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Research Article

ELECTROSTATIC PRECIPITATOR DUST: A SOURCE FOR FUNGI TOLERANT TO HEAVY METALS

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A B S T R A C T

Metal pollution of the environment, especially at elevated concentrations, is known to adversely affect microbial activities. However, microorganisms are a versatile group, as they can adapt and grow under various extreme conditions including high metal concentrations. The aim of this study was the screening and characterization of Fe2+ and Cu2+ tolerating fungal isolates. For this study 13 fungal isolates were isolated from dust sample collected from TISCO(Tata Iron and Steel Company, Jamshedpur) and screened for their capacity to tolerate different concentration of Cu2+ and Fe2+ On the basis of their cultural and morphological study, it is said that the isolates may belong to the genus of Aspergillus spp, Cladosporium spp, Periconia spp., Lacellina spp.,Sadasibania girisa and Aspergillus niger. Among the isolated fungal isolates, Aspergillus niger was the most tolerant to Fe2+ (150 ppm) and Periconia spp.tolerant to Cu2+(75) ppm).The effect of metals on fungal growth was determined together with their ability to grow in different pH and temperature. The fungal isolates grew well between pH 4.0 to 6.0 and temperature between 20 to 30ºC.This study was conducted with an aim to obtain metal tolerant fungi with metal removal ability which could be utilized as a potential tool for detoxification of the polluted sites.

INTRODUCTION

Soil pollution by the heavy metals is one of the most significant environmental problems has its negative impact on human health and agriculture. Iron and copper are metallic chemical element with atomic number 26 and 29 respectively. Iron is commonly used in electroplating industries, steel and ferroalloy units etc.Almost all organism and living cell requires iron for the basic cellular process. Whereas excessive iron causes toxicity(Jaishankar *et al*.,2014).Environmental contamination due to copper is caused by mining, printed circuits, metallurgical, fiber production, pipe corrosion ,metal plating, paper and pulp, petroleum refining and wood preserving industries (Beavington ,1975).Agricultural sources such as fertilizers, fungicidal sprays and animal wastes also lead to water pollution due to copper. In some instances, exposure to copper has

resulted in jaundice and enlarged liver. It is suspected to be responsible for one form of metal fume fever (Wagoner *et al*., 1976). Among all various sources, both living and inactivated biomass of fungi exhibit interesting metal binding capabilities. (Veglio,1997)In general, two mechanisms have been proposed for heavy metal tolerance in fungi:1) Extracellular (chelation and cell-wall binding) sequestration.2) Intracellular (binding to non proteins thiols and transport into intracellular compartments) sequestration.(Yang *et al.,*2017).
In extracellular mechanisms, different organic molecule such as oxalic acid and in particular di and tricarboxylic acids that do not belong to the matrix of the cell wall are excreted by the fungal cell to chelate metal ions and binding onto cell wall components. (Volesky, 2003).In the intracellular mechanism, metal transport proteins may be involved in metal tolerance, either by extruding toxic metal ions from the cytosol out of the cell or by allowing metal sequestration into vacuolar compartment.

This study was conducted with an aim to obtain metal tolerant fungi with metal removal ability which could further be utilized as a potential tool for detoxification of the polluted sites.

MATERIAL AND METHODS

Collection of samples

ESP (dust) sample from the contaminated site were collected from TISCO (Tata Iron and steel Company)Jamshedpur. Samples for microbiological analysis were collected in sterile containers and brought to laboratory and kept in refrigerator at 4^{o} C for further processing.

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Physicochemical analysis of ESP (dust) samples

Physicochemical characteristic of ESP(dust) samples were analyzed for the following properties: Total Organic matter (%), moisture contents, total N (Kg^{-hac}), P (Kg^{-hac}), Ca ((Kg^{-hac}) and K (Kg^{-hac}).Organic carbon was determined following a rapid titration method with some modification provided(Walkey *et al.*1947).Total moisture contents was determined by oven dry methods and calculated employing the following formula(MC(%)=W-*w*w*100) provided by (AWPA,2004).Determination of total Nitrogen content was performed by digestion of the waste with a mixture of acids ($HClO_4$, HNO_3, H_2SO_4) and then using Kjeldahl procedure (Bremner 1960). Total Phosphorous was estimated by Colorimetric method using ammonium molybdate and stannous chloride. Total Sodium and Potassium were estimated by Flame photometric method Calcium was determined by titration, pH was determined using pH meter.

Total metal concentration like Cu, Zn, Mn, Fe and B were analyzed in ESP (dust) sample using Atomic Absorption Spectrophotometer. 0.5 gm of sample was dissolved in 20 ml of (1:4) nitric: percloric acid. The samples were allowed to digest till the white colored solution if formed. When the samples got completely digested distilled water was added to the samples and were allowed to cool. These filtered were directly used for analysis on Atomic Absorption Spectrophotometer model Perkin Elmer Analyst 800 fitted with a multielemental hollow cathode lamp of particular metal using appropriate drift blanks at a different wavelength(249.7,327.4,213.9 and 372.0 nm) at the Bio-Tech Lab Training and Demonstration Centre, Ambikapur, India. International soil reference materials were used to prepare calibration curves for different trace metals and to check the accuracy of the analytical data.

Isolation of and Screening of Fungal Isolates for Tolerance to Fe^{2+} and Cu^{2+}

Fungal isolates were isolated by using standard isolation technique employing spread plate method(Cappuccino and Sharman ,2006) on Potato Dextrose Agar Medium in triplicate, containing 25 ppm of $FeCl_3$ and $CuSO_4.5H_2O$ individually and control plates were set up without the metals. After incubation total number of fungal isolates was counted and the percentage of fungi tolerant to Fe^{2+} and Cu^{2+} was calculated as follows.(Prescott and Harley,2002).

% Metal tolerant fungi = Metal tolerant fungi/Total fungi x 100

Purification and Identification of Fungal ISOLATES

The colonies of predominant genera of fungi were picked up and purified by repeated sub culturing on PDA medium and identified on the bases of their macroscopic and microscopic characteristics of preparation stained with lactophenolcotton blue staining and observing under compound microscope(40 X magnification) and identify on the bases of colonial characteristic features such as color, hyphae,types of spores and appearance of spore head.(Cappuccino and Sharman ,2006,Gilman, 1998;Subramam 1971,1983;Ellis and Ellis 1985).

Effect of Graded Concentration of Iron and Copper on Fungal Growth

The fungal isolates obtained from Potato Dextrose Agar(PDA) media were further tested for their ability to tolerate different concentration of the metal salts($FeCl_3$ and $CuSO_4$)incorporated into minimal salt medium. The ability of the isolates to tolerate different concentration of metal salts ($FeCl_3$ and $CuSO_4$)was determined by incubating the 1 ml of the freshly prepared spores suspension(10^{-6} to 10^{-7} spores/ml)of each fungal isolate were inoculated in 250 ml of sterilize flask with 99 ml of minimal salt medium which is containing different concentration of Fe^{2+} and Cu^{2+} such as 0-200 ppm (0 ,25,75,100 ,125,150 ,200) and put on shaker at 150 rpm at 28 ± 2^o C at Ph 6.5 for 15 days after which the growth of the fungal isolates was determined. The fungal biomass was harvested by filtering through whatmann no.1 filter paper and dried at 80°C until a constant weight was obtained. The wet weight and dry weight of biomass was measured.(Yang *et al*.,2017).

Optimization of Growth Parameters

Batch experiments was performed in Erlenmeyer flasks to determine the effect of different factors such as pH, temperature for accumulation of heavy metal(Fe^{2+} and Cu^{2+}). For optimization of parameters in heavy metal(Fe^{2+} and Cu^{2+}) tolerance study, the 8 day old 1 ml of fungal spores suspension(10^{-6} to 10^{-7} spores/ml)of the fungal isolates *Cladosporium sp.,Periconia sp., Mycelia sterilia 1, Aspergillus niger* and *Aspergillus* sp were inoculated into 99 ml of minimal salt medium containing 100 ppm of Fe^{2+} and Cu^{2+} in 250 ml Enrlimayar flask(Joshi,2014).

pH

The optimum growth condition with reference to pH was carried out at different pH ranging from 3.0 to 8.0 and incubation was carried out 28^o C for 96 hrs. The pH of the medium was adjusted using citrate phosphate buffered and sodium phosphate buffer medium. After incubation fungal biomass was estimated gravimetrically by filtering culture through a preweighted dry Whatmann no.1 filter paper. Mycelium was thoroughly washed with distilled water and dried at 80^oC.

Temperature

Studies on optimal temperature for the fungal isolates were carried out by growth at different temperatures (20, 25, 30, 35 and 40°C) in the presence of 100 ppm of Fe^{2+} and Cu^{2+} concentration. These were incubated on rotary shaker at 120 rpm for 96 hrs.The optimum temperature was evaluated based upon the biomass production by each selected fungal isolates.

RESULTS

Physicochemical Analysis of Sample

The physicochemical properties of the ESP (Dust) sample was investigated in this study are presented in Table:1

Table 1 Analysis of Physicochemical properties of ESP (Dust) samples (Mean is ± S.D; n=3)

S No.	Parameter	Code of ESP(Dust)Sample	
		JHJF 1	JHJF 2
1.	Color	Black	Black
2.	Appearance	Powdery	Powdery
3.	Odour	Odoueless	Odoueless
4.	Moisture(%)	16±0.0	8±0.0
5.	pH	5.42±0.008	5.05±0.004
6.	Conductivity(µS/cm)	6.56±0.402	6.8±0.047
7.	TOC(%)	0.29±0.00	0.36±0.00
8.	Nitrate(Kg^{-hac})	141.0 ±0.012	149.0±0.008
8.	Phosphorous(Kg^{-hac})	10.25±0.047	7.20±0.047
9.	Potassium(Kg^{-hac})	170.03±0.008	110.0±0.004
10.	Boron(mg^{-kg})	0.44±0.012	0.49±0.004
11.	Copper(mg^{-kg})	1.05±0.004	1.02±0.469
12.	Zinc(mg-kg)	0.16±0.012	0.11±0.008
13.	Manganese(mg^{-kg})	44.0±0.402	39.0±0.004
14.	Iron(mg^{-kg})	1.30±0.002	1.22±0.008

The observed moisture contents of samples were found in the range of 16 % to 08%.Observed pH value of samples were found in the range of 5.42 to 5.05.EC(6.0 to 6.9 µS/cm),TOC (0.29 to 0.36%),Nitrate(141.0 to 149.0),Phosphorous(10.25 to 7.20),Potassium (170.03 to 110.0),Boron(0.44 to 0.49),Copper (1.05to1.02),Zinc(0.11 to 0.16),Manganese(44.0 to 39.0) ,Iron(1.30 to 1.22) and microbial count 1.4×10^{-4} to 1.2×10^{-4}). pH, conductivity and presence of nutrients are important parameters that strongly influence the chemical behavior of metal ions present in terrestrial and aquatic environments. They have a direct/indirect effect on the solubility and mobility of metal ions including their potential to form chelates with other soil constituents.(Jan *et al*.,2013).

Heavy metals are typically released by acidic pH. Usually these heavy metals are found at moderate concentration levels in ESP(Dust)sample. From Table 1, it can be observed that the Mn (44.0±0.402 to 39.0±0.004 mg^{-kg})was most abundant metal for the both site followed by Fe(1.30±0.002 to 1.22±0.008 mg^{-kg}),Cu(1.05±0.004 to 1.02±0.469 mg^{-kg}),Zn (0.16±0.012 to 0.11±0.008 mg^{-kg}) and B (0.44±0.012 and 0.49±0.004 mg^{-kg}).

Isolation and Screening of Fungal Isolates for Tolerance to $Fe2^{+}$ and Cu^{2+}

ESP (Dust) containing high level of heavy metals which are potential sources of metal-tolerant fungi. Data from numerous studies suggests that the presence of high concentration of metal ions provides a selective medium favoring the evolution of microbial communities that are more tolerant to heavy metals but with lower diversity. (Clausen,2000).

During the isolation procedure a different fungal isolates(n=12) were isolated and metal tolerant fungal isolates were also observed on PDA media containing 25 ppm of $FeCl_3$ and $CuSO_4.5H_2O$.The number of fungal isolates and the percentages of fungal tolerant are presented in Table-1

Table 1 Number of fungal morphotypes and population of metal tolerant in a given sample

S.N.	Code of (Dust)ESP sample	Control	Fe^{2+}		Cu^{2+}	
			TFC	%Tolerant	TFC	%Tolerant
1	JHJF 1	11	5.0×10^{-4}	45.5	4.0×10^{-4}	36.36
2	JHJF 2	12	8.0×10^{-4}	66.66	5.0×10^{-4}	41.66

TFC= Total fungal count

In the present study the total count appeared on PDA plates with no metal induction to be 12×10^{-4} average of the two samples selected, but varied from 5.0×10^{-4} to 5.5×10^{-4} on the Fe^{2+}(25 ppm) and 4.0×10^{-4} to 5×10^{-4} on the Cu^{2+}(25 ppm)supplemented plate (Table 1).The percentages of Fungal colonies tolerant to Fe^{2+} fluctuated from 45.5% to 66.66% and Cu^{2+} fluctuated from 36.36% to 41.66%.There was a noticeable reduction in growth of fungi when the media was supplemented with metal because toxicity of heavy metals is related to their ability to disrubt enzyme functions and structures by binding with thiol and other groups on proteins or by replacing the natural existing metal co-factors in enzyme's prosthetic groups(Colussi *et al*.,2009;Chen *et al*.,2014).

Several investigators have also reported isolation and characterization of heavy metal such as Cu,Ni,Cr,Zn,Pd,Co,Cd tolerant fungi from contaminated sites in presence of 25ppm concentration.(Swarup *et al*.,2011; Joshi *et al*.,2014; Kumar *et al*.,2014).

Purification of Fungal Isolate

After preliminary screening all the fungal isolates were obtained in pure culture's by using standard technique.(Fig:2)and they were named as JHJF-1,JHJF-2,JHJF3,JHJF-4,JHJF-7,JHJF-11,JHJF-12,CPCB-1,CPCB-3 and AFG.

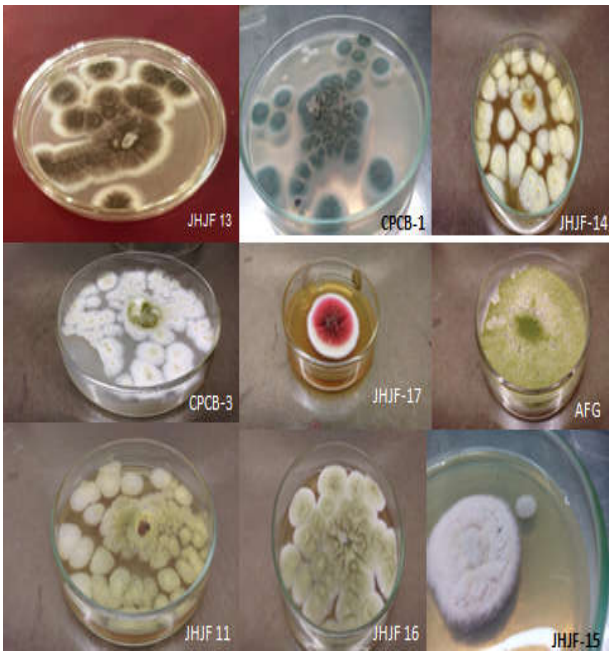

Fig 1 Colony morphology of fungal isolate on Potato Dextrose Agar Media (14 Mpx)

Identification of Fungal Isolate

The culture characteristic and the sporulating structure of pure isolates are presented in fig:2.On the bases of their culture characteristics and the sporulating structure ,the isolates may belong to the genus of JHJF-14-*Periconia sp.*,JHJF-17-*Cladosporium sp*. ,JHJF-10-*Memnoniella* sp,JHJF-13-*Aspergillus niger* ,CPCB-1-*Sadasibania girisa*,CPCB-3 –*Lacellina sp*.and AFG –*Aspergillus sp*.Among all the fungal isolate,*Aspergillus* was the most frequently identified genera.Rests of the isolates such as JHJF-1,JHJF-11 and JHJF-12 were not identified owing to the lacks of sporulating structures under presently used incubation condition. Such strains were designated as Mycelia Sterilia.

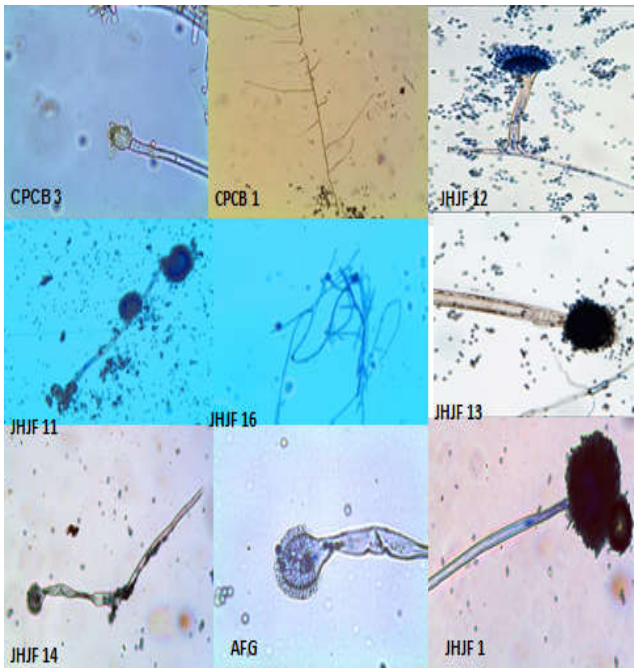

Fig 2 Photomicrograph of fungalmorphotypes at 100X magnification

Growth of the Fungal Morphotypes at Graded Concentration of Iron and Copper

The effect of the heavy metals on the growth and viability of the fungi appeared to vary with the concentrations and type of element. Generally low concentrations of many metals such as Zn,Cu,Mn and Fe appeared to stimulate the growth of fungi, while high concentrations, the metal interacts with nucleic acids and enzyme active site, which can lead to rapid decline in membrane integrity and cell death.(Cervantes and Gutierrez-Corana,1994;Stohs and Bagchi;1985).

The ability of the isolates to tolerate different concentrations of Fe^{2+} and Cu^{2+} were shown in Figure 3 and 4. In this study, the highest biomass yield was observed, for all the isolates, in the medium with no metal ion amendment, which served as the control.

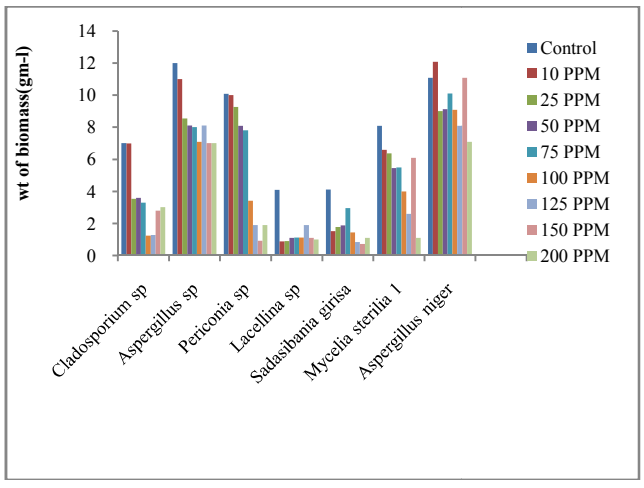

Fig 3 Tolerance of the fungi to different concentrations of Fe^{2+}

Figure:3 Shows the tolerance of the isolates for different concentrations of Fe^{2+}.In present study, the increasing rate of tolerance were found by *Aspergillus* sp,*Periconia sp,Mycelia sterilia 1* and *Aspergillus niger.*Among all four isolates the highest tolerance was observed by *Aspergillus niger* in presence of 150 *ppm of* Fe^{2+} and biomass yield was obtained 11.09 gm^{-1}.

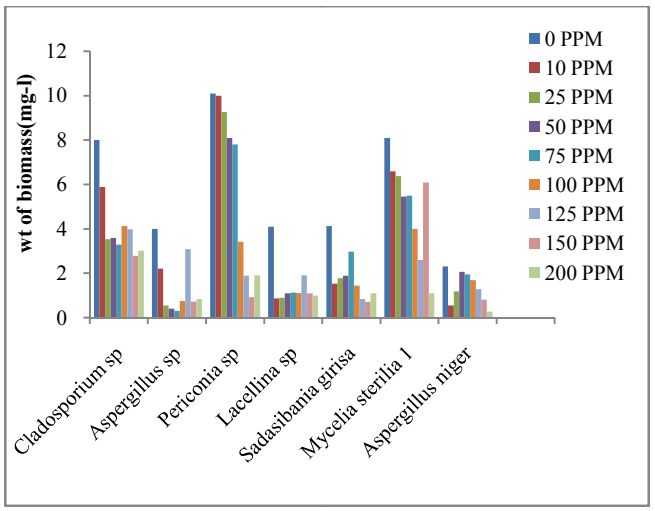

Figure 4 Shows the tolerance of the isolates for different concentrations of Cu^{2+}.

In present study, the increasing rate of tolerance were found by *Periconia sp*, *Aspergillus sp Cladosporium sp and Mycelia sterilia1*. *Among all isolates* the highest tolerance was observed by *Periconia* sp *in the presence of 75 ppm of* Cu^{2+} and biomass yield was obtained 7.35 gm^{-1}.

This adaptation was suggested to refer to two factors. The first one is a gradual decrease in metal availability due to immobilization reaction. The other factor is a gradual change in microbial community structure based on changes in phospholipids fatty acid profiles (Gadd , 1993) which results in more tolerant organisms. Due to this tolerance towards heavy metals, these organisms (*Aspergillus* sp, *Periconia sp, Mycelia sterilia 1,Aspergillus niger and Cladosporium sp*) can be used as indicators and scavengers of heavy metal pollution (Bilgrammi *et al*., 1996).

Optimization of Growth Parameters

On the bases of maximum tolerance of the fungal isolates at graded concentration of metal in Minimal Salt Media the potent isolates were selected for further growth optimization process.

pH

The pH value is an important environmental factor that only affects fungal activity but also the chemical behavior of metal ions in solution. In this study the affect of pH on fungal growth in the presence of 50 ppm Fe^{2+} and Cu^{+2} by selected isolates *Cladosporium sp.,Periconia sp., Mycelia sterilia 1, Aspergillus niger* and *Aspergillus* sp is presented in Fig:6 and 7.

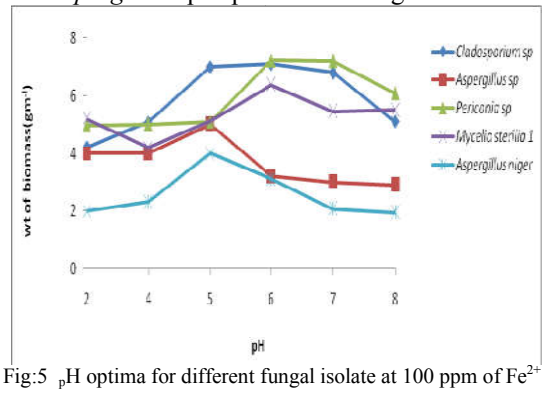

Fig:5 pH optima for different fungal isolate at 100 ppm of Fe^{2+}

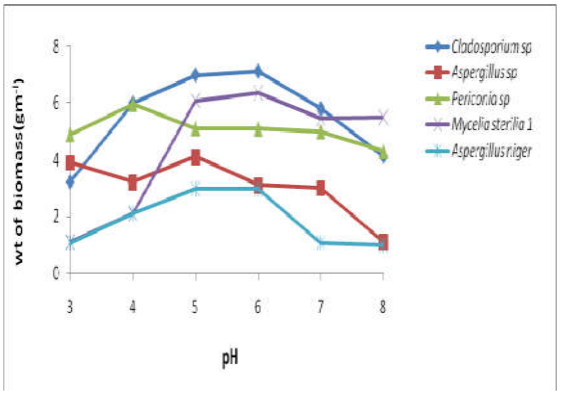

Fig 6 pH optima for different fungal isolate at 100 ppm of Cu^{2+}

Most of the fungi grow well in the pH range of 4.0 to 8.0 but a number can tolerate high acid conditions.Optimum pH for maximum tolerance of Fe^{2+} by *Cladosporium* sp was found to be at pH 6 while isolate *Periconia sp.,Mycelia sterilia 1, Aspergillus niger* Was found to be pH 4,5 and 5.While Optimum pH for maximum tolerance of Cu^{+2} by *Cladosporium sp* was found to be at pH 6 while isolate *Periconia sp.,Mycelia sterilia 1, Aspergillus niger* Was found to be 6,5 and 5. Increased pH can result in precipitation of metal hydroxides or oxides reducing the free metal ions concentration. At pH below 7, metallic cations exist predominately as the free divalent ion. A pH between 4 and 7 is widely accepted as optimal for metal interaction with almost all types of biomasses (Brady & Duncan 1994a). Hydrolysis reactions occur with nearly all the metal cations, and because of this the interaction is facilitated (Baes & Mesner.1976).

Temperature

Studies in the past revealed that the fungal isolates shows tolerance against metal ions is closely related to the temperature of the environment. It is an important factor that has a significant effect on fungal growth in the presence of 50 ppm Fe^{2+} and Cu^{+2} by selected isolates *Cladosporium sp.,Periconia sp., Mycelia sterilia 1, Aspergillus niger* is presented in Fig:8 and 9.

High temperature between 35-40°C did not favor growth of fungal isolates. This can be attributed to a decrease in metabolic activity caused by the increase in temperature above optimum. Likewise low temperature also reduced the metabolic activity of the isolates.(Prescott & Whiteley, 2006).

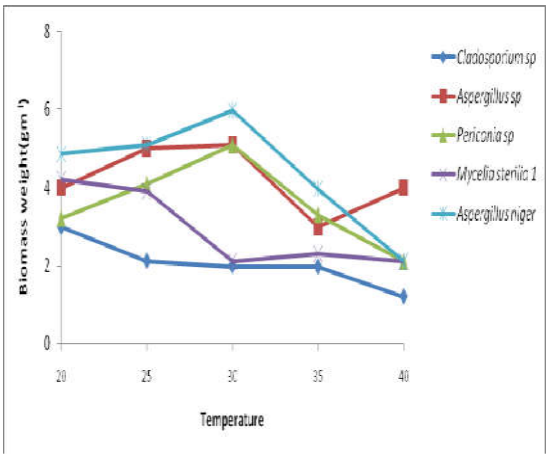

Fig 7 Temperature optimum for different fungal isolate at 100 ppm of Fe^{2+}

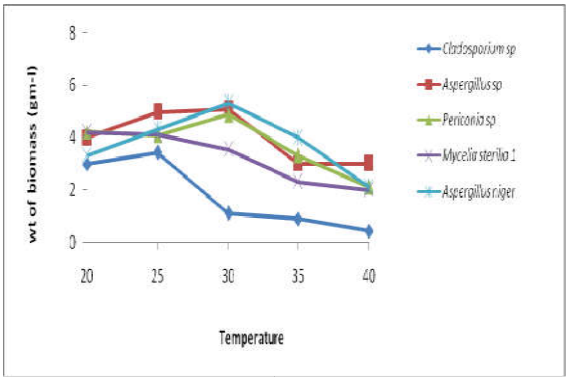

Fig 8 Temperature optima for different fungal isolate at 100 ppm of Cu^{2+}

The result suggested that *Aspergillus sp* and *Periconia sp* preferred an optimum temperature of 25-30°C (Fig:7) for maximum growth in presence of 100 ppm of Fe^{2+}.While presence of 100 ppm of Cu^{2+} ,all fungal isolates except *Cladosporium sp.* preferred an optimum temperature of 25-30°C (Fig:8) for maximum growth.

DISCUSSION

Copper and iron are important trace nutrients for some microorganisms, but at higher concentrations they become toxic. The tolerance and adaptation of metal ions concentration by microbial system of microorganism is the key factor for the study of growth potential and management of microorganism in particular environment. In the present study 13 fungal morphotypes were isolated. On the bases of morphological and microscopic examination of the morphotypes it can be placed to the different genera i.e.*Periconia spp.,Cladosporium spp. Memnoniella spp., Aspergillus niger Sadasibania girisa,Lacellina spp*. The isolates which we obtained exhibit different growth patterns in the presence of different concentration of copper and iron. The isolates showed decrease in growth (measured in terms of biomass production) upon increasing metal concentration compared to the control without metal amendment. The reduction in biomass values revealed that the fungi were affected due to the presence of metal in the growth medium, as the toxicity of heavy metals is related to their ability to disrupt enzyme functions and structure by binding with thiol and other groups on protein or by replacing the natural existing metal co-factor's in enzyme's prosthetic groups.The reduction in growth in the presence of increased concentrations of the metals was evident throughout the experiment compared to the control without metal. However, for the two metals used in this study, Fe^{2+} and Cu^{2+} growth was moderately affected. The isolates *Aspergillus niger* followed by *Periconia sp* were the most tolerant(measured in terms of biomass production) of the copper and iron. It has been also reported by Siokwa *et al*. 2011 that *Aspergillus niger* was the most tolerant to copper and zinc. Environmental factor not only affects fungal activity but also the chemical behavior of metal ions in solution. Studies in the past revealed that the fungal isolates shows tolerance against metal ions is closely related to the temperature and pH of the environment. Variation in external pH can affect the degree of protonation of potential ligan, that contributed to metal binding. According to the Brady and Duncan **1994a, Fourest** and Roux (1992) a pH between 4.0 to 8.0 is optimal for metal tolerance for all types of biomass. the rate of metal tolerance decrease at Extreme of pH. At a pH below 2.0, H^{+} ions compete with metal ions for cellular binding sites and reduce potential metal interaction

with cells.(Gadd,1993). In present study optimum pH for maximum tolerance of Fe^{2+} and Cu^{2+} by *Cladosporium* sp and *Periconia sp* is 6. *and an* optimum temperature was found to be 25-30º C by *Aspergillus sp* and *Periconia sp.* The growth of fungal isolates at high temperature (35-40°C) get reduced due to decrease in metabolic activity caused by the increase in temperature above optimum. Likewise low temperature also reduced the metabolic activity of the isolates

CONCLUSION

In this study bacterial isolates are capable to tolerate heavy metal present in ESP (Dust sample).The results indicate their ability to utilize heavy metal (Fe2+ and Cu2+) for their metabolic activities which can be exploited for environmental as well as health activities.

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