STUDY AND CHARACTERIZATION OF IRON TOLERANT ORGANISMS

AMUDAN R., FATIMA S.

Abstract: Soil and wetlands in urban settings are subject to huge amounts of heavy metal pollution due to discharge from various industries, hospitals, clinics, etc. The micro-organisms present in such environment naturally develop resistance to high concentration of metals. These micro-organisms include many bacteria and fungi. They can be used in bioaccumulation and bioremediation studies of heavy metals. In the present study, the tolerance and bio accumulating capabilities of bacteria and fungi isolated from the Mithi river effluents and soil, against Iron (Fe), a heavy metal was studied and quantified by UV-VIS spectrophotometry and Atomic absorption spectrophotometry. The bacteria and fungus showed growth in the presence of 3mM $FeCl_3$. SEM was also done on the fungal cells to study the ability of the fungus to bio accumulate iron particles. F_1 cells showed the presence of iron particles in the range of 220nm to 520nm.

Key words: bioaccumulation, iron, bacteria, fungi, atomic absorption spectro photometry.

Introduction: Pollution results in changing the physical, chemical and biological characteristics of water and soil in turn leading to deterioration in the water and soil quality and affecting the ecosystem. Heavy metal is a general collective term, which applies to the group of metals and metalloids with atomic density greater than 4000 kg m-3, or 5 times more than water [1]. Although some of them act as essential micro nutrients for living beings, at higher concentrations they can lead to severe poisoning [2]. Iron makes up about five percent of the earth's crust. It can be a soluble or relatively insoluble form, found in water and through water finds its way into the soil. Soluble iron is found in groundwater, oxygen-free reservoirs, dead-ends in water distribution systems, and hard mineral coatings within pipes. The total amount of iron in the human body is approximately 4 g, of which 70% is present in red blood coloring agents.

In order to survive in the heavy metal polluted environments many micro-organisms have developed resistance to toxic metal ions [3]. Bacteria can tolerate heavy metal by regulating transport into and out of their cells compartmentalizing them within cells, as well as other mechanism, including production of siderophores [4]. Resistance systems not only protect the organisms in a harsh environment but also play an important role in the cycling of toxic metals in the biosphere. [5].

Microorganisms possess a variety of mechanisms to deal with high concentrations of heavy metals and often are specific to one or a few metals [6, 7]. Using bacterial and fungal tolerance against metal compounds, one can minimize the effect of heavy metals on total biological activity of the ecosystem. Such tolerant bacteria and fungi can play an important role in bioremediation of heavy metals in environment by converting them into less toxic forms or by accumulating the metal ions from the area of contaminations [8]. Aerobic batch biosorption experiments were carried out for removal of Cr (VI), Fe (III), Cu (II) ions from aqueous metal solutions using *Bacillus Licheniformis* [9]. *Bacillus* species growing in the presence of mercury, lead, silver,

zinc and copper have been isolated from soil [10]. Increased uptake of these heavy metals could lead to their bioaccumulation within the organisms, resulting in their removal from the environment. Also the metals could be bio transformed to less toxic or more volatile forms thereby decontaminating the environment [11].

Materials and methods:

All the components of enrichment and nutrient media were obtained from Hi Media Laboratories Pvt. Ltd. Chemicals were obtained from Loba Chemicals Pvt. Ltd. Deionised water or double distilled water was used for all the experiments. Sterile media used for study of organisms. Aqueous solution of Ferric chloride ($FeCl_3$) was used in the study of heavy metal tolerance.

Sample collection:

The water sample was obtained from Mithi River. The sample was collected during the first week of November. The soil sample was obtained from Wadala near a Metal crap industry (Singh Industry).

Screening and Isolation of microbes from water and soil samples:

On the day of collection, the water sample (1ml) was enriched in 50 ml of nutrient broth for enrichment of bacteria whereas 1 gram of the soil sample was enriched in Sabouraud's broth. The above enriched water sample was then used for isolating iron tolerant organisms by further inoculating them in Nutrient broth and Sabouraud's broth each containing 1mM $FeCl_3$ and then transferred to Nutrient agar and Sabouraud's agar plates each containing 1mM $FeCl_3$.

The isolated iron tolerant bacteria were maintained on nutrient agar slants while the fungal cultures were maintained on Sabouraud's agar slant stored at 4^oC and were regularly sub cultured.

Identification:

The two bacterial isolates X_9 and S_1 were gram stained and on the basis of the staining were subjected to the following biochemical tests for the identification of the isolated species. The tests included, Voges–Proskauer test, glucose, sucrose, mannitol and citrate utilization test, growth at 2%, 5%, 7%, 10% concentration of NaCl, growth at pH 6.8, 7.2 and at 55°C. The pure cultures were identified by means of taxonomic schemes and descriptions as per Bergey's Manual [12]. The morphological characters including the shape, size, colour, arrangement of fruiting bodies and the spores on the fruiting bodies were studied to suggest the taxonomy of the single fungal isolate F_1. The monograph study of the fungus was done at the Agarkar Research Institute, Pune.

Iron tolerance of isolated strains:

Determination of Minimum Inhibitory Concentration of $FeCl_3$ on the isolates:

To examine the ability of the isolates to tolerate the various concentrations of iron, MIC of $FeCl_3$ against the isolates was performed. Cells of overnight grown cultures of X_9 and S_1 and spores F_1 were inoculated in Nutrient broth and Sabouraud's broth containing $FeCl_3$ in the range of 1mM-15mM and incubated for 48 hours at 37ºC and room temperature (around 25^oC) respectively.

Determination of Minimum Bactericidal Concentration and Minimum Fungicidal Concentration:

To confirm whether the organisms were killed or only static in the presence of $FeCl_3$, 10µl of supernatant of X_9, S_1 and F_1 from the MIC tubes that showed no growth were used. The bacteria were inoculated in Nutrient broth and incubated at 37ºC for 24 hrs and the fungus was inoculated in Sabouraud's broth and incubated at room temperature for 48 hrs.

Assay for siderophores:

Assay for the presence of siderophores was done using supernatants of X_9, S_1 and F_1 grown in control medium containing nutrients and medium containing nutrients along with 2mM and 3mM $FeCl_3$ each.1ml of culture supernatant was added to 1ml of Hathway reagent (0.3g of $FeCl_3$ is added to 0.1ml of 0.1N HCl. To the above mixture, 100mlof Distilled water is added followed by addition 0.3g of potassium ferricyanide).

Production of biomass for iron uptake studies:

24 hours old broth cultures of the bacterial isolates X_9 and S_1 (0.1 ml) having an absorbance of 0.05 at 530 nm were inoculated in sterile 1mM aqueous solution of $FeCl_3$ and incubated at room temperature on shaker for 24 hours while the mycelial mass of fungus F_1 was separated from the Sabouraud's culture broth by sterile forceps and transferred into sterile 1mM aqueous solution of $FeCl_3$ and incubated on rotating shaker for 24 hours. The 24hr old supernatant of X_9, S_1 and both intracellular and extracellular extracts of F_1 were then subjected to iron uptake studies by UV spectroscopy, Atomic absorption spectroscopy and Field Emission Gun – Scanning Electron Microscopy

Study of iron uptake by UV Spectroscopy: Three ml of the extracellular supernatant of X_9, S_1, F_1 and intracellular extracts of F_1 was subjected to wave scan between 200-800 nm. The instrument used was Implen Nano Photometer 7122 V2.0, in absorbance mode having path length of 10 mm.

Study of iron uptake by Atomic absorption spectroscopy:

One ml of extracellular supernatants in case of isolates X_9, b_1, F_1 and intracellular extracts of F_1 was subjected to acid digestion with concentrated HNO_3 for four hours on a hot plate till complete dryness was obtained. Four ml of perchloric acid was then added to the residue and digested till the volume reduced to half. This was then reconstituted with 5% HCl to obtain a 1% solution. One ml of this reconstituted solution was subjected to Atomic Absorption Spectra to determine the concentration of the metal. The instrument used for Iron analysis was Schimadzu AA-7000F at a wavelength of 248.15nm.

Study of iron uptake by FEG-SEM (Field Emission Gun – Scanning Electron Microscopy):

After 24hrs, the fungal cells were separated and 0.5gms of the cells was placed on a clean dry cover slip, air dried and then subjected to FEG-SEM imaging to determine the shape and size. The instrument used was JSM-7600F with an accelerating voltage of 1kv to 15kv having a resolution of 1.0 nm to 1.5 nm.

Results and discussion:

Enrichment and Isolation of Micro-organisms: Ten different isolates were obtained on enriching the water sample and soil sample in Nutrient broth and in Sabouraud's broth respectively.

Soil and water are known sources of heavy metal biosorbing bacterial isolates. Reports indicate their successful isolation from industrial soil [10].

Screening for Iron Tolerant Micro-organisms:

It was found that out of the ten isolates obtained from the water and soil sample three isolates had the ability to grow in the presence of aqueous solution of 1mM $FeCl_3$. The three isolates selected for further study, were bacterial isolate X_9 from water sample, bacterial isolate S_1 from soil sample and fungal isolate F_1 from soil sample.

Studies indicate that the presence of iron tolerant isolates in the water and soil sample

might be due to the high amount of heavy metal pollutants in the urban and industrial discharge [13].

Biochemical identification and Morphological characterization of the selected isolates: The selected isolates were biochemically characterized as per Bergey's Manual of Systematic Bacteriology Volume 1 and 2 (2nd Edition) [12]. The bacterial isolates X_9 and S_1 were found to have similar characteristics to Bacillus *sp*p. The monograph study that included the study of macro-morphology and micro-morphology of the fungi identified by Agarkar research institute identified the soil fungal isolate F1 as *Paecilomyces variotii Bain.*

Bacillus species are known to have high resistance levels to heavy metals like cadmium and lead [14].Similarly many genera of fungi also have shown tolerance to heavy metals like lead, chromium, copper and zinc [15].

Determination of Iron tolerance levels:

Minimum Inhibitory Concentration assay of $FeCl_3$ on the isolates: The minimum inhibitory concentration (MIC) of the isolates against the heavy metal Ferric chloride ($FeCl_3$) was studied. Bacterial isolates X_9 and S_1 were able to grow in Nutrient broth with 3mM $FeCl_3$ and F_1 was able to grow in Sabouraud's broth with 3mM. This indicates that MIC of $FeCl_3$ on X_9, S_1 and F_1 is 4mM respectively (Table I).

Table I. Determination of Minimum Inhibitory Concentration of bacterial isolates X_9, S_1 and fungal isolate F_1

Conc. of $FeCl_3$ (mM)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
X_9 in Nutrient Broth	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
S_1 in Nutrient Broth	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
F_1 in Saborauds Broth	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-

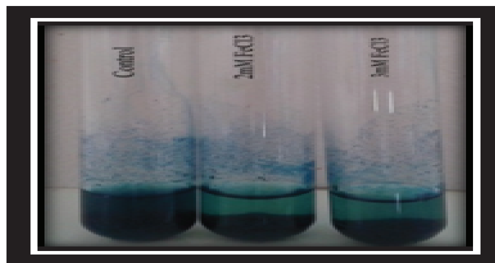
The ability to tolerate upto 3mM of $FeCl_3$ indicates the ability to use these isolates in metal removal and also the theory that contamination with a specific metal increases the level of resistance of the bacterial community to that metal [16]. Also no observable growth of microorganisms at high concentrations explains the theory stated that resistance mechanisms do not offer protection at extremely high levels of free metal ions and a lethal toxic effect is observed [17].

Determination of Minimum Bactericidal concentration (MBC) Minimum Fungicidal Concentration (MFC): X_9 and S_1 isolates showed growth on nutrient media after 24hr incubation which implies that the 4mM-15mM of

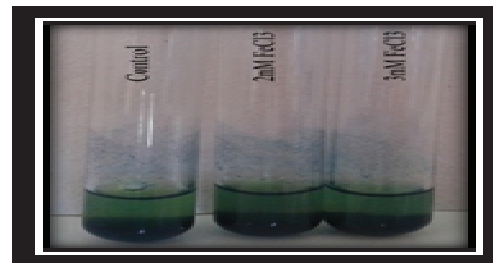
$FeCl_3$ had only bacteriostatic effect on X_9 and S_1 and the effect was not bactericidal. F_1 also showed growth on Sabouraud's media which implies that the 4mM-15mM had a fungi static effect on F_1 and not a fungicidal effect.

Assay for siderophores: A blue and green coloured complex was obtained on treating the intracellular and extracellular extracts of X_9, S_1 and F_1 grown in the presence of enriched medium with Hathaway reagent. This confirms the presence of siderophores in the supernatants. The siderophores may be enhancing the iron uptake by the isolates (Plate1).

Plate I : Observation of presence of siderophores in X_9, S_1 and F_1extracellular extracts



X_9 showing presence of siderophores



S_1 showing presence of siderophores

F_1 showing presence of siderophores

Iron metabolism studies have indicated a potential role of microbial siderophores in facilitating uptake of heavy metals and their mobilisation under certain growth conditions [18].

Production of biomass for iron uptake studies:

On inoculating the isolates in the of presence of the 1mM aqueous solution of $FeCl_3$ only, X_9 showed the production of red colored particles after 24 hrs, while S_1 did not show any significant particle production. F_1 showed complete uptake of $FeCl_3$ after 48 hrs.

This iron uptake and particle production indicates a possible bioremedial activity and nanoparticle producing capability of X_9 and F_1. Studies have indicated that the metabolic activity of microorganisms in the presence of heavy metals lead to precipitation of the metals in the external environment of a cell, the fungi being extremely good candidates for such processes. Formation of magnetite particles proceeds through a sequence of events like reduction of Fe (III) to Fe (II), precipitation of amorphous oxide and subsequent transformation to magnetite. The intracellular methods require a special ion transportation system to transport the metal into the microbial cell [19].

Study of iron uptake by UV Spectroscopy: The UV absorption of 1mM $FeCl_3$ aqueous

solution indicated a peak at 361nm. The absorption wavelength of iron atom is at 248nm. Hence, the U.V. absorbance of extracellular supernatants of X_9, S_1 and F_1 and cell extracts of F_1 sample was compared at 248nm and 361nm. An absorbance reading of 2.5units in 1mM $FeCl_3$, 0.745units in extracellular extract of X_9, 1.380 units in S_1 extracellular extract and 0.18units in F_1 extracellular extracts was observed at 361nm. The absorbance in the cell extracts of F_1 showed an absorbance of 1.335units at 361nm. The comparison in the readings at 361nm indicates an uptake of iron.

Table II: UV absorbance of various samples grown in 1mM $FeCl_3$ for 24hrs at RT

Sr.no	Samples	OD at 248nm	OD at 361nm	Other peaks
1	1mM $FeCl_3$	2.5	2.5	-
2	X_9 extracellular supernatant	0.167	0.7451	244nm,252nm, 269nm,309nm
3	S_1 extracellular supernatant	2.5	1.380	
4	F_1 extracellular supernatant	1.68	0.180	
5	F_1 intracellular supernatant	1.498	1.335	

These readings indicate that the iron uptake is higher in X_9 isolate and F_1 isolate than S_1 isolate. The X_9 extracellular extracts also showed absorbance at 244nm, 252nm,

262nm and 309nm, indicating the presence of certain other oxides of iron. The comparison of absorbance at 248nm also indicated an uptake of iron by X_9 and F_1 but no uptake by S_1 (Table II, Fig1).

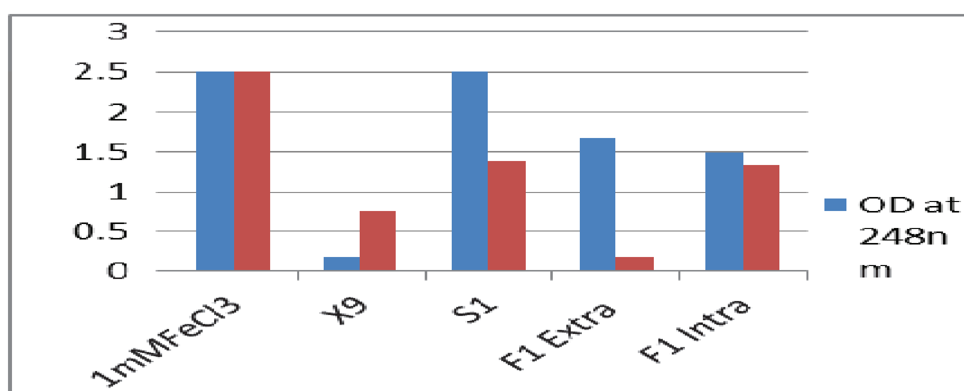


Fig.1: U.V. Absorbance of extracellular samples of X_9, S_1, F_1 and cell extracts of F_1

Extra=extracellular extract, Intra =Intracellular extract

UV Vis absorption studies have been used extensively for studying the iron nanoparticle characterizations and have shown peaks at 240nm and also at 330nm on changing the pH and molar concentrations [20]. The UV absorbance studies indicate a better iron uptake mechanism in isolates X_9 and F_1 in comparison to isolate S_1.

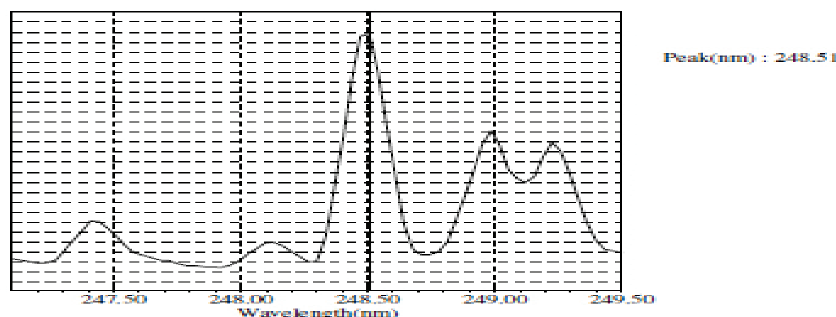
Study of iron uptake by Atomic Absorption Spectroscopy (AAS):

The atomic absorption spectroscopy for iron was performed at 248.15nm. The amount of iron in X_9, S_1 and F_1 extracellular extracts was 2.0118ppm, 2.3581ppm and .0.5765ppm respectively. The amount of iron as read by AAS in the cell extracts of F_1 was 1.6951ppm. These results indicate the uptake of iron by the isolates (Table III, Fig2).

Table III: Concentration of iron in ppm in supernatant of the isolates X_9, S_1 , F_1 and extracellular extract of F_1grown in the presence of 1mM $FeCl_3$

Isolate no	Concentration of Iron (ppm)	Absorbance at 248.15nm
X_9	**2.0118**	0.2374
S_1	**2.3581**	0.2762
F_1(Intracellular)	**1.6958**	0.2020
F_1(Extracellular)	**0.5765**	0.0766

Fig.2:Atomic absorption spectroscopy peak of Iron at 248.51nm



Earlier reports have indicated that AAS is a process that has been used extensively for the estimation of heavy metals like iron and cadmium [21].

The AAS studies of X_9 and S_1 indicate the uptake of large amount of iron as the extracellular extracts after 24 hrs had only about 2ppm while the original iron used in the studies was 1mM (56ppm). Similarly F_1 also showed a larger intracellular uptake of iron. The results do indicate iron uptake by bacterial and fungal isolates.

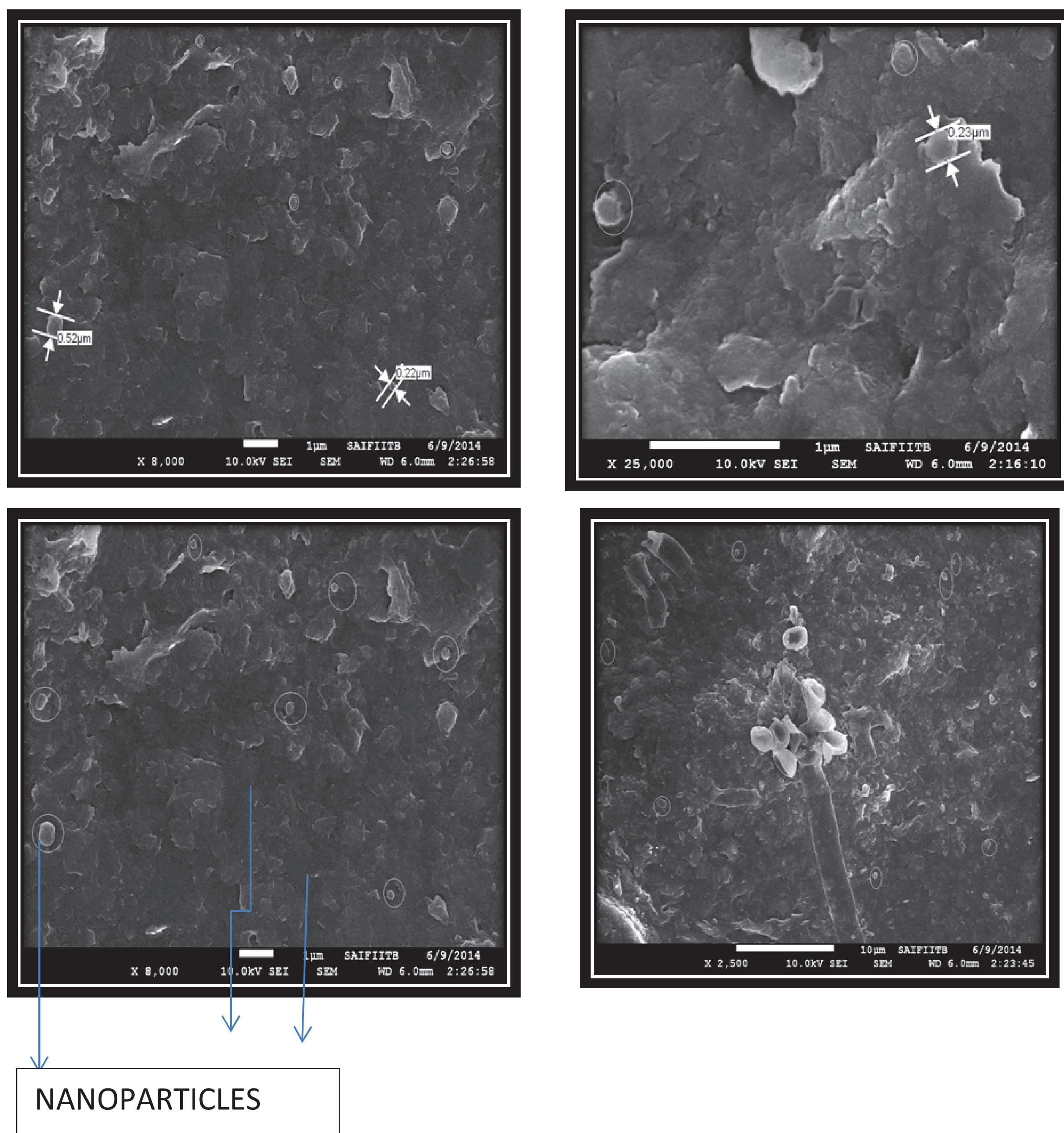
Study of iron uptake by FEG-SEM (Field Emission Gun – Scanning Electron Microscopy):

FEG SEM was used to study the presence of iron

particles on the cell extracts of fungus F_1. The FEG-SEM imaging of F_1 cells showed the presence of iron particles in the range of 220nm to 520nm (Plate2). The particles observed on the cells are spherical in shape, characteristic of certain types of nanoparticles.

This indicates that though nanoparticles of the size below 100nm have not been synthesized by F_1, their synthesis can be achieved by optimising the nanoparticle production conditions using fungi F_1 identified as *Paecilomyces variotii Bain.* Similar work by others have indicated that fungi can be used for the production of nanoparticles. The extracellular mechanism of silver nanoparticles biosynthesis using *Penicillium* fungi has been reported and investigated by UV-Vis spectroscopy, electron microscopy and laser diffraction [22]. Optimization of synthesis of silver nanoparticles using *Fusarium oxysporum has been studied and production of nanoparticles up to 1-100nm size has been achieved [23].*

Plate2 Observation of Iron nanoparticles by FEG-SEM (Field Emission Gun – Scanning Electron Microscopy) in F_1 cells:



Conclusion: Iron tolerant bacteria and fungi were isolated from the water and soil samples. The bacteria were identified as *Bacillus spp* and the fungus was identified as *Paecilomyces variotii Bain*. From the MIC data, it can be said that organisms growing in waters polluted with heavy metals develop a natural resistance towards these metals Isolate X_9, S_1 and F_1 showed growth in presence of 3mM aqueous $FeCl_3$ solution.

The presence of siderophores in all isolates indicates a possibility that the microorganisms may be using siderophores in the uptake of iron. The UV spectroscopic and Atomic absorption studies also indicated the uptake of $FeCl_3$ by X_9, S_1 and F_1. The uptake of iron was more efficient in X_9 and F_1 than in S_1. The FEG SEM imaging indicates formation of particles of 22nm-520nm size which indicates the possibility that the fungal isolate may be converting the $FeCl_3$ into finer particles. This gives the strength to the theory that varying microbial resistance levels to heavy metals may be attributed to a variety of resistance mechanisms in uptake or transport of the toxic metal and also the iron tolerant organisms may be converting them into smaller particles and in a way bio accumulating and bio remediating them to a large extent.

Acknowledgements: We would like to express my gratitude to Dr. Prabhakar Dongre, HOD, Department of Biophysics, Mumbai University and Ms. Jessy John for their invaluable help with UV spectrophotometer studies. We would like to thank of the staff of Dr PS Ramanathan Advanced Instrumentation centre for the Atomic Absorption studies. We would also like to thank the Instrumentation centre at IIT Mumbai for the FEG-SEM imaging and analysis.

References:

1. J.R.Garbarino, H.C. Hayes, D.A.Roth, R.C. Antweiler, T.I.Brinton, H.E. Taylor "Contaminants in the Mississippi river", U. S. Geological Survey Circular, Virginia, U.S.A. 1133, 1995.
2. V.N. Kavamura, E. Esposito, "Biotechnological strategies applied to the decontamination of soils polluted with heavy metals". Biotechnology Advances, Vol. 28, pp.61–69, 2010.
3. D.H. Nies,"Microbial Heavy Metal Resistance", Appl. Microbiol. Biotechnol,Vol51,pp.730-750,1999.
4. I.J.Schalk, M. Hannauer, A.Braud, "New roles for bacterial siderophores in metal transport and tolerance", Environmental Microbiology, Vol.13, No11, pp.2844–2854, 2011.
5. C. Clausen," Isolating metal-tolerant bacteria capable of removing copper, chromium, and arsenic from treated wood",Waste Management and Research, Vol. 18, No.3, pp.264–268, 2000.
6. D.H. Nies, "Efflux-mediated heavy metal resistance in prokaryotes". FEMS Microbial Rev, Vol.27, pp.313–339, 2003.
7. L.J.V. Piddock, "Multidrug-resistance efflux pumps–not just for resistance", Nat Rev Microbial, Vol.4,pp. 629–636, 2006.
8. Q.Affan, E. Shoeb,U. Badar, J.Akhtar, "Isolation and Characterisation of Bacterial Isolates having Heavy Metal Tolerance". Journal of Basic and Applied Sciences,Vol. 5, No.2, 55–60, 2009.
9. D.P.Samarth, C.J. Chandekar and R.K. Bhadekar,"Biosorption of Heavy Metals from Aqueous Solution using *Bacillus Licheniformis"* Int. J. Pure Appl. Sci. Technol., Vol, 10, No2 pp. 12-19 , (2012).
10. M Hookoom and D.Puchooa. Isolation and Identification of Heavy Metals Tolerant Bacteria from Industrial and Agricultural Areas in MauritiusCurrent Research in Microbiology and Biotechnology, Vol. 1, No. 3

pp.119-123, (2013).

11. S. Silver, “Bacterial resistance to toxic metal ions a review”. J. Env. Health Perspective,Vol.105, No.1, pp.98-102, 1996.
12. K.Kersters,M. Vancanneyt,“Bergey's Manual of Systematic Bacteriology”, Springer Verlag, Vancouver, 2005.
13. R.S. Lokhande, P.U.Singare, D.S.Pimple, “Quantification Study of Toxic Heavy Metal Pollutants in Sediment Samples Collected From Kasardi River Flowing Along the Taloja Industrial Area of Mumbai, India”. New York Science Journal, Vol.4,No.9, pp.66–71, 2011.
14. S.Nath, B.Deb, I.Sharma,“Isolation and Characterization of Cadmium and Lead Resistant Bacteria”,Global Advanced Research Journal of Microbiology Vol.1, No.11 pp.194-198,2012.
15. L.Ezzouhri,L. Castro, M.Moya, F.Espinola , K.Lairini “Heavy metal tolerance of filamentous fungi isolated from polluted sites in Tangier, .Morocco”,African Journal of Microbiology Research Vol. 3 ,No.2, pp. 35-48, 2009.
16. U.Badar, R. Abbas, N.Ahmed, “Characterization of copper and chromate resistant bacteria isolated from Karachi tanneries effluents”, J. Ind. Env. Bio, Vol. 39,pp.43-54. 2000.
17. A. Konopka, T. Zakharova, M.Bischoff, L.Oliver, C.Nakastu,R.F.Turco, “Microbial biomass and activity in lead contaminated soil”, J. Appl. Env. Microbiol,Vol 65, No.5, pp.2256-2259, 1999.
18. Y.Chen, E.Jurkevich, E.B. Ness, Y. Hadar,“Stability constant of pseudobactin complexes with transition metals”, Soil Sci. Am. J,Vol.58, pp 390-396,1994.
19. S. Yaghmei, S. Anahid, Z. Ghobadinejad, “Heavy metal tolerance of Fungi”, Scientis Iranica , vol18, N03, pp.502-508,2011.
20. B.Kumar, K.Smita, L. Cumbal, A. Debut, “Biogenic synthesis of iron oxide nanoparticles for 2-arylbenzimidazole fabrication”, Journal of Saudi Chemical Society,Vol. 18, No.4, pp. 364–369, 2014.
21. A.Louhi, A.Hammadi, M.Achouri, “Determination of Some Heavy Metal Pollutants in Sediments of the Seybouse River in Annaba, Algeria”, Air, Soil and Water Research, Vol.5, pp.91–101, 2012.
22. Z. Sadowski, I. H. Maliszewska,B. Grochowalska, I. Polowczyk, T. Koźlecki, “Synthesis of silver nanoparticles using microorganisms” Materials Science-Poland, Vol. 26, No. 2, 2008.
23. H.Korbekandi, Z.Ashari, S. Iravani S.Abbasi, “Optimization of Biological Synthesis of Silver Nanoparticles using *Fusarium oxysporum”,* Iranian Journal of Pharmaceutical Research, Vol.12, No.3, pp.289-298, 2013.

***

Amudan R./ Fatima S. /Department of Biotechnology/

S.I.E.S. College of Arts Science and Commerce/ Sion (West)/ Mumbai 400022/INDIA

ramudhan@gmail.com/ corresponding author