and at 1025cm⁻¹ attributed the C=O of carboxylic acids and alcohols, at 500 cm⁻¹ stretching of Co-O, similar findings were reported for different metals (Simonescu & Ferdeş, 2012). Biomass surface of *F. solani* contained hydroxyl, carboxyl and amine groups. The stretching vibrations at above values indicated the chemical interactions between hydroxyl groups of biomass and metals.

Figure 7 FTIR spectra analysis where A= control, B= fungus grown in cadmium, C= fungus grown in lead, D= fungus grown in nickel and E= fungus grown in chromium containing culture media

Conclusion

It was thus found that *F.solani* isolated from polluted soil was efficient in accumulating different toxic heavy metals. It could sequester heavy metals from the culture medium to a less toxic level. It could also tolerate very high concentration of heavy metals. Maximum metal tolerance was found for chromium. Among the various tested temperature and pH, highest bioaccumulation was reported at 28°C and at pH6. FTIR spectrum presents the stretching vibrations at the values ranged from 4000-400 cm⁻¹ indicated the chemical interactions between heavy metal ions and functional groups of biomass. This fungus needs to be exploited for bioremediation of heavy metals from contaminated soil and industrial effluents.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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