Mycoremediation Potential of *Aspergillus niger* against Heavy Metals of Amanishah Nallah, Jaipur

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Abstract
Developing countries face pollution from industrial waters, which are not treated before release. Mycoremediation, a practical and economical solution, can be effective using filamentous fungal species like Aspergillus sp., secreting organic acids. The main objective of the study is to study concentration of heavy metal and to identify filamentous fungal species which have ability to detoxify the HMs. The location of the sampling area is: Amanishah ka Nalah in Sanganer City which was earlier known as Dravaywati river because vegetables were cultivated in the field of industrial and sewerage waste water. Grab sampling methodology adopted to take samples from the different locations of flow of nallah i.e. residential, non-residential and industrial area to analysed heavy metals.

The atomic absorption spectroscopy (AAS) shows that waste water contains cadmium 1.0 to 1.20, copper 1.0 to 1.20 and nickel 0.94 to 1.21 milligrams/liter. Fungal sp. Were isolated from freshly collected soil on microbial culture media (PDA). Microbial identification was done by using PCR and sequencing to identified fungal sp. i.e. Aspergillus niger. It shows a decrease in the toxicity of mainly two heavy metals Ni and Cu whereas Cd is able to inhibit the growth of Aspergillus niger to some extent. The analysis underscores the significance of A. niger of heavy metals remediation, emphasizing requirement for further analysis and technical improvements to fully utilize their metabolites.

Keywords: Waste water, Fungus, *Aspergillus sp*, Mycoremediation; Heavy Metals, Atomic Absorption Spectrophotometer, Genome sequencing, PCR.

Introduction

In the emerging Nation, particularly in India, water pollution is a main concern due to industrialization[4]. USEPA has considered heavy metal as primary concerned toxic metals such as chromium, nickel, cadmium, lead, arsenic, zinc and copper that have severe threatening remarks on the human health and environment. Waste water in Amanishah nala contains huge amount of metallic elements having relatively high density because the discharge of water from the printing and textile industries. These industries use metal based synthetic chemicals as dye which are hazardous for living beings i.e. humans, plants and animal health. The metal complex dye contains Zn, Cr and Co and wool dying agents contains Cr salts that cause major metal pollutant[7].

The oral exposure of cadmium harms human kidney, so it is known as human carcinogen[13]. Nickel can cause lung cancer, cardiovascular diseases, nasal and allergy in human body through breathing the contaminated air[9]. Dravaywati river which was source of usable water century ago, has deteriorated by sewage waste and industrial waste known as Amanisha nalla. There are numbers of textile dying and printing industries in Sanganer city of Jaipur allocated at the periphery of nalah discharging effluent water that contains heavy metals such as Cr, Cu, Pd, Fe and Zn without any treatment. These dissolved toxicants are taken up by plants and soil. This waste water brought numerous issues such as ground water contamination and detrimental impacts on environment and human health[12].

Mycoremediation is a viable and long term strategy in a cost effective and ecofriendly approach to overcome the issues arising from the industrial waste water discharge into the natural water bodies. The natural metabolites have the enough capabilities to degrade and detoxify the heavy metals from the waste water. Mycoremediation is a futuristic technology that can transform or degrade toxic elements into non or less toxic forms. Each fungal strain has a specific activity against selected heavy metals so mycoremediation is effective when specified fungal sp. targets the heavy metal. It can be assumed that mycoremediation could be affordable and environment friendly solution for the water pollution. *Aspergillus niger* creates acids to solubilize metals like Ni and Cu and transform in less hazardous elements which can be expelled from the environment.

Material and Methods

Site description: Water sampling was done from the different locations of Nalah earlier known as Dravyavati River that originates in western slope of Amber hills passing from north to south direction in Jaipur city.The length of the river is only 57.5 km. It is smallest river in India supplying the water to the city. The river merges with Dhund river which is close to Santoshpura. The major portion of the Jaipur is living within the 10 km of periphery of this river. By the time passing and rapid industrialization, the river deteriorated and get damaged due to discharge of the untreated waste water to transform into a nallah. Sewerage from the urban areas was diluted with domestic wastewater and industrial effluents drains into the river from Jawahar

Nallah and Nahri ka nallah. In July 1981, major flooding occurred in the river. Due to urbanization and industrialization, encroachment caused pollution that changed the quality of the river. There are so many projects to rejuvenate the river. There are so many projects to rejuvenate the river.

Water sampling: Water samples were collected from three distinct locations on the Amanishah Nallah. Seven samples were collected from the polluted sites which are RIICO (Industrial area), Manasarovar (Urban area) and Khatipura (common area) of Amanishah Nalla using high-density, screw-capped, well-labeled, pre-sterilized polyethylene terephthalate (PET) bottles. Before the sampling, all PET bottles were rinsed with water of selected locations. Each source of water sampling was at about 500m to 1500 m far from the waste water discharge point of industries. The samples were collected away from the edge of nalah and below 100 m from the surface. After that samples were labeled for identification of locations and preserved for laboratory analysis. The water samples were preserved by adding HNO_3 to maintain pH <2 for 7 days at 40℃ in a fridge. All the protocols for preserving and analysis were followed[3].

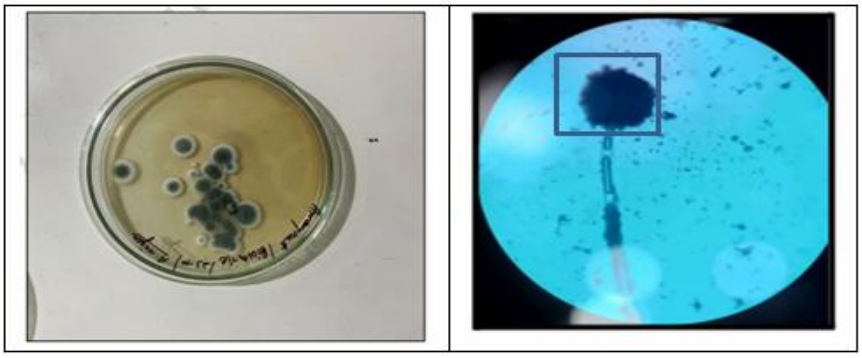
Study of heavy metals by Atomic Absorption Spectroscopy: Atomic absorption spectrometry (AAS) was used to examine for heavy metals in the lab. Analysis was done on the chosen heavy metals Cd, Cu and Ni. Standards of Cd and Cu of 0.2, 0.4 and 0.8 PPM and Ni of 1.0, 2.0, 4.0 PPM were used to standardize the instrument. Take 100 ml of water sample in a beaker and add 2.0 ml of nitric acid. After that concentrate the sample by heating to approx 25 ml. Cool and filter the sample filter through Whatmann no. 41 filter paper and make upto 25 ml with distilled water. Finally, these solutions were analyzed by AAS (AAS software analysis i.e. Thermo scientific AA 303 Ver. 6.1).

World Health Organization (WHO) has recommended guidelines on water quality for human health worldwide whereas Central Pollution Control Board (CPCB) has guidelines for quality of drinking water that specified the acceptable limits and permissible limits (in absence of alternative source) by the Bureau of Indian Standards (BIS).

Isolation and characterization of fungal strain: Freshly collected soil was dissolved in sterilized distilled water and spread on to microbial culture media (PDA). The process commenced with the meticulous preparation of 50ml Potato Dextrose Agar (PDA), coupled with the thorough autoclaving of essential glassware to ensure a sterile environment. Subsequently, the PDA was poured into plates and spores from established culture were uniformly dispersed across the agar surface using a sterile loop. The inoculated plates were placed into an incubator set at a temperature of 25^0C, initiating an optimal climate for microbial growth. Over the course of 7 days, the incubation period allowed for the gradual development and proliferation of the culture. Spore staining was conducted using lacto phenol cotton blue (dye) to facilitate the microscopic observation of the fungus. The box area highlights the conidiophore with a large vesicle indicating the particular fungal strain is *Aspergillus niger* (Fig. 1).

Preparation of PDA plates supplemented with heavy metals: Two 50 ml volumetric flasks containing Potato Dextrose Agar (PDA) media (25 ml) were prepared and autoclaved with distilled water for sterility.

Preparing stock of 1500 ppm in 1 ml volume: Dissolve 1.5 mg of metal power (Cadmium, Copper and Nickel) in 1 ml of distilled water (autoclaved). This solution was added to one PDA flask containing 25ml media so as to create 1.5 pp. After thorough mixing, the PDA from the flasks (one flask with only PDA and no metals and 3 with cadmium, copper and nickel metals in them separately) was poured into plates, designated as 0 ppm (with no metal) and 1.5 ppm (with metal). Spores from the culture plates were then evenly distributed onto Petri dish, which were incubated for 2, 5 and 7 days at 25℃. This method facilitated the creation of distinct metal concentrations in the PDA plates for comprehensive experimentation and analysis. Spore staining was conducted using lactophenol cotton blue (dye) to facilitate the microscopic observation of the fungus (Fig.2).

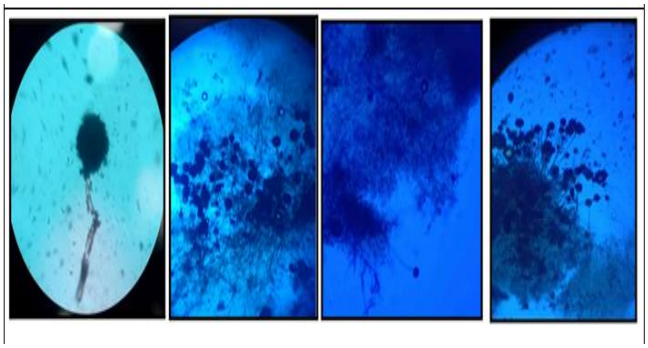


A **B**

Figure 1: Fungus strain was inoculated on PDA media and after 7 days incubation period (A) spore was stained The microscopic observation box area highlights the conidiophore with a large vesicle indicates (B) the particular fungal strain as *Aspergillus niger.*

Identification of Fungal Strain by Genome Sequencing: Genome sequencing was completed by Biokart India Pvt. Ltd. DNA extraction was done by adding 1 ml of Xploregen gDNA Extraction Buffer™ 1 to the beaded vial. Add the sample in the beaded vial containing buffer. Vortex horizontally the vial at maximum speed for 10 minutes (Figure 3).

PCR Amplification of ITS Gene: The analysis of PCR products was done by electrophoresis on agarose gel. To visualize, the bands staining was done by adding ethidium bromide. 166 ng of extracted DNA was taken for amplification along with 10pM of each primer. ITS Forward primer sequence is 5'-TCCGTAGGTGAACCTGCGG-3' at the 57°C and ITS reverse prime is 5'-TCCTCCGCTTATTGATATGC-3' at 53°C. The ~0.7 kbp, ITS-DNA fragments were amplified by using high fidelity PCR polymerase. Aligned sequence data of sample *Aspergillus niger* (625bp) were examined. The amplification reactions for 30 cycles were denaturation, 94°C, 1 min; annealing, 50°C, 1 min; extension 72°C, 2 min and final extension 72°C, 7 min. The cycling and PCR Amplification conditions heve been detailed in figure 3.



A B C D

Figure 2: Staining images of fungal growth at (A) 0 and 1.5 ppm concentration of (B) Nickel, (C) Cadmium and (D) Copper respectively

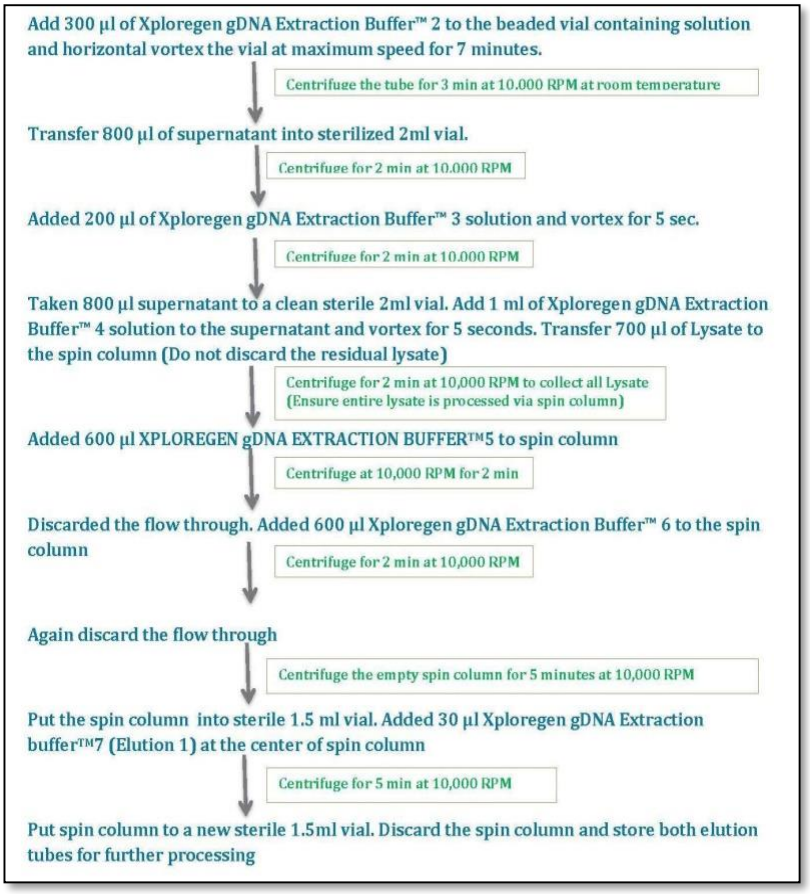


Figure 3: Process of DNA extraction by using Buffer™ solution and centrifuging at 10,000 RPM at different time period

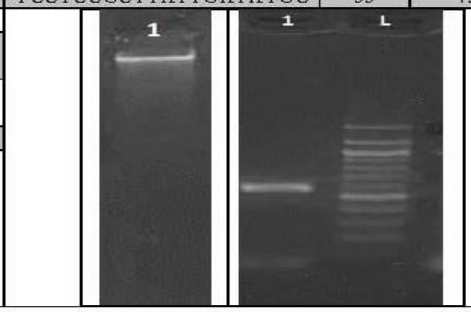
PCR Amplification conditions	Volume	Sequence (5`à 3`)	Tm (°C)	GC-Content
DNA	1 ul			
ITS Forward Primer	2 ul	TCCGTAGGTGAACCTGCGG	57	63.15%
ITS Reverse Prime	2 ul	TCCTCCGCTTATTGATATGC	53	45%
dNTPs (2.5mM each)	4 ul			
10X Taq DNA polymerase Assay Buffer	10 ul			
Taq DNA Polymerase Enzyme (3U/ ml)	1 ul			
Water	30 ul			
Total reaction volume	50 ul			

Figure 4: PCR Amplification conditions to get genomic DNA

Results and Discussion

Atomic absorption spectrometry shows that waste water contains cadmium 1.0 to 1.20 milligram/litre, copper 1.0 to 1.20 milligram/litre and Ni 0.94 to 1.21 milligram/litre. According to WHO guidelines, the comparison with standard limits shows that Cu was discovered within the acceptable limit, the concentrations of Cd and Ni in the sample were excessively high. However, the CPCB guideline comparison revealed that while Cu is within the permissible limit, the other three criteria are all outside of it. Noteworthy growth of *Aspergillus niger* was discernible on both plates of nickel and copper, designated as 0 ppm and 1.5 ppm, indicating the adaptability of the fungus to varying nickel and copper concentrations (Figure 2). The concentration of Ni and Cu by *A. niger* on day 7th of incubation was decreased up to 0.21 ppm and 0.34 ppm respectively.

At this point of experiment found that the *A.niger* strain produces large amount of metabolites that have the ability to develop on HC sources[16], whereas Cd inhibits the growth of *Aspergillus niger*. The phylogenetic tree was generated by using phylogentic tree builder that uses sequence aligned with software. Jukes-Cantor corrected distance model used to generate distance matrix. The tree is created using Weighbor with alphabet size 4 and length size 1000. 166 ng/µl DNA was extracted by followed the steps of DNA extraction. The microbe was found to be *Aspergillus costaricensis* isolate PM7 small subunit r-RNA gene with sequence ID: MK910049.1. The next homologue was close to *A. Tubingensi* strain CMV00A3 18s r-RNA gene with sequence ID MK450659.1.

Amanishah nallah is being polluted by industrial discharge that contains heavy metals. During the research we targeted three heavy metals i.e. Cd, Cu and Ni. Analysis of Cd, Cu and Ni was dons by using AAS. The analysis results shows that industrial effluents have more content of HMs, that are enough to pollute the surface water body. The conclusion is that the cropping pattern is consuming these metals which affect the food chain and are hazardous for animal's health as well as human also. This study assessed the fungus *Aspergillus niger* capacity to eliminate copper and nickel ions in some concentration whereas Cd inhibits the growth of *Aspergillus niger*.

Conclusion

Aspergillus niger is able to grow in the presence of Ni and Cu up to a concentration of 1.5 ppm and the same 1.5 ppm concentration of cadmium is able to inhibit the growth of *Aspergillus niger* to some extent. The phylogentic tree builder shows that the identified fungus is *Aspergillus niger*. Basic Local Alignment Search Tool (BLAST) indicated that the resembling of fungus strain is very similar to 4 *Aspergillus niger* that are *Aspergillus niger* isolate MFD4-1, *A. niger* strain T4 SSr-RNA gene, *A. niger* isolate SMP2 SSr-RNA gene and *A. niger* isolated ANE1 18S r-RNA gene.

To improve the efficacy of heavy metal tolerance and inhibition, modified *Aspergillus niger* functional groups and biomass should be introduced. Mycoremediation is not only an eco-friendly alternative wastewater management program for human health, but it is also cost-effective to preserve the survival of many biodiversity components of the riverine ecosystem. Using fungi to remove heavy metals from water, myco-remediation is an emerging *in situ* bioremediation technique that is both environmentally friendly and commercially successful.

Acknowledgement

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