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Research Article

HEAVY METAL TOLERANCE POTENTIAL OF FUNGUS ISOLATED FROM COPPER SMELTING INDUSTRY

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ABSTRACT

This study investigates the tolerance potential of different species of indigenous fungi that isolated from solid waste at copper smelting industry. The heavy metals concentration in the solid waste was characterized by using Atomic Absorption spectroscopy. The concentration of heavy metal in the solid waste from copper smelting industry was found to be 220 ppm of copper (Cu) and 43 ppm of Iron (Fe). Two different species of fungi such as *Coprinellus xanthothrix* and *Alternaria tenuissima* were isolated from this copper contaminated sample using dilution techniques. They were screened for their resistance to copper (Cu) in potato dextrose agar plates amended with various concentrations ranging from 50-250 ppm of Cu. The isolates were resistant to Cu, among these strain *Coprinellus xanthothrix* was the most tolerant against the tested heavy metal at higher concentration, shows the tolerance index of 1, 0.738, 0.7, 0.355 and 0.288 and the species *Alternaria tenuissima* exhibit 1, 0.78, 0.59, 0.56 and 0.21 respectively. The isolate *Coprinellus xanthothrix* exhibit higher colony diameter and better growth phase for 50-200 ppm of Cu, while compared to the isolate *Alternaria tenuissima*. The results revealed that these initial adaptive behaviors are reflections of the strains tolerance development with increasing metal concentration.

Keywords: Solid waste, Fungi, Heavy metals, 18S rRNA, Minimum Inhibitory Concentration.

INTRODUCTION

In today's world Industrialization and urbanization is confronted with the contamination of soils, water sources and air with hazardous and toxic xenobiotics. Due to the need of generating cheap forms of energy, they cause the continuous release of various harmful chemicals into the Environment. The rapid development of many industries, such as mining, fertilizers, surface finishing, energy and fuel production, pesticides, electric appliance manufacture, and others activities, wastes containing metals are directly or indirectly discharged their waste into the biosphere, they producing a significant threat to the health of humans and also posing a serious environmental pollution[1,2]. The wastewater from metal processing industries is also polluting the environment as well as from other pollutant routes. Virtually, any industrial activity using metals have a metal disposal problem[3]. Heavy metals have drastically increased in the environment and also found in nature. Elements or compounds having different properties, such as Zn, Cu, Ni, Fe, and Mn are essential trace elements in living organisms and non-essential metals such as cadmium, lead, mercury, and nickel they are toxic even at low concentration[4,5]. Heavy metals are non-biodegradable and persistent into the environment and accumulate into living tissues and posing a serious threat to the environment and public health. Due to this reason, they are not able to purify by the environmental compartments (soil and water). Theses harmful substances are cytogenic, carcinogenic and mutagenic in nature and are causing threats to the living beings. Many conventional methods are used for the removal of heavy metals including chemical precipitation and sludge separation, chemical oxidation or reduction, ion exchange, reverse osmosis, filtration, adsorption using activated charcoal, electrochemical treatment and evaporative recovery[6,7]. These techniques are too costly and their metal-binding properties are non-specific[8]. Bioremediation is an integrated management of a polluted ecosystem where different organisms are employed to catalyze the natural process that decontaminates the environment. It is defined as "the utilization of microorganisms to reduce or eliminate environmental contaminants using chemical reactions or physical processes"[9]. These microorganisms convert toxic organic and inorganic compounds to harmless products, mostly carbon dioxide and water. The removal of heavy metals is taken more into consideration while using microbial biomass[6]. Further surface soil is a rich habitat of all major groups of microorganisms, i.e., bacteria, actinomycetes, fungi, and algae and are natural recyclers. The contaminated sites are the provenance for heavy metal resistant micro-organisms[10]. In naturally polluted environments, the heavy metal toxicity of microbes depends largely on the concentration and availability of metals[11]. The most common heavy metal tolerate microbes are fungi and yeast[12,13]. They are a versatile group, as they can adapt and grow under various extreme conditions of pH, temperature and nutrient availability, as well as high metal concentrations[14]. They offer the advantage of having cell wall materials which shows excellent metal-binding properties[15]. In the same way researchers reported the promising biosorption for Cd and Cr by two filamentous fungi, *Aspergillus* sp. and *Rhizopus* sp., isolated from metal-contaminated agricultural soil[2]. About 32 fungal species were tested for their resistance to metals, which was isolated from polluted water in Egypt. In that, the species *Cunninghamela echinulata* biomass could be act as biosorbent of heavy metals in wastewater[16]. The laboratory study was conducted for finding the MIC of *Aspergillus niger*, *Phanerochaete chrysosporium* and *Trichoderma* were from 75 to 100 mg L -1 chromium and for nickel (50 to 100 mg L-1), which depended on the fungal

isolate[17]. It has been shown that copper is toxic to most microorganisms, *Aspergillus niger*,*Penicillium chrysogenum* and *Rhizopus stolonifer* could able to sustain in a liquid medium containing up to 500 ug\ml of Cu. It appears that copper must have been transported in high amounts into the fungal cells that the tested fungi can accumulate Cu upto 6000,5300 and 3250 ug g-1 respectively[18]. The fungi strains including such as *Penicillium funiculosum, Aspergillus foetidus, Penicillium simplicissimum* were tolerant to heavy metals, which could be leached from nickel laterite ores (Ni, Co, Fe, Mg and Mn). These strains were exposed to heavy metals upto 2000 ppm. Training and isolation of tolerant strains were conducted in plate tests by repeated subculturing. The objective of this study is to isolate and identify the fungal strains with various tolerance towards heavy metals. In particular focusing on the effect of various concentration of heavy metals and adaptive behavior of each type of strains.

MATERIAL AND METHODS

Sampling Site

The solid wastes from copper smelting industry were collected from thoothukudi, Tamilnadu. The solid waste sample was air dried and sieved, to remove the homogeneity and stored in the plastic container for subsequent experiments. The chemical characterisation of the sample was carried out by using Energy-dispersive X-ray spectroscopy (EDS). The total heavy metal concentration in the solid waste sample were determined by an acid digestion method. In this method, the sample of 1.0 g was treed with 15 ml HCl and 5 ml 5 ml HNO_3 (ratio 3:1). The mixture was heated until decomposition was complete and volume reduced by evaporation to about 5ml. The digested liquid was filtered through Whatman No. 42 paper and washed with distilled water, transferred quantitatively to a 100 ml volumetric flask. The filtrate was analysed for the heavy metals content using an Atomic absorption spectrophotometer (AAS) [19].

Isolation and Identification of Fungi

The fungal strains were isolated from solid waste by serial dilution method using potato dextrose agar (PDA) in order to avoid overlapping colonies. The dilution technique was made by placing one gram of the sample in the test tube containing 10 ml of sterile distilled water and tenfold serial dilution was made by transferring 1 ml of the suspension to another test tube containing 9 ml of distilled water. This step was repeated ten times to obtain a dilution of 10^{-10}. An amount of 0.1 ml from the test tubes (10^{-3}) was taken and placed on the plate containing PDA[20]. Streptomycin was added to mediums after autoclaving at 15 psi for 15 min and 121 ºC to arrest bacterial growth. After incubation distinct colonies were counted and identified. The isolated fungi were sent to YAAALLL for genomic identification. Molecular identification of the isolates was achieved by 18S rRNA sequence analysis. First, the fungi were grown in flasks containing Sabouraud's dextrose broth and incubated at 25∘C for several days. After colony growth, the culture was filtered and the fungal mass was washed with distilled water for several times and stored in a freezer (−20 ºC) for further processing. The frozen mycelium mass was smashed by mechanical pressure using sterile pounder and liquid nitrogen. Genomic DNA was extracted from colonies and mixed with 450 µl of "B Cube" lysis buffer in a 2 ml microcentrifuge tube and lyse the cells by repeated pipetting. The neutralization buffer such as RNAse A and B Cube of 4 µl and 250 µl were added. The sample was shaked for 30 minutes at 65˚C in water bath using Vortex. Then the sample was Centrifuge for 15 minutes at 14,000 rpm at 10 ˚C. The supernatant were transferred into a new microcentrifuge tube. The DNA concentrations were measured by running aliquots on 1% agarose gel. Then the isolated DNA of 5 µL is added to the 20 µL of PCR reaction solution (1.5 µL of Forward Prime and Riverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix). The PCR is performed using the thermal cycling conditions. ITS1 and ITS4 were the two primers used to amplify, the fungal DNA region segment. The sequencing conditions included a denaturation process carried out at 94°C during 5 min, followed by a cycle at 94°C during 30 s, and an annealing step at 58°C (for ITS) and at 63°C (for 26S), both during 30 s. An extension was carried out at 72°C for 1 min for a total of 36 cycles and a final extension at 72°C and 4°C for 7 and 5 min, respectively. The PCR product was sequenced using the ITS1/ITS4 primers. Sequencing reactions were performed using a ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems). The 18s r RNA sequence was done by using blast, NCBI blast similarity search tool. The program phyML 3.0 aLRT and Tree Dyn 198.3 was used for phylogeny analysis and for tree rendering.

Screening For Heavy Metal Tolerant Fungi

The tolerance potential among the isolated fungal strains were evaluated by the PDA medium and it was amended with various concentrations (50,100,150,200,250 ppm) of heavy metals. Media was autoclaved and the plates were incubated at room temperature for 7 days. The growth of fungi was monitored from the point of inoculation or centre of the colony. Tolerance of the fungi was measured by observing minimum inhibitory concentration (MIC) and tolerance index. Streaking of fungal isolates on normal PDA medium served as control (normal growth) for comparison of growth of fungal isolates on PDA medium containing different concentration of heavy metals.

$$\text{Tolerance} = \frac{Diameter\ of\ the\ colony\ in\ presence\ of\ heavy\ metals}{Diameter\ of\ the\ colony\ in\ the\ absnce\ of\ heavy\ metals}$$

Isolation and acclimatisation were conducted in petri plate, 9.0 cm in diameter. The growth was monitored by measuring the spread of the colony from the point of inoculation to the end of the longest hypha. The minimum inhibitory concentration of the isolates was determined as the lowest concentration of metals that inhibits visible growth of the isolates. If there is no apparent growth of fungi was observed after ten days on the plates, the metal concentration was considered as the highest metal concentration tolerated by the tested fungus.

RESULTS AND DISCUSSION

Solid Waste Characteristics

Solid waste sample were collected from copper smelting industry at Thoothukudi, Tamilnadu. The pH of the sludge was slightly alkaline (8.46). The elemental analysis were done by using EDS and found as O (44.39 wt %), Fe (31.29 wt %), Si (12.35 wt %), C (3.35 wt %), Al (2.51 wt %), Ca (1.47 wt %), Ti (1.01 wt %), Mg (0.89 wt %), K (0.88 wt %) and Cu (0.70 wt %) respectively. The heavy metals like total copper (220 ppm), total iron (43 ppm) were quantified in the dye industrial sludge.

Isolation of Heavy Metal Tolerant Fungi

Two heavy metal tolerant fungus species were isolated from solid waste sample from copper smelting industry using standard method. Some of the most long time exposure of soil fungi to heavy meals can lead to physiological adaptation or considerable modification of their microbial populations[21]. The identification of the isolate was carried out by 18S rRNA Sequencing were performed at Yaazh Xenomics Technologies Pvt. Ltd., Tamilnadu, India. The 18S rRNA gene of selective isolate of each tissue extract was sequenced and presented in FASTA format. Finally 18S rRNA sequence of the isolate was compared with that of other fungi sequence by way of BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). The result was compared with the sequence of GenBank based on partial 18 S rRNA sequence to check the relationship and similarity with the isolates. BLAST and the phylogenetic trees were constructed for the isolated fungus, were identified as *Coprinellus xanthothrix*, *Alternaria tenuissima* respectively. The figure 2 and figure 4 revels the molecular sequencing of the strain *Alternaria tenuissima* and *Coprinellus xanthothrix*. the figure 3 and Figure 5 shows the phylogenetic trees of the isolates *Alternaria tenuissima* and *Coprinellus xanthothrix*.

Minimum Inhibitory Concentration

The effect of heavy metals on the growth of fungus was assessed on the basis of mycelia diameter. The MIC values suggested that the resistance level against individual metals was dependent on the isolates. The isolated tested fungal species such as *Coprinellus xanthothrix and Alternaria tenuissima* showed the strong radial growth, which exhibit the high metal tolerance. At lower metal ions concentrations, the both the isolated fungal species were found to be resistant and exhibit strong radial growth. While in higher metal ion concentrations, they caused a reduction in growth and increased the length of the lag phase compared to the control. A diminution in the growth rate is a typical response of fungi to toxicants[22], whereas the lengthening of the lag phase is not always present. MIC for heavy metals was determined and found to be in the range from 50 to 250 ppm for two the isolated fungus. Copper is a co-factor in numerous enzymatic processes and represents the third most abundant transition metal found in living organisms[23]. As shown in figure 6 and figure 7, *Coprinellus xanthothrix and Alternaria tenuissima* strains showed the strong mycelia growth on Cu media ranges from 50-250 ppm in comparison with control. The several strains of *Antrodia vaillantii* tested, some were able to tolerate up to 40 mM of Cu, whereas others were not able to grow at 3 mM of Cu in the medium. All the tested strains showed strong colony growth on Cu media at 50 ppm in comparison to the control[24]. Even at 50 ppm Cu concentration the strain grows equal to the length of control. *Coprinellus xanthothrix* showed higher degree of Cu tolerance which could show minimal growth in media with 250 Cu concentration. The next highest level of resistance exhibited by another strain *Alternaria tenuissima*. In figure 6, reduction in mycelium diameter were observed from 100-250 ppm of Cu concentration, were due to the inhibition of copper at increasing concentration. Similarly, figure 7, is also depicts the same phenomena. Metals are able to exert harmful effects by their strong coordinating capabilities. Heavy metals affect microorganisms in natural environment by reducing numbers and diversity of microbes and selecting a metal resistant population. It is also commonly assumed that metal exposure leads to the establishment of a resistant and tolerant microbial population[10]. There were morphological and physiological differences between fungal genera, species and strains, and therefore, their response was not same to the concentrations of the heavy metal ions. As Figure 6 depicts, that *Coprinellus xanthothrix* shows the strong radial growth of 9, 6.65, 6.3, 3.2 and 2.6 cm for the various concentration of Cu ranges from 50, 100, 150, 200 and 250 ppm. Similarly *Alternaria tenuissima* reveals the colony diameter of 7.4, 5.8, 4.4, 4.15 and 1.6 cm respectively.

The morphology of strain *Alternaria tenuissima* was changed in the presence of copper. Several authors were observed the colorful mycelia in the presence of heavy metals on agar media due to the increase of copper concentration [25]. Because of the detoxification tolerance mechanisms of the each isolated strain, the different morphological changes were occurred. The isolates have different tolerance capacity against heavy metals, due to the incidence of different types of tolerance strategies or resistance mechanisms exhibited by different fungi[2, 26].

Isolates of the same genus could even show a difference in the level of resistance to metals[27]. Fungi exhibiting high tolerance to toxic metals may be useful in remediation process[2]. The microorganism isolated from the heavy metal contaminated environments, which have the capacity to adapt to such environments[28]. These results indicated that the isolated fungal strains were responded differently to different metal concentration of copper. From figure 6 & 7, it reveals that the strain, *Coprinellus xanthothrix* shows the higher colony diameter, when compared to the strain *Alternaria tenuissima* . It exhibit the tolerance potential of idegious fungus against heavy metals.

Growth Behaviour On The Tolerance Development

The growth pattern of the fungi is represented in Figure 1, which is characterized by five stages:
(a) Lag phase which occurs at the beginning of the inoculation; very little or no growth was observed.
(b) Rapid growth in which the initial growth of fungi occurs.
(c) Retarded growth in which the growth rate begins to decline.
(d) Similar growth in which the rate of growth in the presence of heavy metals and control is similar.(e) Enhanced growth in which the absolute growth rate often exceeds the control (TI > 1)[29].

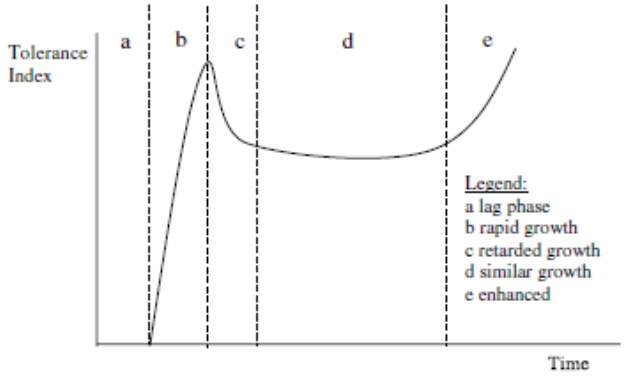

Figure 1: Growth phase of fungi

CAGCCCGCCTTCATATTTGTGTAATGATCCTTCCGTAGGTGAACCTGCGGAGGGATCATTAC
ACAAATATGAAGGCGGGCTGGAACCTCTCGGGGTTACAGCCTTGCGGAATTATTCACCCTTG
TCTTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACATAAACCTTT
TGTAATTGCAATCAGCGTCAGTAACAAATTAATAATTACAACTTTCAACAACGGATCTCTTGG TTCTGGCATCGATGAAAAAC

Figure 2: Identification of *Alternaria tenuissima* species based on 18S rRNA sequences

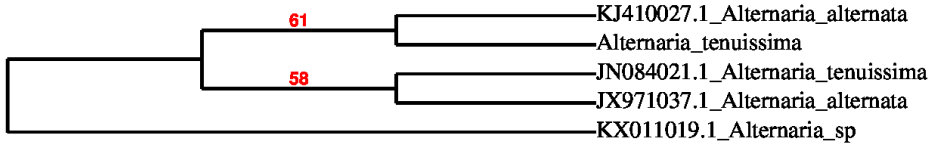

Figure 3: Phylogenetic tree of isolated fungi strains *Alternaria tenuissima* and related strains

TGAACCTGCGGAAGGRKCMTTAACGAATAACTATGGTGTCTTGGTTGTAGCTGGCTCCT
CGGAGCAATGTGCACGCCCGCCATTTTTATCTTTCCACCTGTGCACCGACTGTAGGTCT
GGATACCTCTCGCCCTTTCACGGGGGCGGATGCGAGGGTTGCTCGTAAGGGCTCCCCTC
GAACTTCCAGGCTCTACGTCTTTTTACACACCCCAATAGTATGATGCAGAATGTAGTCA
ATGGGCTTCTCAGCCTATAAAACACTATACAACTTTCAGCAWCGGATCTCTTGGCTCTC GCATYGATGAAAAAC

Figure 4: Identification of *Coprinellus xanthothrix* based on 18S rRNA sequences

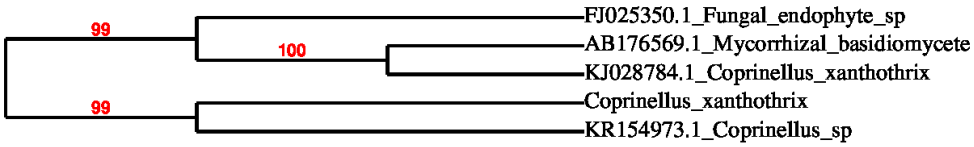

Figure 5: Phylogenetic tree of isolated fungi strains *Coprinellus xanthothrix* and related strains

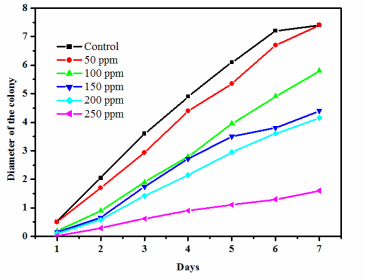

Figure 6: Effect of Cu concentrations on growth of *Coprinellus xanthothrix* strains isolated from contaminated sample, up to 7 days in PDA medium

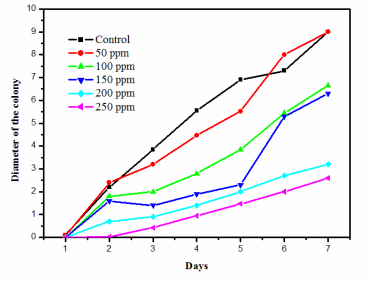

Figure 7: Effect of Cu concentrations on growth of *Alternaria tenuissima* strain isolated from contaminated sample, up to 7 days in PDA medium

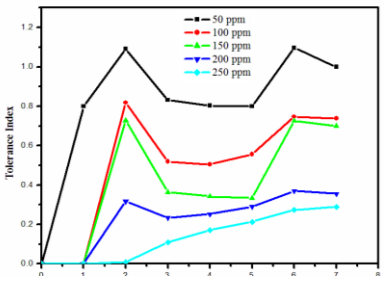

Figure 8: Effects of growth phase of fungi *Coprinellus xanthothrix* in the presence of heavy metal (cu)

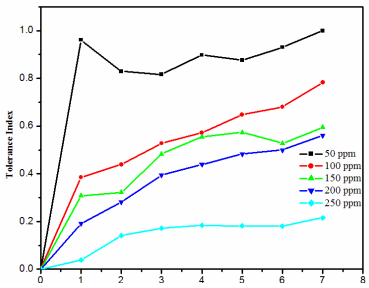

Figure 9: Effects of growth phase of *Alternaria tenuissima* fungi in the presence of heavy metal (cu)

This growth pattern depicts the tolerance potential of fungi. All these five stages are not exhibited in all fungi. In lag phase, the tolerance index remain 0 for 1 or more days. It is evident that the presence of heavy metals retarded the growth of fungi strains. TI varies with time of exposure to the heavy metals and type of strain. The slope of tolerance with time, give the rates of growth and death of the microbes[29]. The growth rate at phase b and the death rate at phase c are related to the tolerance index developed at phase d. A high growth rate and a relatively low death rate led to a higher tolerance index at phase d, indicating a better adaptive tolerance behaviour of fungi in heavy metals. With an increase in metal concentration, the tolerance index at the d phase may begin to increase. This would indicate a development in fungi tolerance with increasing metal concentration. In some cases, however a decrease in TI at phase d with an increasing concentration of heavy metal may be due to the poor adaptive nature of fungus, but it does not mean that it cannot tolerate high concentration of heavy metals.

From figure 8, The *Coprinellus xanthothrix* fungal strain exhibits the better tolerance index and also shows the five stages of growth phase in various concentrations of heavy metals (50-150 ppm). At the concentration of 200 ppm, and 250 ppm, the strain exhibiting a longer lag phase of 3 days. it revealed that, a higher concentration, the adaptive behavior of fungi in heavy metal were reduced. It can be concluded that by increasing copper concentration from 50-250 ppm, *Coprinellus xanthothrix* showed good tolerance index. The TI at phase d for concentration of 50-150 ppm are higher than the *Alternaria tenuissima* , so *Coprinellus xanthothrix* starin showed a higher tolerance for 50-150 ppm and shows a poor tolerance for 200 and 250 ppm. In figure 9, *Alternaria tenuissima* showed a better tolerance for 100-250 ppm of copper. But the strain showed a higher tolerance at concentration of 50 ppm respectively. The results predicted in figure 9, suggested that *Alternaria tenuissima* had not fully adapted to 100 -250 ppm of copper, because growth pattern could not be characterized by five stages and the TI remained at less than 1. In this condition, *Alternaria tenuissima* at higher concentration (200, 250 ppm) does not exhibit the longer lag phase in comparison to three days of strain *Coprinellus xanthothrix* . The strain *Alternaria tenuissima* revealed the better tolerance towards copper at higher concentration. This shows the evident that the presence of heavy metals does not retard the growth of fungi strains in the beginning of the inoculation. Copper is essential for fungi growth and their metabolism, but it can exert toxicity when it is present above a certain threshold concentration. It is evident that, from figure 8 and figure 9 the fungus *Coprinellus xanthothrix* at the concentration of 250 and the *Alternaria tenuissima* at the concentration of 100-250 has not been fully adapted to the copper. The strain *Coprinellus xanthothrix* could tolerate up to 200 ppm of Cu. A relatively high growth rate does not necessarily lead to lower death rate.

CONCLUSION

The present study concludes that solid waste from copper smelting industry contains the two heavy metal tolerant fungus. These fungus were screened for their tolerance towards two heavy metal (Cu) in PDA medium containing heavy metals from 50 to 250 ppm. These fungi showed the various resistance strategies towards the heavy metals of different concentration. It exhibits the potential growth in presence of Cu. *Coprinellus xanthothrix* showed remarkably differed in detoxification behavior of Cu from other isolated fungi in this study. Similarly the strain *Alternaria tenuissima* showed the moderate tolerance towards the heavy metals. There was decrease in growth of the fungi, for their tolerance to heavy metal with increase in concentration from 50 to 250 ppm. The growth pattern of fungi in the presence of heavy metals, characterised by five stages, showed the toxic effects of heavy metals on fungi growth. Among the strains acclimatized, *Coprinellus xanthothrix* was the most tolerate species and show high growth rate even at high concentration (250 ppm) of Cu. When compared to the growth rate of strain *Alternaria tenuissima*. If selection is based on tolerance, this strain will be most suitable for the bioremediation process. The adaptive behaviour of fungi strains to heavy metals reflected the growth and death rate of strains could be used to indicate the tolerance behaviour or adaptation with increasing metal concentration. Biological treatment based on living fungi, which is cheaper and more environmental friendly, has emerged as a good alternative technique to existing conventional methods for metal removal.

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