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Determination of Bio-Sorption Potential of Microorganisms against Heavy Metal Lead

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Abstract

Environmental pollution is currently a global problem. The presence of heavy metals in lake water released by industries causes serious danger to the environment and to many other life forms which are in frequent contact with it. The objective of the study was to isolate organisms and check for their bio-sorption potential towards heavy metal lead. Among the isolates, bacteria – Bacillus sp, Pseudomonas sp and fungal strains – Aspergillus niger, Aspergillus flavus, Penicillium sp, and Cladosporium sp were found to be resistant to lead. For bacteria, Maximum Tolerable Concentration (MTC) was performed up to 500ppm of lead concentration and checked colorimetrically. All the fungal isolates showed growth in all concentrations of lead but there was decrease in growth as the concentration increased. The bio-sorption potential was investigated by atomic absorption spectrophotometer (AAS), FTIR and Scanning Electron Microscope (SEM). The results of AAS showed that Penicillium sp had higher bio sorption ability of 26.6% whereas Cladosporium sp showed minimal adsorption of 7.2%. SEM micrographs revealed localization of lead on the mycelial mat of the organisms. These results suggested that Penicillium sp. has potential application in the bioremediation of lead contamination in water.

Kaywords: *Maximum Tolerable Concentration, Bio sorption, Bioremediation, Heavy Metal Tolerance*

1.0 Introduction

Heavy metals are the metals or metalloids that are highly toxic or damaging to the environment. They are found in the earth's crust and are non-biodegradable therefore are persistent in the environment. The frequently found heavy metals are lead (Pb), cadmium (Cd), mercury (Hg), arsenic (As), chromium (Cr), thallium (Tl), zinc (Zn), nickel (Ni), copper (Cu), barium (Ba), selenium (Se) etc. They have a relative density ranging from 3.5 to 7gcm-3. The presence of air, water and soil therefore causing pollution (Ravindra K Gautam, et al., 2015).

Heavy metal contamination is predicament for the environment. These contaminants are discharged into the environment by various human activities including industrial discharge, wastewater treatment, mining, agricultural runoff (fertilizers and pesticides – erosion) etc. Air, water and soil are highly affected by heavy metal pollution (Vhahangwele Masindi, et al., 2018). The uptake of heavy metals by plants and successive accumulation in human soft tissues and bio magnifications through the food chain leads to adverse health issues in living organisms (Singh, et al., 2011).

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However, Indian cities are prone to industrialization and urbanization resulting in rapid increase of pollution. Hence, the fresh water bodies are contaminated due to unscientific disposal of wastes or long-term discharge of untreated domestic and industrial wastewater, solid dumping of waste etc. which has caused immense problems not only to humans but also to aquatic environment[3].

Peenya is regarded as the oldest and largest industrial area of Southeast Asia, situated in Bengaluru, India. Presence of about 5000 small scale industries and 30 large scale industries, this place has turn to be hazardous to the water bodies surrounding it. The release of toxic effluents and heavy metals hinder the natural environment. Working in or living near an industrial site which utilizes these metals and their compounds increase one's risk of exposure, as does living near a site where the metals have been improperly disposed (Sabine Martin, et al., 2009).

2.0 Material and Methods

2.1 Isolation of Organisms

The water sample was obtained from Shivapura kere in Peenya industrial area. The heavy metal resistant microorganisms were isolated from the water sample by serially diluting the sample with sterile distilled water and plating using pour plate method. For plating the samples were plated on nutrient agar and potato dextrose agar containing heavy metal lead for bacteria and fungi respectively. The organisms were identified by performing staining and biochemical tests.

2.2 Media

The culture media contains essential nutrients that supports the growth and development of microorganisms. Based on consistency, it can be a solid medium (agar) or a liquid medium (broth). Different types of synthetic media are available for the isolation and growth of specific organisms.

Nutrient agar, potato dextrose agar were used for culturing of bacteria and fungi respectively. Pseudomonas agar was used for the specific isolation of Pseudomonas sp. For performing experiments, nutrient agar and Sabouraud's dextrose broth were used along with different concentrations of heavy metal salts to determine the growth and heavy metal resistance of the organisms.

2.3 Source of Heavy Metal

In this study, the chemical salt of lead (Pb) from HiMedia as a source of heavy metal was used.

2.4 Identification of Isolated Heavy Metal Resistant Microorganisms

Basic identification of microorganisms was done based on their morphological characteristics. Staining is used to visualize the morphology of the microorganisms. Staining is a technique for enhancing the contrast between organism and the background with the help of chemical dyes.

In present study, gram staining and endospore staining techniques to identify bacteria and lactophenol cotton blue staining for identification of fungi was used.

2.5 Determination of Maximum Tolerable Concentration of Heavy Metal

Maximum tolerable concentration (MTC) is the highest concentration of heavy metals which has no effect on the growth of test organism. MTC can be determined using different methods.

2.5.1 Agar Well Diffusion Technique

In this technique suitable media (nutrient agar for bacteria, potato dextrose agar for fungi) was prepared and autoclaved at 121ºC for 15 minutes at 15 psi pressure. After autoclaving media was poured into sterile petri plates and allowed to solidify. Lawn culture of organisms were done by swabbing technique. Wells of 6mm were bored using the sterile well borer. To each well 50µl of heavy metal solution of different concentrations was added and incubated at respective temperatures and time, i.e.,37ºC and 24 hrs for bacteria; 25ºC and 5-7 days for fungi. Similarly disc diffusion technique was also done using sterile filter paper disc dipped in heavy metal solutions.

2.5.2 Dilution Technique

Sabouraud's dextrose broth containing different concentrations of lead from 50ppm to 100ppm was prepared and spore suspension of organism was inoculated and incubated at 25ºC for 5-7 days. Calculation for amount of Heavy Metal to be added in the media $Pb(NO_3)_2$ salt was used as a heavy metal source for lead (Pb). The concentration of lead was calculated and added in terms of ppm. The formula used for calculation:

$$\text{Gram equivalent of Pb} = \frac{\text{Molecular weight of } Pb(NO_3)_2 \times 100}{\text{Atomic weight of Pb} \times \text{percentage purity}}$$

2.6 Determination of Tolerance Potential of Different Bacterial Species Towards Lead

Sterile nutrient broth tubes were prepared along with different concentrations of lead [$Pb(NO_3)_2$] (25ppm, 50ppm, 75ppm and 100ppm). To these tubes 0.1ml of the isolated

bacterial strains were inoculated. The nutrient broth tube without organism and heavy metal was used as a blank. The tubes were incubated at 37^0C for 24 hours. The OD values were taken after incubation using colorimeter at 540nm wavelength.

2.7 Determination of Tolerance Potential of Different Fungal Species Towards Lead

Sterile sabouraud dextrose broth with different concentrations of lead (25ppm, 50ppm, 75ppm and 100ppm) heavy metal were prepared. To these tubes fungal spores were inoculated. The broth without heavy metal with spores was used as control. The conical flasks were then incubated at 27ºC for 7 days. The inoculated flasks were observed for growth of the test organism by comparing against the organism control.

2.8 Sample Preparation for AAS, Sem and Ftir Analysis

75ppm and 100ppm concentrations of heavy metal lead were prepared. 2 gms of mycelial mat was weighed and inoculated into the sterile heavy metal solution aseptically. Similarly, the mycelial mat was inoculated into the sterile distilled water without heavy metal and was used as control. The inoculated flasks were then incubated for 7 days at 27ºC. the mycelial mat was removed by filtration and the filtrate was used for residual heavy metal concentration and the mycelial mat was dried and powdered and used for AAS, FTIR and SEM analysis.

3.0 Results

3.1 Isolation of Microorganisms

Out of 2 samples, 22 bacterial isolates (Fig.1) and 25 fungal isolates (Fig.2) from different genera were isolated by spread and pour plate technique Second round of screening was done by growing the microorganisms in varying concentrations of lead and 3 bacterial and 4 fungal isolated were screening depending upon there tolerance to lead.

The cultures were observed for colony morphology and the bacterial colonies were subjected to gram staining, while the fungal colonies were stained with lactophenol cotton blue. Bacterial isolate 1 was found to be gram positive rods with central endospores; Bacterial isolate 2 was observed as gram positive rods with terminal endospores; bacterial isolate 3 was gram negative short rods.

Based on cultural characteristics, colony morphology and microscopic observations the fungal species were identified as: Aspergillus niger – the upper surface of the colony

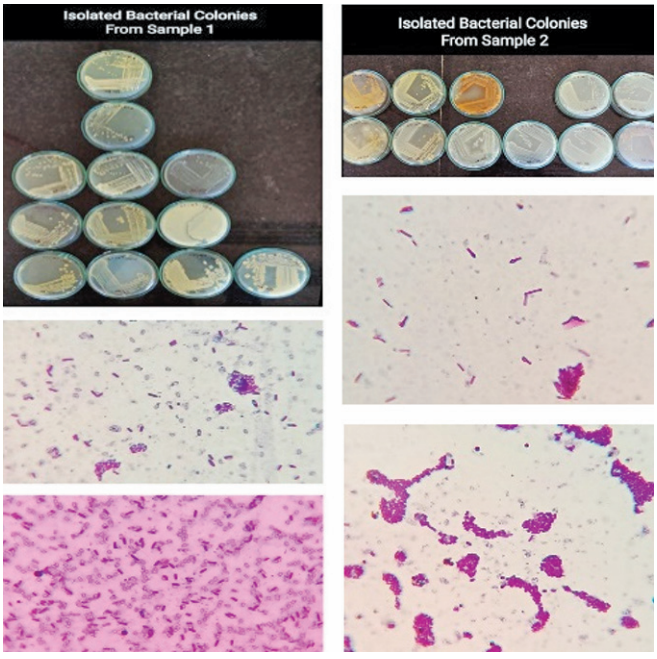

Figure 1: Isolated colonies of bacteria from the samples and staining of the bacterial isolates

Figure 2: Isolated fungal colonies from the samples

Table 1: Morphological characteristics of fungal isolates

	Culture	Media	Colony morphology	Organism identified
1	Fungi-1	Potato dextrose agar	Black upper surface, white lower surface	Aspergillus niger
2	Fungi-2	Potato dextrose agar	Dark green upper surface, pink lower surface	Aspergillus flavus
3	Fungi-3	Potato dextrose agar	Green cottony upper surface, light orange lower surface	Penicillium sp.
4	Fungi-4	Potato dextrose agar	Light green upper surface, blackish lower surface	Cladosporium sp.

Table 2: Biochemical tests conducted for bacterial isolates

Pseudomonas	Bacillus 2	Bacillus 1	Organism
Gram negative short rods	Gram positive rods	Gram positive rods	Gram staining
No endospore	Terminal endospore	Central endospore	Endospore staining
+	+	+	Catalase test
+	+	+	Oxidase test
-	-	-	Indole test
-	-	+	Methyl red test
+	-	+	VP test
-	-	-	Citrate test
-	-	+	Glucose Fermentation
-	+	+	Mannitol
+	+	+	Nitrate Reduction
+	+	+	Motility

appeared black and lower surface was white in colour. It contanins black round vesicle surrounded by chains of conidiospores arranged radially. Aspergillus flavus – the colony was dark green on the upper surface while pink in the lower surface. The mycelium appeared blue in colour having round vesicle and radially arranged conidiospores. Penicillium spp – green cottony upper surface with light orange lower surface. It has brush like spore bearing structures on which conidiospores are arranged in chains. Cldosporium spp – the colonies produced appear olive green upper surface with dark or blackish lower surface. The hyphae and conidia are darkly pigmented and has simple or branching short chains of conidiospores (Table 1).

3.2 Biochemical tests

The bacterial species were subjected to different biochemical test. All the organisms showed negative test for indole and citrate. All the test organisms were positive for catalase, oxidase, motility and nitrate reduction. Both the Bacillus spp were positive for mannitol fermentation while Pseudomonas spp was negative. Bacillus 1 was positive for methyl red, glucose fermentation whereas Bacillus 2 and Pseudomonas spp were negative. Bacillus 1 and Pseudomonas spp were positive while Bacillus 2 was negative (Table 2).

3.3 Well Diffusion Method

Agar well diffusion method was carried out for the test organisms to check the resistance or sensitivity of organisms against the heavy metal provided. Initially, the bacterial cultures were sensitive to lead (concentration ranging from 250mM-1500mM) due to more precipitation of lead into the media. Later, when the concentration of lead was reduced to 50mM-250mM, the test organisms (both bacteria and fungi) showed visible growth and are resistant to all the concentrations of lead (Fig.3).

3.4 Disc Diffusion Method

The discs containing 20microliters of lead were used in the media. This method was followed to overcome precipitation of lead into the media. The test organisms showed visible growth in the medium and therefore are resistant to lead of concentration from 50mM-250mM in respective media (Fig.4).

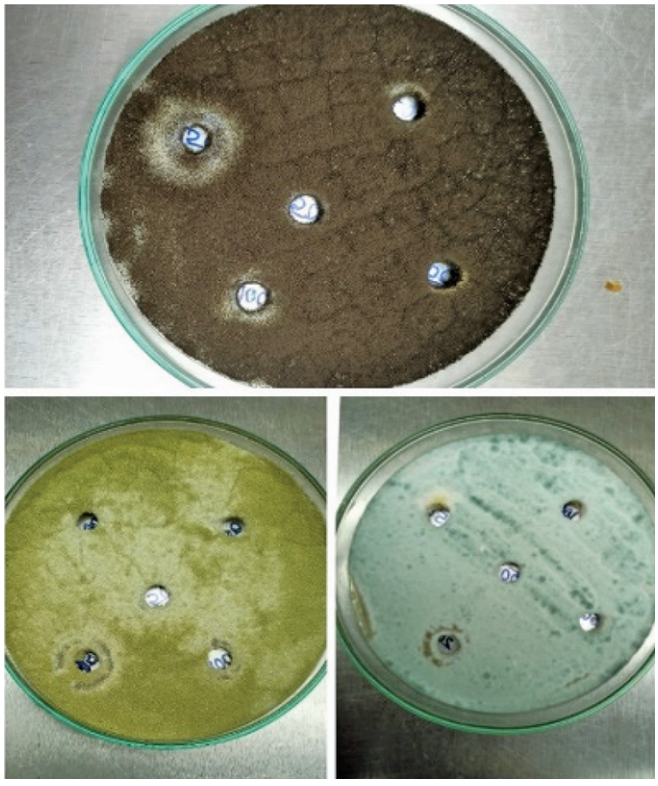

Figure 3: Well diffusion test for the fungal isolates

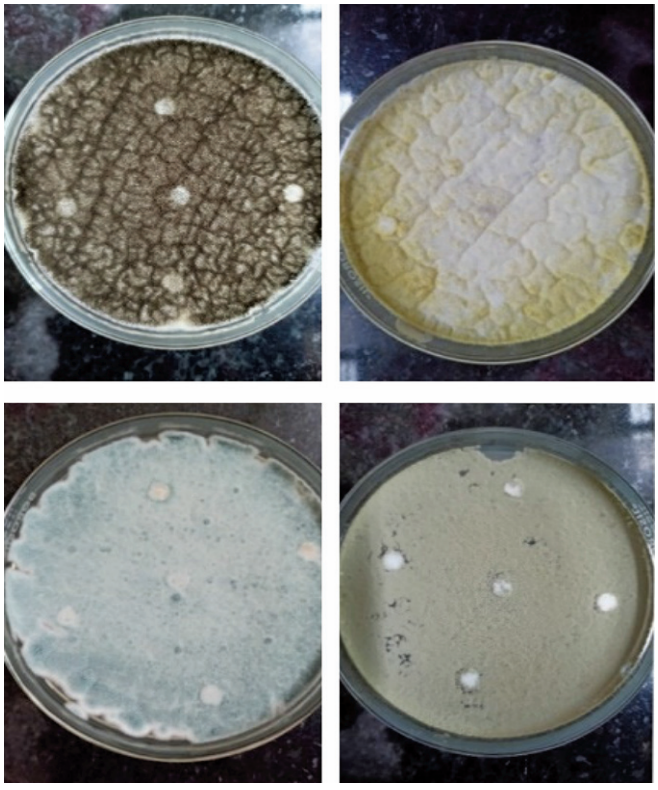

Figure 4: Disc diffusion test done for fungal isolates

3.5 Biosorption of Lead (Pb) by Bacteria

The test organisms were grown in nutrient broth containing heavy metal Pb of different concentrations (25ppm, 50ppm, 75ppm and 100ppm). The organisms showed good growth in all the concentrations after 24 hrs of incubation. The absorbance of these tubes were observed at 540nm and recorded. There was decrease in the growth from 25ppm to 100ppm. The maximum growth was found at 50ppm for Bacillus 2 (0.35), and minimum growth was at 100ppm for Pseudomonas (0.07). The results are tabulated in Table 3:

Maximum tolerable concentration (MTC) was found to be 500ppm for all the test organisms. The maximum growth was seen in Bacillus 2 (0.56) at 100ppm. The least growth was observed for Pseudomonas spp (0.12) at 500ppm (Fig.5).

3.6 Biosorption of Lead (Pb) by Fungi

The test organisms grown in SD broth in the presence of different concentration of Pb (25ppm, 50ppm, 75ppm and 100ppm) showed formation of mycelial mat in decreased manner from 25ppm to 100ppm compared to the mycelial mat formed in the media without heavy metal (Table 4).

The mycelial mat showed good biosorption activity when again inoculated into heavy metal solution which was analysed by atomic absorption spectrophotometer (AAS). The results showed that there was considerable reduce in Pb

Table 3: Absorbance values at 540nm for the test organisms at 25ppm to 100ppm concentrations of heavy metal lead

Organism Concentration of Pb (ppm)	Heavy metal control	Bacillus 1	Bacillus 2	Pseudo-monas
25	0.02	0.28	0.19	0.26
50	0.03	0.28	0.35	0.21
75	0.07	0.24	0.17	0.15
100	0.07	0.15	0.14	0.07

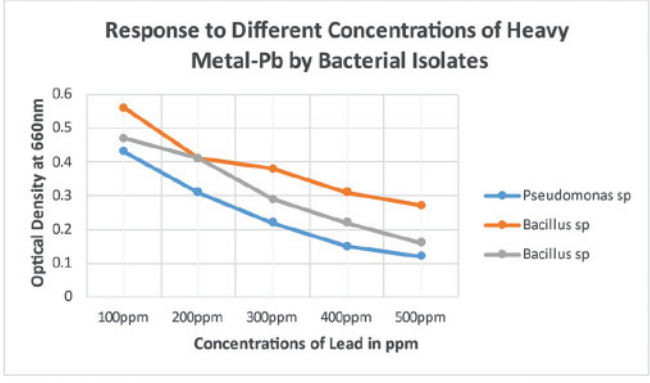

Figure 5: Absorbance values at 540nm for the test organisms at 100ppm to 500ppm concentrations

Table 4: Residual concentration of lead measured by AAS

	Organism	Concentration of Pb (in ppm)	Residual Pb (in ppm)	Percentage of adsorption (%)
1	Aspergillus niger	75	67.2	10.4
2	Aspergillus niger	100	84.6	15.4
3	Aspergillus flavus	75	61.8	17.6
4	Aspergillus flavus	100	80.0	20.0
5	Cladosporium spp	75	69.6	7.2
6	Cladosporium spp	100	88.2	11.8
7	Penicillium spp	75	58.1	22.53
8	Penicillium spp	100	73.4	26.6

concentration in all the samples. The percentage adsorption was calculated and the results are shown in Fig.6.

Penicillium spp showed higher adsorption potential of 26.6% for 100ppm and Cladosporium spp showed minimal adsorption of 7.2% for 75ppm concentration (Fig.6).

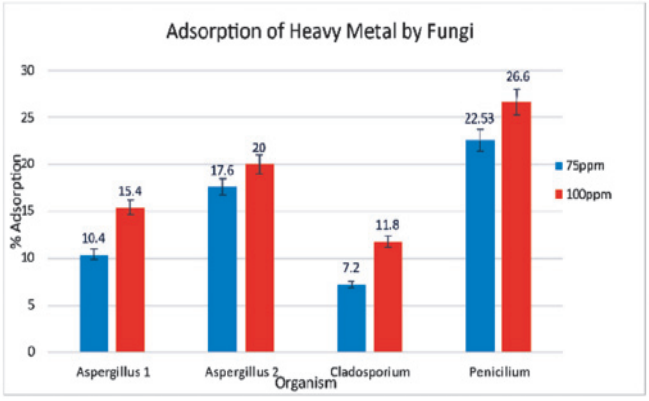

Figure 6: Adsorption of heavy metal by fungi

3.7 Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

FT-IR spectra of fungal mat before and after adsorption of lead is checked and represented in Figs.4.5a to 4.5d for different fungal isolates. The IR spectra used for scanning was in the range of 400 to 4000 cm^{-1}. Different characteristic peaks were observed in the sample with and without heavy metal. This result indicated there is change in the wave peaks due to the interaction of heavy metal and active sites on the biomass. The biosorption mechanism analyzed using Fourier Transform Infrared Spectroscopy (FTIR) showed the presence of N-H stretching vibrations at 3257 cm-1 and 3272 cm-1 in Aspergillus cultures, these cultures also showed C=C stretching vibration of alkene functional groups at 1600cm^{-1} to 1700cm-1. In case of Cladosporium it showed the presence of CH_2 stretching vibrations at 2936cm^{-1}, the band also represented C=O stretching vibrations of carboxylic acid at 2856cm^{-1}, some peaks in the range of 1040 to 1234 cm^{-1} represented stretching vibrations of C-O bonds. Penicillium showed O-H vibration at 3285cm^{-1} and also represented C=O stretching vibrations of carboxylic acid at 2927cm^{-1} also some peaks in the range of 1040 to 1234cm^{-1} represented stretching vibrations of C-O bonds. Penicillium showed significant shifts of these specific peaks to the higher wave number after adsorption of lead indicating chemical interactions between lead ions and amide groups, alcoholic groups, carboxylic groups (Fig.7).

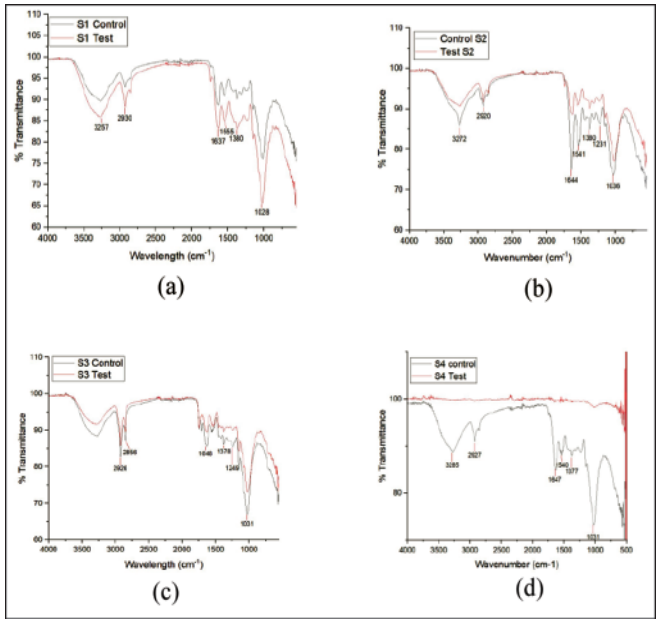

Figure 7: FT IR spectra of a. Aspergillus niger, b. A. flavus, b. Cldosporium sp. d. Penicillium sp

3.8 Scanning Electron Microscopy

SEM-EDX spectroscopy was used to examine the fungal biomass in presence of heavy metal lead (Chao Zhang et.al., 2019). Image intensity is related to the average atomic number, with brighter image areas indicating higher atomic number. These techniques show topographical and elemental information of bio-sorbent characteristics of the organism. Different sections of fungal mycelial mat were observed for bio-sorbent characteristics towards lead. There were many small particles were adhered to the surface of the cell wall. The area of bright spots in the micrograph exposed to lead indicated the area of higher atomic number which indicated presence of lead.

4.0 Discussion

The industrialization and anthropogenic activities has increased the environmental pollution rate by letting out large quantities of effluents containing toxic heavy metals (such as

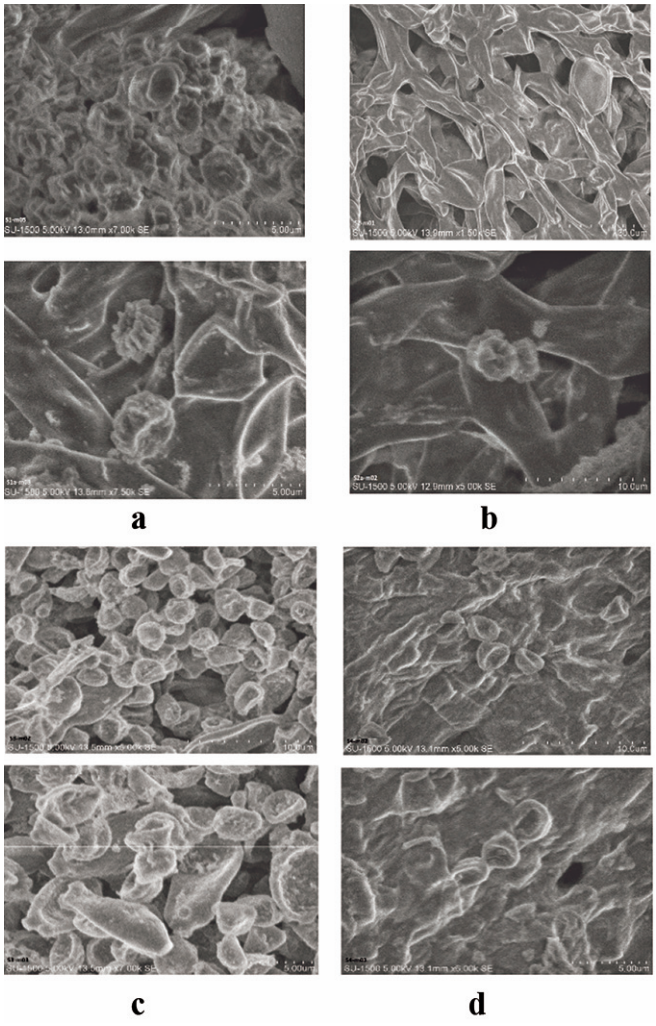

Figure 8: SEM Micrographs of (a) Aspergillus niger, (b) A. flavus, (c) Cldosporium sp. (d) Penicillium sp

Pb, Cd, Hg, Cr, and Zn). This is due to unscientific disposal of waste or long term discharge of untreated industrial and domestic wastewater. This has caused immense problems not only to humans but also to other living organisms. Bioremediation is the one of the effective methodology and is defined as "the technology using microorganisms for removal of these toxic elements from polluted samples" and it is effective compared to other conventional techniques. The bioremediation technique is cost-effective, safe and eco-friendly.

In this study we isolated microorganisms from lake water sample and screened them for tolerance of heavy metal lead. The organisms that showed growth in media containing heavy metal lead were subjected to identification by Gram's staining for bacteria and lactophenol cotton blue staining for fungi. From the microscopic observation, bacteria 1 was Gram positive rods with central endospores and hence was identified as Bacillus sp 1; bacteria 2 was Gram positive rods with terminal endospores and hence identified as Bacillus sp 2; bacteria 3 was Gram negative short rods and hence identified as Pseudomonas sp. Also, biochemical tests were performed for further identification of bacterial strains. Bacillus 1 was positive for Catalase, Oxidase, Methyl Red, Voges-Proskauer, Glucose fermentation, Mannitol, Nitrate reduction and Motility; negative for Indole and Citrate utilization test. Bacillus 2 was positive for Catalase, Oxidase, Mannitol, Nitrate reduction and Motility; negative for Indole, Methyl Red, Voges-Proskauer, Glucose fermentation and Citrate utilization test. Pseudomonas sp was positive for Catalase, Oxidase, Voges-Proskauer, Nitrate reduction and Motility; negative for Indole, Methyl Red, Glucose fermentation, Mannitol and Citrate utilization test.

From the microscopic observation and colony characteristics the fungal isolates were identified as Aspergillus niger, Aspergillus flavus, Penicillium sp and Cladosporium sp.

Initially the biosorption experiment was performed with mM concentration of lead (250mM -1000mM) for bacteria and fungi. Well diffusion and disc diffusion methods were carried out to check the resistance of organisms. All the test organisms showed resistance towards all the concentration of lead. But as there was excess of precipitation in the liquid media after addition of lead nitrate solution the growth of bacteria was difficult to measure. Hence the experiment was carried out by changing the concentration of lead nitrate from mM (mili Molar) to ppm (parts per million). The bacterial isolates were grown in nutrient broth containing different concentration of heavy metal lead i.e., 25ppm, 50ppm, 75ppm and 100ppm. The growths of the organisms were then measured colorimetrically at 540nm after 24 hours of incubation. The maximum growth was found for bacillus 2 at 50ppm (0.35) and the minimum growth was at 100ppm for Pseudomonas (0.07).

To estimate the maximum tolerable concentration (MTC) of the bacterial isolates, they were inoculated into broth containing higher concentrations of lead 100ppm, 200ppm, 300ppm, 400ppm and 500ppm. Then the absorbance was measured colorimetrically to check the growth of organisms after 24 hours incubation. The absorbance values indicated that 500ppm was the MTC for all the test organisms and the optimum growth was found to be at 100ppm. The growth of organisms decreased due to increase in concentration of lead and there was least growth at 500ppm hence it was considered to be the MTC.

The fungal isolates were inoculated in 50ml of sabourauds dextrose (SD) broth with different concentrations of heavy metal lead (25ppm, 50ppm, 75ppm and 100ppm) and without any heavy metal (control). After 7 days of incubation, it was observed that the formation of mycelial mats was visibly decreasing as the concentration of heavy metal increased. The mycelial mats re-inoculated in 300ml of heavy metal solution (100ppm) was dried and sent for FTIR and SEM analysis. And the heavy metal solution of re-inoculated mat (75ppm and 100ppm) was sent for AAS to check for the residual lead present in it. From the results of AAS, the percentage adsorption was

calculated. It indicated that Penicillium sp. exhibited highest adsorption of 26.6% at 100ppm (73.4ppm of residual lead). FTIR and SEM analysis will help us to know the groups and cell components involved in the mechanism of adsorption. Results of FTIR and SEM analysis are awaited. The dried fungal mat inoculated in heavy metal solution should be further analyzed.

5.0 Conclusions

Heavy metal contamination is predicament for the environment. These contaminants are discharged into the environment by various human activities including industrial discharge, mining, waste water treatment and agricultural run out etc. Working in or living near an industrial site discharges heavy metals with proper treatment can contaminate the waterbodies and also increase one's risk of exposure therefore causing serious health issues. Bioremediation is one of the effective methods in removal of heavy metals from the contaminated environment compared to other conventional methods. The use of microorganisms in bioremediation process has many advantages like cost effective, eco-friendly and easier to handle. So, in this study we tried to identify the organism for better biosorption potential and its mechanism. Firstly, the organisms that showed resistance to heavy metal lead, were isolated from the lake sample. These isolates were identified as Bacillus 1, Bacillus 2, Pseudomonas, Aspergillus niger, Aspergillus flavus, Cladosporium sp., and Penicillium sp. by performing Gram staining, endospore staining and biochemical tests for bacteria and Lactophenol cotton blue staining for fungi. The isolated were then checked for biosorption potential by inoculating them in media containing different concentrations of heavy metal Pb. The biosorption potential of bacteria was determined colorimetrically. The fungal samples were analysed by atomic absorption spectrometry (AAS) to calculate the % adsorption of lead. Fourier Transform Infrared (FTIR) Spectroscopy was used to analyse the functional groups present on the surface of different fungal isolates and also the functional groups which are responsible for the interaction of fungal surface and the heavy metal-lead. Scanning Electron Microscopy (SEM) revealed the presence of adsorbed heavy metal-lead was sequestered on the mycelial mat. From the absorbance values for bacteria, it was observed that though all the organisms were capable of growing even at higher concentrations of lead, there was decrease in growth as the concentration increased. But it can be inferred that Bacillus 2 was more efficient that other two isolates. From the results of AAS it was observed that all the test organisms were efficient in removal of lead from the solution. Among the four fungal strains, Penicillium was much more efficient in removal of lead. The results suggested that the isolates Bacillus 2 and Penicillium sp. has potential application in the bioremediation of lead which can be further investigated to develop a novel strategy for removal of lead from contaminated water.

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