World Journal of Pharmaceutical ReseaRch
SJIF Impact Factor 5.045
Volume 3, Issue 8, 597-611. Research Article ISSN 2277 – 7105

HEAVY METAL LEACHING BY A NOVEL *Aspergillus* sp. ISOLATED FROM A POLLUTED SITE

Nilika Bhattacharya*, Apala Pal, Preeti Khan, Moumita Basu , Sohini Chakraborty, Nayanika Sengupta and Dr. Arup Kumar Mitra

Department of Microbiology, St. Xavier's College (Autonomous), Kolkata-700016.

Article Received on
05 August 2014,

Revised on 01 Sept. 2014,
Accepted on 25 Sept.2014

***Correspondence for Author**
Nilika Bhattacharya
Department of Microbiology, St. Xavier's College (Autonomous), Kolkata-700016.

ABSTRACT

In this investigation, a polluted soil from a metal industry of Kharagpur was collected; serially diluted and plated in a microbiological media. The organism isolated was found to be the blue green mould *Aspergillus sp.* Atomic absorption specificity analysis of soil shows maximum presence of Cu (7.47mg/kg) and Fe (61495.21mg/kg) and the organism also shows appreciable tolerance to heavy metals. This heavy metal stress also brought about certain morphological variations, percentage shrinkage in vesicular size with respect to control is 30.23%, 66.00%, 27.30% for Fe-750ppm, Fe-1000ppm and Cu-10ppm respectively , but the size of conidia remained invariant. On the basis of precipitates collected from the culture tube, the change of oxidation state from 2-3 for both the metals was observed. Accordingly Cu and Fe dependent oxidases of the organism was estimated. Iron Oxidase showed an increase by 0.20u/mg of total protein in presence of Fe and copper dependent oxidase showed an increase by 0.25u/mg of total protein in presence of Cu. Phase contrast microscopy distinctly proved the morphological modification. Hence we propose that the fungus not only is tolerant to Cu and Fe but also removes it from the cyclical pool to reservoir pool having adequate potential for biomining.

KEYWORDS - *Aspergillus* sp., Metal Tolerance , Morphological variation , Enzyme Assay, hase Contrast Microscopy.

INTRODUCTION

Environmental pollution caused by heavy metal ions have acquired increased importance in modern day research. Biosorption is based on the principle of bioremediation that utilizes natural biological sources like bacteria, yeast, fungi etc. The relative high density of the

heavy metals have considerable toxic effects at high and even at lower concentrations. Varying biosorption results are obtained from species to species as the process depends on factor like fungal species, metal concentration, solution, pH, incubation time and ionic composition. Being widespread in soil, fungus contribute significantly to heavy metal dynamics in soil, subjected to their high metabolic activity and large surface area to volume ratio. This has been proved by several experiments on tolerance of filamentous fungi isolated from polluted sites in Tangier (Ezzouhri L et al. 2009).

In our investigation the site from where the polluted black soil was brought is a metal industry, at Kharagpur. Due to pollution from the metal industry the soil in that area turned black and enabled the growth of various microorganism in the soil more importantly fungus as they have greater potential for remediation by virtue of their aggressive growth, greater biomass, production and extensive hyphal reach in soil. The purpose of the present investigation was to investigate different isolate's absorption behaviour towards various heavy metals toxic . The similar experiments were done in a soil irrigated with industrial wastewater (Iram et al. 2012), also in a contaminated agricultural soil (Zafar S et al.2006). The variation in the metal tolerance may be due to the presence of one or more strategies of tolerance or resistance mechanisms exhibited by fungi.

About the organism: *Aspergillus* is a filamentous, cosmopolitan and ubiquitous fungus found in nature.It is commonly isolated from soil, plant debris, and indoor air environment. While a teleomorphic state has been described only for some of the *Aspergillus* spp,others are accepted to be mitosporic,without any known sexual spore production.It belongs to:

Family : Trichomaceae

Genus : *Aspergillus*

The genus *Aspergillus* includes over 185 species. Around 20 species have so far been reported as causative agents of opportunistic infections in man. Among these, *Aspergillus fumigates* is the most commonly isolated species, followed by *Aspergillus flavus* and *Aspergillus niger* , *Aspergillus clavatus, Aspergillus glaucus, Aspergillus nidulans, Aspergillus oryzae, Aspergillus terreus, Aspergillus ustus,* and *Aspergillus versicolor*are among the other species less commonly isolated as opportunistic pathogens.

Major macroscopic features: The major macroscopic features remarkable in species identification are the growth rate,color of the colony and thermotolerance .Except for

Aspergillus nidulans Aspergillus glaucus, the growth rate is rapid to moderately rapid.While *Aspergillus nidulans* and *Aspergillus glaucus* grow slowly and reach colony size of 0.5-1cm following incubation at 25^0 C for 7 days in Czapek Dox Agar ,those of the remaining species are 1-9 cm in diameter in the specified setting. These variations in growth rate help in species identification. *Aspergillus* colonies are downy to powdery in texture .The surface color may vary depending on the species .The reverse color may be purple to olive in some strains of *Aspergillus versicolor. Aspergillus fumigatus* is a thermotolerant fungus and grows well at temperatures over 40^0 C .This property is unique to *Aspergillus fumigates* can grow at a temperature range of 20-50^0C.

Major microscopic features: The basic microscopic morphology is same for all species. However, some other microscopic features are unique to certain species and constitute the key features for species identification together with the surface color of the colony.

Features common to all species include: Hyphae that are septate and hyaline. The conidiophores originate from the basal foot cell located on the supporting hyphae and terminate in a vesicle at the apex. Vesicle is the typical formation for the genus *Aspergillus.*The morphology and colour of the conidiophore vary from one species to another. Covering the surface of the vesicle entirely("radiate head") or partially only at the upper surface ("columnar head") are the flask-shaped phialides which are either uniseriate and attached to the vesicle or are biseriate and attached to the vesicle via a supporting cell, metula. Over the phialides are the round conidia (2-5 micrometer in diameter) forming radial chains.

Other microscopic features that are uniqe to certain species only include sclerotia, cleistothecia, aleuriconidia, andhulle cells .These structures are of key importance in identification of some *Aspergillus* species. Cleistotheium is a round, closed structure enlclosing the asci which carry the ascospores. The asci are spread to the surrounding when the Cleistothecium bursts. Cleistothecium is produced during the sexual reproduction stage of some *Aspergillus spp.* Aleuriconidium is a type of conidium produced by lysis of the cell that supports it. The base is usually truncate and carries remnants of the lysed supporting cell. These remnants form annular frills at its base. Hulle cell is a large sterile cell bearing a small lumen. Similarly to cleistothecium, it is associated with the sexual stage of some *Aspergillus species. Aspergillus molds* thrives best in oxygen rich environments .*Aspergillus* molds also

grows well on materials rcih in carbon which they feeds off for nutrients. However some species of *Aspergillus* molds can survive in environments with very little nutrients and can survive off very little moisture such as the humidity in the air(known as xerophilic).

MATERIALS AND METHODS

1. Determination of physical properties

a) Determination of the pH of soil

1 gm of the provided soil sample was dissolved in 10 ml of distilled water and allowed to stand for some time. Once the sedimentation of the soil sample took place,it was filtered using Whatman's no. 1 filter paper.The pH was accordingly measured by using pH paper and confirmed using a pH sensitive device.

b) Determination of the moisture content of the soil

1 gm of the provided soil was taken in an aluminium foil.It was placed in the hot air oven for round about 10-15 minutes. Then after drying, the soil sample was again weighed.The difference in the initial weight and the final weight gave the moisture content/gm of the soil.

c) Determination of the Total Dissolved Solids (T.D.S) and Electrical Conductivity (E.C) of the soil

1 gm of the provided soil sample was dissolved in 10 ml of distilled water and allowed tostand for some time. Once the sedimentation of soil took place, it was filtered by Whatman's no. 1 filter paper and E.C and T.D.S were accordingly measured by conductometric device.

2. Pure culture isolation

Pour plating of soil by serial dilution method.

i) Serial isolation of the soil – 1 gm of the provided soil sample was dissolved in 9 ml of distilled water to prepare 10^{-1}dilution. Subsequently, 1 ml of the 10^{-1} dilution was pipette out using a micropipette and transferred into another vial containing 9 ml of distilled water to prepare 10^{-2} dilution. Subsequently, 10^{-3} and 10^{-4} dilutions of the provided soil sample were prepared.

ii) Media making – Preparation of PDA and NA plates were done.

Composition of NA

Peptone = 5 g

Beef extract = 3g

Nacl (Sodium Chloride) = 5 g

Agar = 20 g

Distilled Water = 1000 ml

pH = 7.3±0.2(pH adjustment is done by addition of 1N NaOH (if acidic) and 1N HCl (if basic)).

Composition of PDA

Potato = 200 g

Dextrose = 20 g

Agar powder = 20 g

Distilled Water = 1000 ml

pH = 5.6±0.2 (at 25°C)

After the making of PDA and NA , they were respectively autoclaved; pour plating was done.

iii) Pour plating – 0.1 ml of 10^{-3} dilution of soil was pipetted out using a micropipette on to the Petri plate. About 20 ml of the prepared NA and PDA were respectively added to the Petri plates. They were covered and rotated clock wise and counter-clock wise to ensure uniform mixing of the media with the inoculum. (Here 0.1 ml of soil sample).

iv) Incubation – The PDA plates were incubated at room temperature for 3 to 4 days and the NA plates were incubated at 37°C for 24-48 hours.

Organism isolated:-The colonies were taken from the fungal plate i.e. PDA plate and Lacto phenol Cotton blue wet mount was done. The microscopic observation (under 45X magnification) revealed the presence of *Aspergillus spp.*

From the NA plate 3 bacterial colonies were isolated and gram staining was performed with them. Microscopy revealed the presence of gram positive long rods and gram negative short rods respectively. However, we proceeded our work with the *Aspergillus spp.*

4. Metal detection in soil (that was done by 2 methods)

a) Flame test: The flame test was performed using Platinum wire loop. Small amount of soil sample was taken on the tip of platinum wire loop after it was dipped in concentrated Hydrochloric acid. It was held in the burner flame for quite some time and shows bluish

green flame indicating the presence of Copper. And since the soil was isolated from a metal industry in Kharagpur , we presumed the presence of Iron too and therefore the metals detected form the soil sample were supposedly Copper(Cu) and Iron(Fe).

b) 200 gram of the provided soil sample was used for the metal detection method and the procedure for metal detection was –

As per USEPA 3052/3051A for Copper (as Cu) and USEPA 3050B/3052 for Iron (as Fe).

5. Biomass Reduction Test

This test was performed to detect the ability of the micro-organism to survive in presence of various concentration of metals , Fe and Cu respectively and then to obtain their dry weight and to check for metal uptake.

Method

a) In 7, 250ml sterile conical flasks 50 ml of solution was taken, 5 conicals contained broth and varying metal concentration of Cu and Fe and 1 conical contained only the PDA broth that was the control for the experiment. Previously inoculated *Aspergillus spp*. plates were taken to serve as the inoculum for this experiment. In each of the conicals containing broths and varying metal concentration (which were Fe 1000 ppm, Fe 750 ppm, Cu 100 ppm, Cu 50 ppm, Cu 10 ppm), a particular amount of inoculums was added by means of a cork borer , from the previously isolated *Aspergillus spp*. plated , and the same was done for control as well. Then all the conical were kept in incubation for 10 days at room temperature to see the following observation.

b) After the incubation for 10 days , the mycelia mesh of the organism in each conical was filtered in a cheese cloth the filtrate was kept in test tubes and each of the residues were taken in aluminium foils , their initial masses were measured , then they were kept in hot air oven , and finally after drying their masses were again measured. These dried masses were treated with concentrated Nitric Acid , they were heated , diluted with water and then it was filtered and the filtrate was sent for observing metal uptake.

6. Metal Uptake

Method

With the above filtrates of control, Iron 750ppm and Copper 10ppm , metal uptake was carried out as per APHA 22nd.EDN.:2012-3120B. And the observations were noted.

7. Enzyme Assay

The filtrates which were kept in test tubes (in the biomass reduction test 2nd method) showed the presence of precipitates at the bottom of the test tubes , the precipitates were taken in apendroffs and enzyme assay was carried out for both the samples of Iron , Copper and control also. Specifically Copper oxidase and Iron oxidase enzyme assays were done (as the metals detected from the soil sample were Iron and Copper) in the following manner.

Copper Oxidase

In the presence of a suitable amine substrate, amine oxidase enzymes generate hydrogen peroxide, which then drives the peroxidase-dependent oxidation of 4-aminoantipyrine. A subsequent interaction with vanillic acid generates stoichiometric amounts of a red quinoneimine dye, the appearance of which is monitored at 498 nm.

Fe-oxidase

The iron containg cytochrome oxidase test--- reagent--*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine(TMPD) -The reagent is a dark-blue when oxidized, and colorless when reduced. Oxidase-positive bacteria possess cytochrome oxidase or indophenol oxidase (an iron-containing hemoprotein)—the color intensity is measured at 520nm.

8. Morphological Charaterization

Method

From each of the samples of the above experiment (from the conicals in which the sample were incubated for 10 days) few microlitres of the inoculum (black cottony mass) were taken in a slide and was watched under the microscope , the percentage shrinkage in the vesicular size in each of the samples were observed with respect to control. The sizes of conidia were also compared with respect to control. The observation was noted in a tabular form with respect to standard error.

RESULTS

Isolation of micro organism from Metal Industry soil (Kharagpur)

1. Physical properties of the soil such as ph, electrical conductivity,total dissolved solids, moisture content were studied and the results are given in Table -1.

Table 1 : Physical characteristics of soil

SAMPLE	OBSERVATION			
Soil	pH	Electrolytic Conductivity	Total Dissolved Solid	Moisture Content
	7.0	3816 ms	189.1 ppm	0.049%

2. The organism isolated from pure culture was found to be fungal in origin with colonies growing rapidly producing mycelia.The detailed results are shown in Table- 2.

Table 2: Nature of organism and colony characteristics

NATURE OF THE ORGANISM	COLONY CHARACTERISTIC	SITE FROM WHERE SAMPLE WAS TAKEN	MICROSCOPIC FEATURE
Fungi	Colonies growing rapidly, white cream mycelium that turn black during spore formation.	Metal Industry of Kharagpur	Micro-conidia are usually abundant and small spore were also present.

3. From the initial serial dilution and pour plating technique the organism isolated was fungi and it was *Aspergillus spp.*

4. The metals in the soil sample were detected and it gave 7.47 and 61495.21 mg/kg of Cu and Fe respectively.The results are shown in Table -3.

Table 3: Metal detection

AMOUNT OF SOIL USED FOR TESTING (in gm)	METALS DETECTED	AMOUNT in mg/kg
200	Cu	7.47
	Fe	61495.21

5. The biomass reduction tests were performed and it showed a reduction in biomass of 86.18%,99.00% ,2.30 % for Cu having concentrations of 10,50,100ppm respectively and 97.9% and 98.4% for Fe having concentration 750and 1000ppm respectively. The results are tabulated in Table-4.

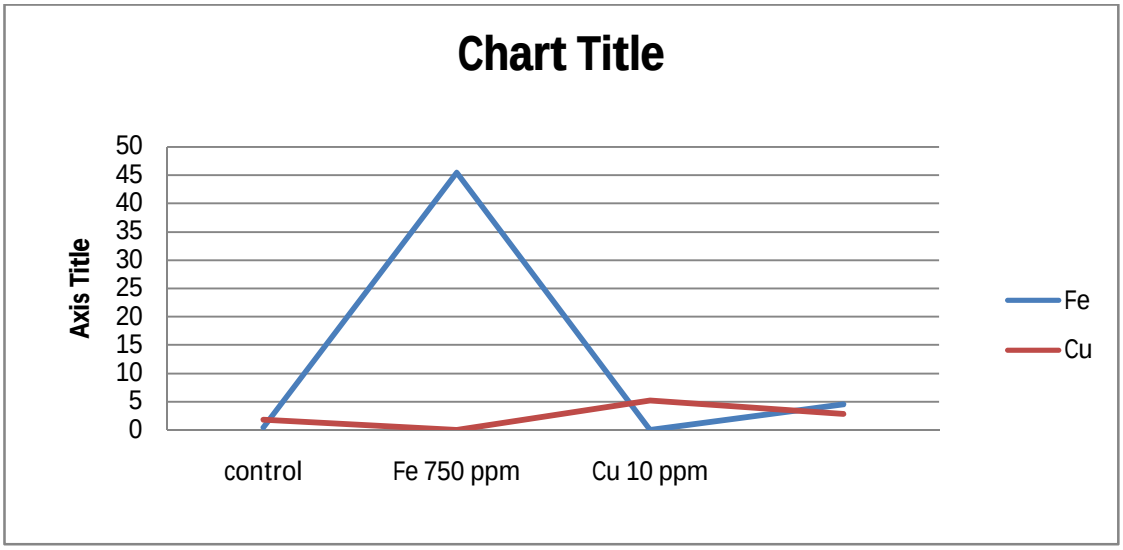
Table4: Biomass reduction of the organism

Concentration	Weight of Soil (in gm)	Weight of Organism +Foil(in gm)	Weight of Organism (in gm)	Reduction In Weight of Organism (in %) W.R.T Control
control	0.554	1.1206	0.5666	_
Cu,50ppm	0.552	0.6306	0.0783	86.18
Cu,100ppm	0.492	0.4969	0.0049	99.00
Cu,10ppm	0.452	1.0055	0.5535	2.30
Fe,750ppm	0.453	0.4646	0.0116	97.9
Fe,1000ppm	0.501	0.5096	0.0686	98.4

6.Tests were performed to estimate the amount of metal taken up by the organism and it was found that in the control in presence of both the metals the amount of Cu taken was 1.83 mg/L that is higher than the amount of Fe taken up which is 0.46mg/L.In presence of Fe 750 ppm and Cu 10 ppm ,the organism being incubated in the two concentrations separately it was found that the amount of Fe and Cu taken up were 45.49mg/L and 5.24mg/L respectively.The results are tabulated in Table-5.(continued to next page)

Table 5: Metal uptake

TEST SAMPLE	AMOUNT OF METAL TAKEN UP BY THE ORGANISM	
	Iron (Fe)	Copper (Cu)
Control	0.46 mg/L	1.83 mg/L
Fe 750ppm	45.49 mg/L	-
Cu 10ppm	-	5.24 mg/L

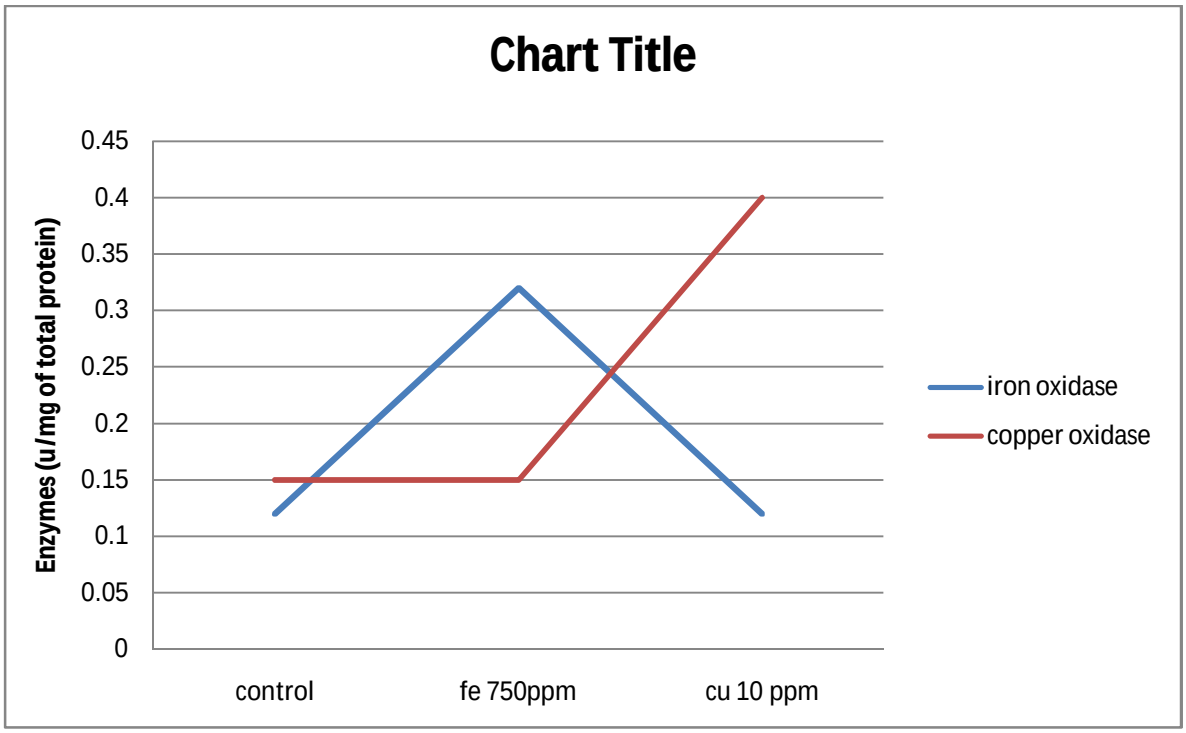


Graph 1.-Showing metal uptake by control and samples of Fe 750ppm and Cu 10ppm respectively

7. The enzyme assay showed that in control the amount of Fe and Cu-oxidase was 0.12 and 0.15 u/mg of total protein respectively whereas in presence of Fe 750 ppm the amount Fe-0xidase was higher than that of Cu oxidase i.e 0.32u/mg of total protein and in presence of 10 ppm Cu the presence of Cu-oxidase was higher than that of Fe oxidasei.e 0.40 u/mg of total protein. Results are given in Table-6.(continued in the next page).

Table 6: Enzyme assay

TEST SAMPLE	Fe- oxidase u/mg of total protein	Cu-oxidase u/mg of total protein
Control	0.12	0.15
Fe 750 ppm	0.32	0.15
Cu 10 ppm	0.12	0.40

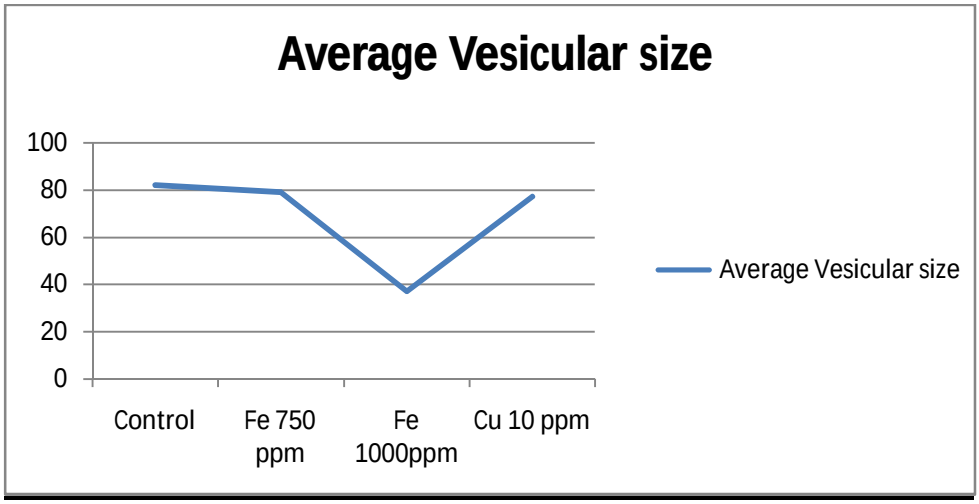


Graph 2.- Showing the amount of enzymes present in various test samples (control , Fe 750ppm and Cu 10 ppm respectively)

8. The various morphological characteristics of the organism such as size of conidia, no. of conidia, volume of culture used, vesicular frequency and percentage shrinkage were estimated and tabulated in Table-7(continued to the next page).

Table 7: Morphological characteristics of isolated *Aspergillus* sp.

Concentration	Size of Conidia (µ)	Averagesize of Conidia with Standard Error(µ)	No. of Conidia	Average No. of Conidia with Standard Error	Volume of Culture Given on Slide(µL)	Density of Conidia with Standard Error	Vesicular Size (µ)	Average Vesicular Size with Standard Error	Vesicular Frequency	Average vesicular frequency with standard Error	Percentage Shrinkage of Vesicular Size W.R.T Control
Control	3.30 3.30 3.30 3.30 3.30	3.30±0.00	>100 >100 >100 >100 >30	>86±14	50	3 ± 0.54	79.2 99.0 66.0 89.1 79.0	82.46±5.52	1.0 1.0 1.0 1.0 1.0	1 ± 0.00	_
Fe 750ppm	3.30 3.30 3.30 3.30 3.30	3.30±0.00	160 90 130 140 150	134±12.08	50	2.68± 0.24	112.2 99.0 66.0 75.9 42.9	79.2±12.2	1.0 1.0 3.0 1.5 3.0	2.9±0.4716	30.23%
Fe 1000ppm	3.30 3.30 3.60 3.30 3.30	3.312 ±0.06	78 100 100 99 100	95.4±4.35	50	2.308 ±1.47	49.5 19.8 26.4 49.5 42.9	37.62±6.13	1.0 2.0 1.0 2.0 1.0	1.4 ±0.245	66.0%
Cu 10ppm	3.30 3.30 3.30 3.30 3.30	3.30±0.00	20 25 31 50 20	29±5.580	50	0.58 ±0.1116	66.0 79.2 100 .7 72.6 66.0	76.9±6.43	1.0 1.0 4.0 1.5 2.0	1.8 ±0.557	27.3% (osmotic shock)



Graph 3.-Showing the average shrinkage in vesicular size of the fungi in presence of various metal concentrations, with respect to control.

PICTURES OF SAMPLE ORGANISM AS A CONTROL AND IN PRESENCE OF CU – 10ppm

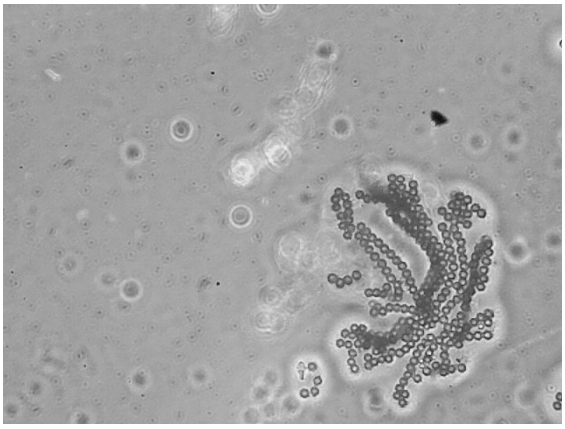


Fig.1-Control sample seen under Phase contrast Microscope

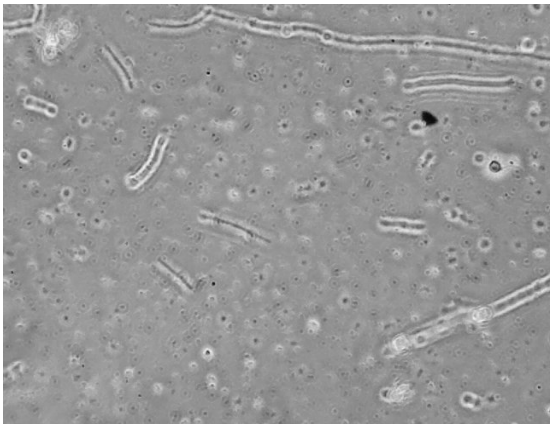


Fig.2-Cu 10ppm sample seen under Phase contrast Microscope

DISCUSSION

The heavy metal concentration is rapidly increasing in the environment with industrialization. Micro-organisms play an important role in controlling metal pollution by biosorption of heavy metals from polluted soil. In the present study it is observed that the micro-organism isolated from soil plays an important role in bioremediation.

Bioremediation is a waste management procedure involving micro-organisms to remove or neutralize pollutants from the contaminated site. Bioremediation may occur on its own (natural attenuation or intrinsic bioremediation) or may only effectively occur through

addition of fertilizers or oxygen and so on that help encourage the growth of pollution eating microbes within the medium (biostimulation).

Some of the earlier works of IramShazia, Uzma et al of Kasur, Pakistan include studying heavy metal absorption capability of Aspergillus and its subsequent role as a biosorbent. The main finding of our experiment is the relative tolerance of the fungus to Copper (Cu) and Iron (Fe) and its possible role in removing them from the cyclic pool to the reservoir pool showing potential role in biomining.

In a recent study conducted in North Carolina, United States, repeated application of swine waste resulted in increased accumulation of Cu and Zn in the soil. Through subsequent experiments, it was proved that *Aspergillus niger* was most resistant to Copper and best able to remove Cu from culture media and swine waste water. *Aspergillus* removed 91% of Cu and 70% of Zinc from hog waste water thereby serving as a promising candidate for removal of Cu and Zn from swine waste water.

Another important finding in the experiment was the ability of the organism to produce enzyme Cu and Fe oxidase obtained by the enzymatic assay of the organism grown in high concentration of Cu and high concentration of Fe respectively with respect to the control. In presence of high concentration of Iron, the increased activity of the enzyme iron oxidase resulted in the conversion of the oxidation state of Fe from +2 to +3 which was visible in the form of red precipitate. Whereas for Cu oxidase basically they are the Multicopper oxidases(MCOs) from a family of redox enzymes that catalyze the reduction of molecular oxygen into water by a four-electron transfer process. In presence of copper the enzymatic activity of copper oxidase has been found to increase with respect to control. However in certain studies copper is shown to have been involved in increased iron uptake by microbial oxidation. Fungal MCOs are usually involved in delignification, morphogenesis, pigment formation , pathogenesis , competitor interactions and transport of metal ions , so they are very important for sustainable industrial processes like bioremediation.

In the present study, AAS analysis of the soil showed maximum presence of the Cu and Fe and organism showed appreciable tolerance to both the heavy metals. This heavy metal stress also brought about certain morphological variations indicating osmotic shock which was further confirmed by phase contrast microscopic studies.

Certain species of aspergillus such as *Aspergillus fumigatus* have reductive iron acquisition mechanism for uptake of iron that is conversion of iron from Fe^{+3} to Fe^{+2} and subsequent uptake by an iron permease. This ability was exhibited by the *Aspergillus* spp. in our study.

CONCLUSION

In this investigation the atomic absorption spectrophotometric analysis of the soil showed the presence of the iron and copper and the corresponding potential of the organism to tolerate both the heavy metals. Also the fungus isolated from the soil showed the potential of secreting iron and copperoxidase and thereby exhibiting possible role in biomining by removing the metals(iron and copper) from the cyclic pool to the reservoir pool.

ACKNOWLEDGEMENT

We would take this opportunity to express our profound gratitude and deep regards to Fr.J.Felix Raj, Principal, Dr. Kasturi Sarkar, Head of the Microbiology Department, Dr. Arup Kumar Mitra , Former Head of the Microbiology Department, Dr.SudeshnaShyam Choudhury, Assistant Professor, and Ms. Debanjana Banerjee , Research Scholar, St.Xavier's College ,Kolkata for their cordial support, valuable information and guidance ,which helped us in completing this project through various stages.

REFERENCES

1. Niermanetal. Genomic sequence of pathogenic and allergenic filamentous fungus *Aspergillusfumigates* . Journal – Nature. 2005; 438:1151-6.
2. ShaziaIram, KousarParveen, Jawaria Usman1, Kinat Nasir1,Noreen Akhtar1,Sana Arouj1,Iftikhar Ahmad .Heavy metal tolerance of filamentous fungal strains isolated from soil irrigated with industrial wastewater. BIOLOGIJA. (© Lietuvosmokslųakademija, 2012), 2012; 58(3):107–11.
3. ShaziaIram, Iftikhar Ahmad, BariraJaved, SaeedaYaqoob, Kulsoom Akhtar, Munawar Raza Kazmi and Badar-Uz-Zaman, Fungal Tolerance to heavy metals.Pak. J. Bot., 2009; 41(5): 2583-2594.
4. Ezzouhri L, Castro E, Moya M, Espinola F, LairiniK.Heavy metal tolerance of filamentous fungi isolated from polluted sites in Tangier, Morocco. Afr J Microbio Res., 2009; 3(2): 035-48.
5. Zafar S, Aqil F, Ahmad L , Metal tolerance and biosorption potential of filamentous fungi isolated from metal contaminated agricultural soil. Bioresour Technology, 2006; 98:2557-61.

6. Navarro M.C., Pérez-Sirvent C., Martínez-Sánchez M.J., Vidal J., Tovar, P.J. and Bech, J., Abandoned mine sites as a source of contamination by heavy metals: A case study in a semi-arid zone, J. Geo. Explor.,2008; 96(2-3), 183-193.
7. ShaziaIram , KousarPerveen , NailaShuja , KanwalWaqar, Ijaz Akhtar , Iftikhar Ahmad.Tolerance potential of different Species of Aspergillusas bioremediation tool - Comparative analysis.E3 Journal of Microbiology Research, June, 2013;1(1): 001-008.
8. Djukic, D. and L. Mandic. 2000. Microorganisms and technogenic pollution of agroecosystem. Acta. Agricul..Serbica., 5(10): 37-44.
9. Gupta R, Ahuja P, Khan S, Saxena R K, Mohapatra H. (2000). Microbial biosorbents meeting challenges of heavy metal pollution in aqueous solutions, Current Sciences 78: 967-973.

   Melgar M.J., Alonso J. and Garcia M.A., Removal of toxic metals from aqueous solutions by fungal biomass of Agaricusmacrosporus. Sci. Total. Environ., 2007; 385, 12–19 .
10. Ahalya N, Ramachandra TV, Kanamadi RD. Biosorption of heavy metals. Res.J. Chem. Environ. 7: 71-78.