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IN-VITRO STUDY ON BIOACCUMULATION AND TOLERANCE OF HEAVY METALS BY ENDOPHYTIC FUNGI *Alternaria alternata* ISOLATED FROM *Cupressus torulosa* D.DON

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Abstract: Microorganisms play a significant role in bioremediation of heavy metal contaminated soil and wastewater. Microorganisms including fungi have been accounted to remove heavy metals from contaminated sites through bioaccumulation at low cost and in eco-friendly way. In this study, an attempt was, therefore, made to check a strain of *Alternaria alternata*, isolated from leaves of *Cupressus torulosa* D.Don located in Garhwal, Himalayan region, has been studied for tolerance to various heavy metals (copper, lead, silver and mercury) and for its capacities to uptake these metals. It was found that endophytic fungus is capable of surviving on high metal concentrations, apparently as a result of the natural selection of resistant cells. Fungal endophyte was identified as *Alternaria alternata* by morphotypic and genotypic study. *A. alternata* exhibited removal of varying amount of heavy metals with respect to Cu^{+2}, Pb^{+2}, Ag^{+}, Hg^{+2}. Tolerance ability of fungus to heavy metals was determined by cultivating on Potato dextrose agar at concentration ranging from 50-1400ppm. The tolerant growths were studied for removal of heavy metals from liquid media at concentration from 50-250ppm of Cu^{+2}, Pb^{+2}, Ag^{+}, Hg^{+2}. In this paper we discussed that *A. alternata* was most efficient in removal of varying amount of heavy metals with respect Cu^{+2}, Pb^{+2}, Ag^{+}, Hg^{+2} which showed the maximum uptake of lead of 80% and the least uptake of Ag of 63.1% at 50 µg/mL concentration. This indicated the potential of these fungi as bioaccumulation for removal of heavy metals from wastewater and industrial place effluents containing higher concentration of heavy metals which makes them attractive potential candidates as bioremediation agents. The results of this investigation could provide a basis for applying the endophytic fungi for an environmentally friendly and economically feasible decontamination of pollutants. Finally, the results indicated that *A. alternata* could be a prospective candidate for bioremediation processes.
Keywords: Bioaccumulation; bioremediation; Endophytic fungi; Heavy metals tolerance.
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INTRODUCTION

Human run toward industrialization and comfort life is a leap into environmental pollution and consequently deterioration of human health. Heavy metals constitute a noteworthy risk for the biological system and particularly human health. The environment is polluted by substantial metals from industrial wastewaters during metal processing as well as other contamination routes. For all intents and purposes, any industrial action using metals has a metal disposal problem (Das *et al.*, 2008). Nature of substantial metals is non-biodegradable and persevering; therefore, environmental compartments (soil and water body) are not able to decontaminate themselves from these harmful contaminations. Substantial metals can be characterized into essential metals such as copper, manganese, zinc, and iron, and nonessential metals such as cadmium, lead, mercury, and nickel (Grąz *et*

al., 2011). Heavy metal pollution is a most important apprehension, as it prompts danger, risk to human survival and disturbs ecological balance (WHO, 2007). Heavy metals are toxic to all groups of life form from microbial to plant and animal. Due to their smaller size and direct exposure to the environment, microorganisms are more influenced by toxic heavy metals (Patel *et al.*, 2007). Toxic heavy metals entering into the ecosystem prompts to geo-accumulation, bio-aggregation and bio-amplification) processes which have intense ecological and public health implications (Jarup, 2003).

As modern advancement train is not stoppable, struggle with heavy metal contamination requires novel remediation methods. Conventional treatment systems such as chemical precipitation, filtration, ion exchange, electrochemical treatment, layer advances, adsorption on activated carbon, evaporation, have failures which incorporate insufficient metal sequestration, high expenses, high reagents and/or vitality necessities, and generation of toxic sludge or other waste products that require transfer. Re-establishing metals in an effective and conservative technique has required the utilization of various choices in metal-separating methods. Microorganism uptake metals either bioaccumulation *i.e.* active metabolism-dependent forms, which may incorporate both transport into the cell and partitioning into intracellular components and/or biosorption *i.e*. the binding of metals to the biomass by procedures that do not require metabolic energy (Hussein *et al.*, 2003). Hussein *et al.*, (2004) reported that bioaccumulation of heavy metals by organisms has been successful to some extent .Bioaccumulation has pulled in as an optional technique to conventional process involved in toxic metal removal from mechanical squandered streams (Kiran *et al.*, 2005). Bioaccumulation can be defined as the uptake of toxicants by living cells. The toxicant can transport into the cell across the cell membrane and accumulate intracellular. The aggregated metals are detoxified through cell metabolic cycle (Raghavan and Sang, 2008). Bioremediation of overwhelming metals from aqueous solutions is a relatively new process that has been affirmed as a promising procedure in the removal of heavy metal pollution (Melgar *et al.*, 2007). The microbial population present in metal polluted environment have adjusted and gotten to be impervious to toxic concentration of heavy metals (Leung *et al.*, 2000). The aim of this study is analysed the heavy metals resistance pattern and the bioaccumulation potential to find out their efficiency to remove heavy metals from liquid media under laboratory conditions by using fungal endophytes.

EXPERIMENTAL

Strain used: The strain used in this study was an endophytic fungus *Alternaria alternata* which was morphotypically and genotypically characterized as A. alternate (Rajput *et al.* 2016) and isolated from adult and healthy needle of *Cupressus torulosa* D.Don (Family: Cupressaceae) from Pauri, Garhwal region.

Growth medium: The fungi were cultured in Potato Dextrose Agar (PDA) composed of 1 g/L, K_2HPO_4, 3 g/L $NaNO_3$, 0.5 g/L $MgSO_4$. $7H_2O$,0.5 *g/L* KCl, 0.01 *g/L* $FeSO_4.7H_2O$, 5 *g/L* yeast extract, 30 g/L sucrose, 15g/L agar and trace elements.

Heavy Metal tolerance by using solid agar media: To test the effect of different metal on varying concentration of heavy metal, the heavy metal of different concentration were added to the PDA media range varying from 50-1400ppm. Fungal isolates on normal PDA medium served as control. The metal ions treated plates were inoculated with 6mm agar plugs from young fungal colonies grown on normal PDA medium and were incubated at 27˚C for at least 7 days. Tolerance of fungal endophyte was studied by the determination of tolerance index and minimum inhibitory concentration (MIC).

Metal Tolerance Index: Fungal endophyte were checked for their copper (Cu^{+2}), lead (Pb^{+2}), mercury (Hg^{+2}) and silver (Ag^{+}) tolerance. PDA plates supplemented with 50-1400ppm of heavy metal were inoculated with fungal isolate. The inoculated plates were incubated at 27˚C for 7 days. The effect of each heavy metal on the growth of the isolates was

estimated individually by measuring the radius of fungal growth extension against the control (without metal). Metal Tolerance Index (Ti) was calculated as the ratio of the extended radius of the treated colony to that of the untreated colony (Akhtar *et al.*, 2013).

$$Ti=D_t/D_u$$

Where D_t is the radial extension (cm) of treated colony and D_u is the radial extension (cm) of untreated colony.

Determination of MICs (Minimum inhibitory Concentrations): To test the heavy metals resistance design, the heavy metals Cu^{+2}, Pb^{+2}, Ag^{+}, Hg^{+2} used as Copper sulphate, Lead acetate, Silver nitrate, Mercuric chloride respectively were added to PDA media at concentrations covering the range from 50-1400ppm. Plates were inoculated with fungus agar plug of 6mm and incubated at 27˚C for 7 days. The minimum inhibitory concentration of the isolate was determined as the lowest concentrations of metals that inhibit visible growth of the isolates. Streaking of fungal isolate on normal PDA medium served as control (normal growth) for comparison of various growth of fungal isolate on PDA medium at different concentration of heavy metals (Dwivedi *et al.,* 2012).

Bioaccumulation of heavy metals in Liquid media: Eight day old agar plug of *A. alternata* was inoculated into series of 250mL Erlenmeyer's flask contained 50ml of Potato dextrose broth added with different concentration of different heavy metals Cu^{+2}, Pb^{+2}, Ag^{+}, Hg^{+2} ranging from 50-250ppm. The inoculated media added with heavy metals were incubated on rotary shaker in 150rpm speed at 27°C for 7 days with control contained medium having heavy metal without fungal inoculation. Fungal growth was harvested after 7 days through filtration using Whatman's filter papers, then filtrate are centrifuged at 12,000 rpm for 20 min. The concentration of the heavy metals before and after development of organisms was determined by UV spectrometry (Greenberg *et al.,* 1985). At the same time, dry weight of the fungus was determined. Values are expressed as the averages of three determinations did in parallel. Bioaccumulation of the metal in the biomass was expressed as the amount expelled from solution containing the metal based on the following equation.

$$\text{Metal removal } (\%)=(C_0-C_t)/\ C_0\times100$$

where C_0 is metal initial concentration in the solution (µg/ml), C_t is metal concentration after incubation in the solution (µg/ml).The most tolerant fungal strain isolates at different heavy metals were evaluated for uptake of heavy metals in potato dextrose broth medium which contain different concentration of different heavy metals Cu^{+2}, Pb^{+2}, Ag^{+}, Hg^{+2}ranging from 50-1400ppm. The Potato dextrose broth each containing different concentration of one of that prepared heavy metals was dispensed in 50 ml lots to 150 ml conical flasks and sterilized at standard condition (15 lbs/psi) for 15 min. These flasks were inoculated with one agar plug of 7mm of fungal isolate and kept on shaker at 150 rpm at 27˚C for 7 days. Control flasks having only PD broth of each concentration of different heavy metals served as control.

Bioassay Procedure: The fungal isolate were bio assayed for their capacity to resist and grow in the presence of 50-250 µg/ml of the test heavy metal ions *in-vitro*. Eight day old spore suspension of *A. alternate* were inoculated into series of 250mL Erlenmeyer's flask contained 100mL of specific growth medium (PDB) supplemented with 50-250ppm, of each heavy metal. The medium contained heavy metals are incubated on rotary shaker in 150rpm speed at 27˚C for 7 days with control contained spore inoculated medium without contained heavy metal. After incubation the fungal matt was harvested from the aqueous growth medium by sieving through Whatman's filter paper and the filtrate was collected. Fungal matt was washed twice with distilled water to remove media and dried in an oven at 80˚C for 12 hr. The yield of dry mycelia biomass was obtained by subtracting the weight of the filter paper alone from the weight of the filter paper and the mycelia biomass and constant dry weight was taken (Jaeckel *et al.*, 2005).

RESULTS AND DISCUSSION

Analysis of Fungal Isolate for Tolerance to Heavy Metals: The impact of substantial metals on fungal development was evaluated

on the basis of mycelia diameter. The responses of the test isolate were found to vary when grown at the different concentrations of the test metals. The isolated fungal endophyte *A. alternata* was tolerant to Cu^{+2}, Pb^{+2}, Ag^{+}, Hg^{+2} at 50-1400ppm. Fungal isolate showed maximum growth at low concentration of heavy metals. Observation showed decrease in growth of isolates at higher concentration of heavy metal. Fungal isolates exhibited tolerance to Pb at 1200 mg/L, and it could tolerate to Ag at 800 mg/L (Figure 1). Similar observation was seen in their tolerance to Cu and Hg. This observation indicates that inhibition of growth of fungal isolates at higher concentration of heavy metals due to the various biological factors. Similar result has also been reported in Akhtar *et al.*, (2013). All the species of genus *Aspergillus, Pythyme* and *Curvularia* showed high metal tolerance. All the tested strains showed strong colony growth on Cu media at 30 m*g/L* in comparison to the control. The ability of microbial strains to grow in the presence of heavy metals would be helpful in the waste water treatment where microorganisms are directly involved in the decomposition of organic matter in biological process for waste water treatment, because often the inhibitory of heavy metal is a common phenomenon that occurs in the biological treatment of waste water and sewage (Filali *et al.*, 2000; Agrawal *et al.,* 2016).

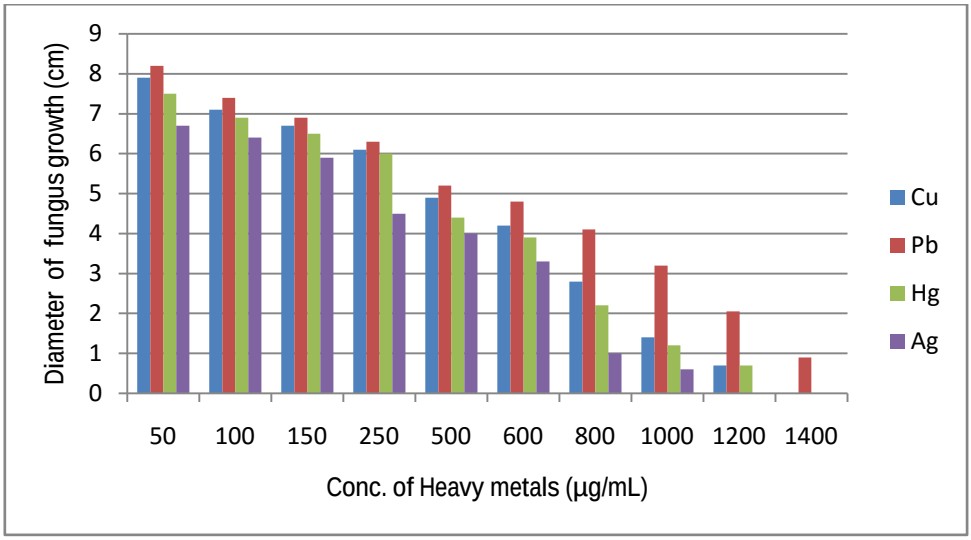

Figure 1. Effect of heavy metals concentrations on growth of Fungal strain

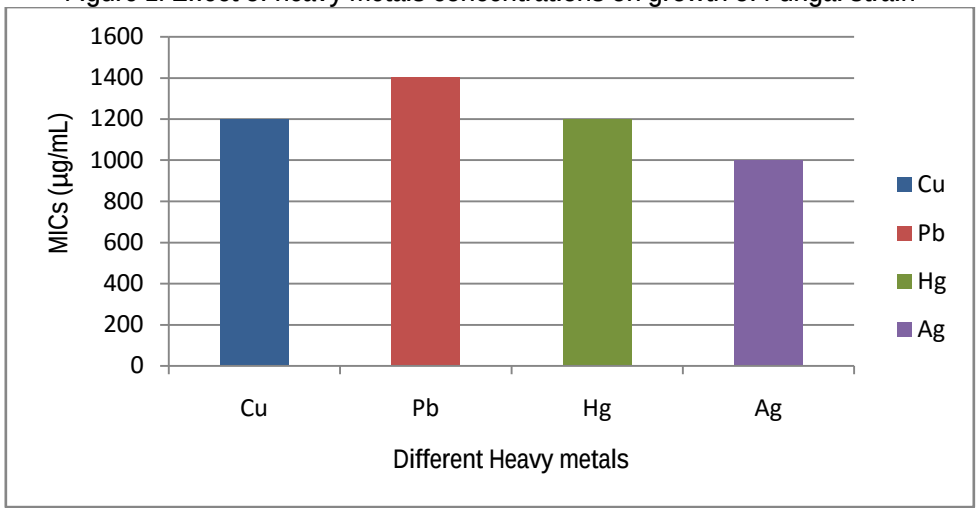

Figure 2. Minimal inhibitory concentrations (MICs) of Fungal isolate at different Heavy metals

Minimum Inhibitory Concentrations (MIC): The MICs of the Pb, Cu, Hg, and Ag against the fungal endophyte *A. alternata* have been studied In the presence of heavy metal relative to the control, the growth rate of the fungi exhibited a lag, retarded, similar, and enhanced

rates of growth. The development pattern appears to suggest tolerance development or adaptation of the fungi to the presence of heavy metals. At lower metal ions concentrations, the fungal isolates were very resistant and exhibited strong growth. Higher metal ion concentrations caused a reduction in growth and increased the length of the lag phase compared to the control. A decrease in the development rate is a typical response of fungi to toxicants, whereas the lengthening of the lag phase is not always present. The same reduction or change in growth pattern was observed during study on fungi, which belonged to the genera *A. alternata*, and were more resistant to Pb at higher metal concentrations, and suddenly the growth pattern changed as concentration of heavy metal increases. The tolerant isolate shows the MIC (µg/mL) of 1400 for lead, 1200 for copper, 1200 mercury and 1000 silver (Figure 2). *A. alternata* showed that better to grow or tolerate heavy metals at low concentration of heavy metal. They were less tolerant to silver heavy metal present in sample. The growth rate of fungi tested was reduced. The MIC has also been reported in the study conducted by Akhtar *et al.*, (2013). The highest MIC of Cu was 444 m*g/L Aspergillus niger* and ranged from 32 to 444 mg/L Cu was showed. Further, Parameswari *et al.* (2010) relatively reported high Cu MIC values ranging between 50 and 75 m*g/L*. The similar MIC values reported by Ahmad *et al.* (2005). They reported MIC values 100-600 m*g/L* for Cu.

Copper ions appeared more toxic in comparison with the lead. Isolates shows different MIC when utilising different heavy metals. Kamika and Momba (2011) found that higher concentrations of lead were toxic for bacteria and fungi. Isolates showed a difference in their tolerance to metals; however, the growth of fungus isolate on agar media containing a high lead concentration was higher as compared to the presence of remaining heavy metal. This is probably due to a period of adaptation where cells of the *A. alternata* isolate synthesized some enzymes vital for the uptake of lead. The results obtained affirmed that the response of the isolates to heavy metals depend on the metal tried, its focus in the medium, and on the isolate considered. Isolates were tolerant to some heavy metal, while on others responded adversely even at low metal particle focuses. The variety in the metal resistance might be because of the presence of one or more techniques of tolerance or resistance mechanism exhibited by fungi. Findings of the present study indicate that fungal isolated have the ability to resist higher concentrations of metals. The tolerance and the resistance of the isolates depended substantially more on the organism tried than on the destinations of its isolation. This variety might be clarified by the development of tolerance or adaptation of the fungi to heavy metals. *A. alternata* isolate were resistant to the metals tested, which may make them promising candidates for further investigation with respect to their capacity to evacuate metals.

Tolerance Index: Lead, copper, mercury and silver tolerance indices of the tested fungal isolate are presented in Figure 3. Growth in diameter of *Alternaria alternata* was observed in the presence of various concentration of heavy metal ranging from 50-1400 µg/ml. Fungal endophyte showed the maximum growth in presence of lead followed by copper, mercury and then silver. The graph shows that fungus isolate were most tolerant to metal concentrations of lead, but it were too sensitive to silver at same conc. On the other hand, as the concentration of heavy metal increase it reduces the fungal growth. *A. alternata* showed high tolerance index for lead followed by copper, mercury, and silver. Fungal endophyte was quite sensitive to concentrations of heavy metal. The level of resistance contrasted for various heavy metals for isolates. The most probable reason for the difference in resistance levels could be the variation in the mechanism of resistance (Ezzouhril *et al.*, 2009, Sani *et al.*, 2003).The resistance against individual metals was much more dependent on the isolate than on the sites of its isolation. Major differences in Ag and Pb tolerance have been found by isolates. These finding was supported by Akhtar *et al.*, (2013). Enhanced growth (diameter) of various species of *Aspergillus* isolates *i.e. A. flavus* (SF-1), *A.*

niger (SF-5), *A. flavus* (GF-5), *A. bervipes* (GF-7) and *A. flavus* (SF-4) ranging from 6.5 to 100% was observed in the presence of 1 Mm Cu concentration. The variation in the metal tolerance resilience might be because of the nearness of various sorts of tolerance processes or resistance mechanisms exhibited by isolates. The detoxification of silver by *A. alternata* may be mediated by an enzymatic antioxidant system such as peroxidase, catalase. From this preliminary test, heavy metal- resistant filamentous fungi were selected and the minimal inhibitory concentration (MIC) to Pb, Cu, Hg and Ag was determined.

Bioaccumulation and Bioassay Procedure: To survive and develop at the tested concentrations of Pb, Cu, Hg, and Ag, the test isolates ought to have developed mechanisms by which the poisonous quality of metals is circumvented. Several of such mechanisms have been reported to be employed by organisms developing in situations containing hoisted levels of heavy metals. These include metal exclusion by permeable barriers (Ezeonuegbu *et al.*, 2014) extracellular sequestration or biosorption and enzymatic alteration of the metal ions to less lethal form (Nilanjana *et al.*, 2008). In addition, fungi are reported to possess particular genes for resistance to heavy metal ions. The genes encoding the synthesis of metal binding proteins such as metallothionein are a good example (Valavanidis and Vlachioganni, 2010). These form the basis of bioaccumulation as a mechanism of resistance to heavy metal ions among fungi. Metal accumulation in this study was carried out using *Alternaria alternata* after simple preparing of fungi in aqueous growth medium containing metal ions. Fungi in this study have unique metal accumulation characteristics and simple to culture. Tolerance and accumulation of Cu^{+2}, Pb^{+2}, Ag^{+}, Hg^{+2}heavy metals were determined in aqueous growth medium. Heavy metal tolerance of fungal isolate was in the order of Pb>Cu >Hg>Ag, in the concentration ranging from 50-250ppm (Figure 5). Growth of isolate was inhibited by higher conc. of heavy metal. In heavy metals bioaccumulation study, *A.alternate* showed high at Pb (80%) accumulation followed >Cu (76.4%) >Hg (68.5%) >Ag (63.1%) at concentration of 50ppm and they showed lowest at Ag (54.3%)followed by <Hg (61.7%) <Cu (66.6%) <Pb (68.5%) (Figure 4). These finding has also been supported by Sulaimon *et al.*, (2014). In this selected isolates *Micrococcus luteus, Bacillus subtilis,* and *Trichoderma harzianum* used for the bioaccumulation studies were found to completely accumulate lead (Pb). *Bacillus subtilis* had the highest percentage accumulation for copper (86.67% and 90.4%). Amount of heavy metals bioaccumulation was calculated by the difference between initial metal concentration in media and the metals concentration of heavy metals remained in media after the fungal growth. *A.alternata* reduced the content of Cu^{+2}, Pb^{+2}, Ag^{+}, Hg^{+2}.

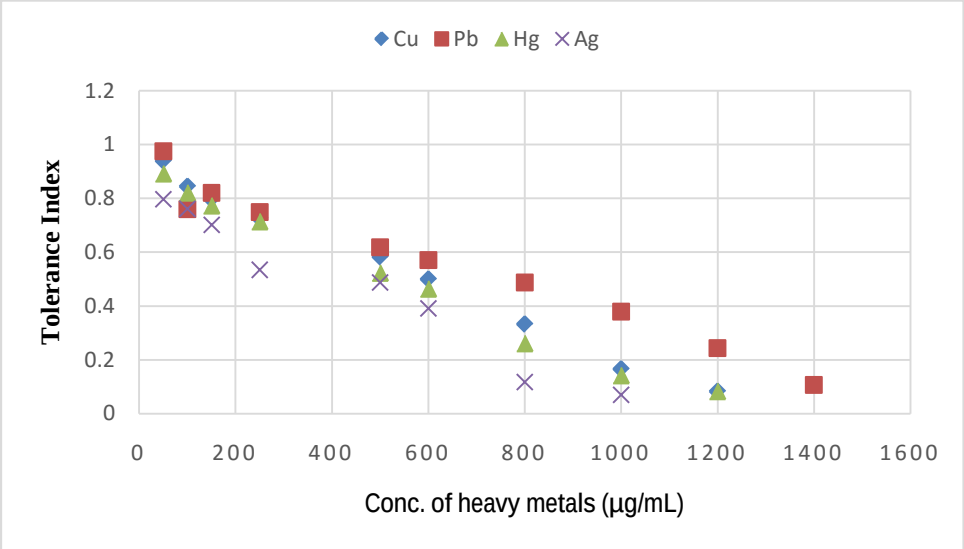

Figure 3. Heavy metal Tolerance index of fungal endophytes

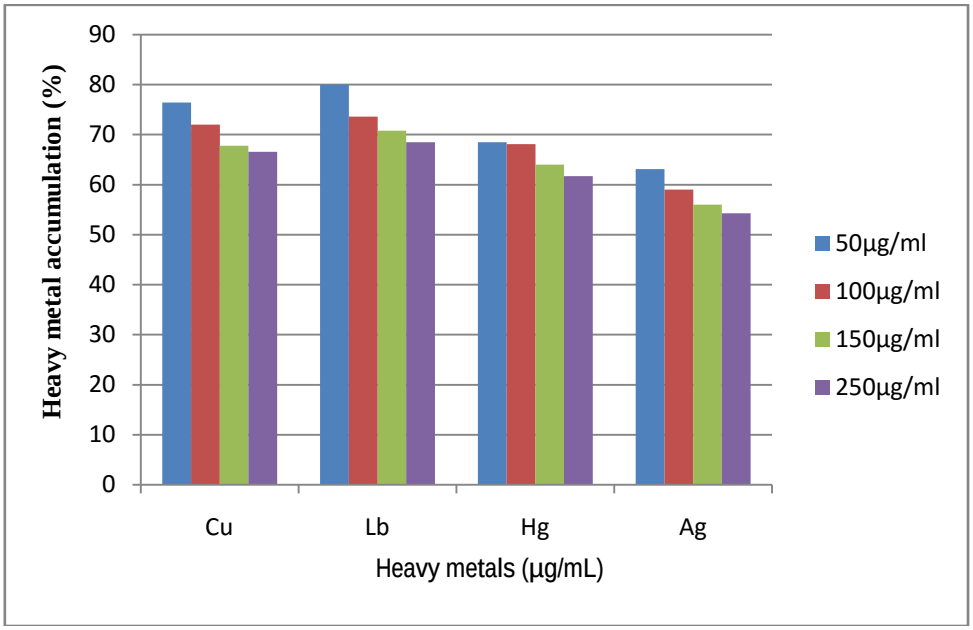

Figure 4. Effect of different metal Concentrations on Bioaccumulation Capacity of *A. alternata*

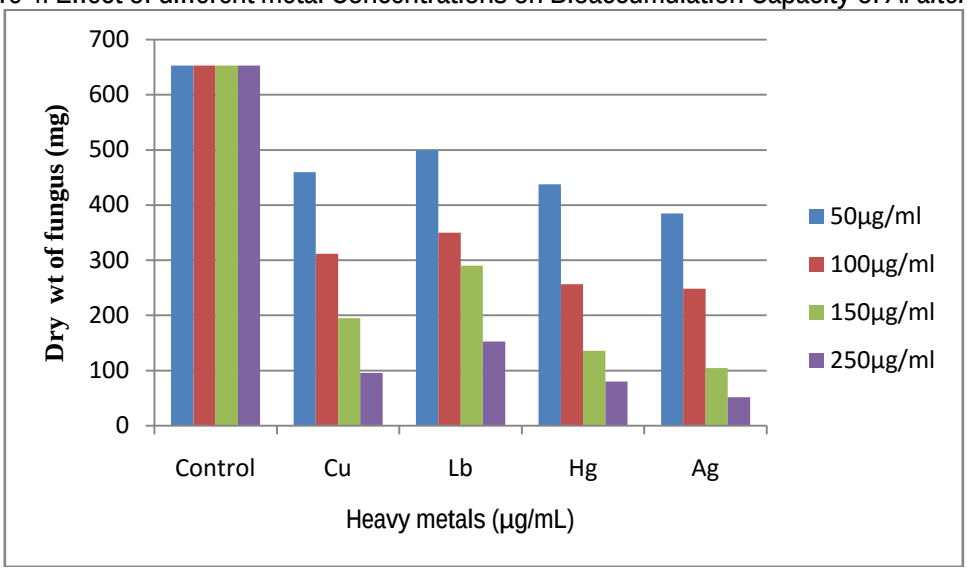

Figure 5. Dry weight of *A. alternata* treated with Heavy metals

CONCLUSION

It is evident from this work that *Alternaria alternata* have potential roles in the bioremediation of heavy metals. The ability of *A. alternata* biomass to remove heavy metals, i.e. Cu^{+2}, Pb^{+2}, Ag^{+}, Hg^{+2} was investigated. Fungi were screened for their tolerance to four heavy metals (Cu, Pb, Ag and Hg) in PDA medium containing heavy metal from 50-1400ppm. There was decrease in growth of fungi for their tolerance to heavy metal with increase in concentration of heavy metal. The tolerance of fungal endophtes for heavy metals depends upon the type of metal and its concentration. The heavy metal tolerant fungi were further tested for removal of heavy metals by adsorb/accumulate them from PD broth. Data revealed that the fungi removed substantial amount of substantial metals and this will greatly help to make environment free of poisonous heavy metalThis demonstrated the potential bioaccumulation capacity of these fungi to remove heavy metals from contaminated site. This made application of these fungi in bioremediation of heavy metals.

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