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Screening and Characterization of Heavy Metal Resistant Fungi for its Prospects in Bioremediation of Contaminated Soil

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A B S T R A C T

Developing new bioremediation processes for soils and effluents polluted by heavy metals is one the serious concern. For the same, extensive screening for the fungal resistance to heavy metals such as lead, nickel, cobalt, chromium, copper, manganese and zinc was attempted. During this SG1 isolate was obtained having maximum threshold level of tolerance to manganese (60 mg/ml), copper (5 mg/ml), cobalt (5 mg/ml), lead (4.5 mg/ml), nickel (6 mg/ml), chromium (3.9 mg/ml) and GK1 isolate was obtained having maximum threshold level of tolerance to zinc (80 mg/ml). Maximum growth of the isolate SG1 at threshold concentrations of metals was found to be at pH 5 and temperature 30°C in presence of manganese, pH 5 and 32°C with copper, pH 5 and 30°C with cobalt, pH 5 and 31°C with lead, pH 5 and 30°C with nickel, pH 5 and 29°C with chromium while isolate GK1 showed at pH 4 and 30°C with zinc. Besides metal tolerance further antibiotic resistance pattern of these isolates were also studied. The results revealed that antibiotic resistance and metal resistance were highly coupled. GK1 was found to have multiple antibiotic resistances. UV mutagenesis has been found to be productive in terms of the increase in resistance against the metal ions. A mutant of GK1 was found to show improved resistance against Zn up to 82 mg/ml. Based on the morphological characterization, it was found that the isolates SG1 and GK1 were of *Neurospora sp.* and *Penicillium sp.* respectively.

Introduction

Heavy metals and trace elements in subsurface environments come from natural and anthropogenic sources. The intense petrochemical works, rapid industrialization and urbanization during last two decades increase this pollution enormously. Heavy metal pollution of the biosphere has received huge concern due to its toxicity, abundance, persistence and subsequent accumulation in environment. These toxic heavy metals in air, soil and water are global problems that are a growing threat to humanity (Dong *et al.*, 2010).

Typical sewage contains oxygen-demanding materials, grease, oil, scum, pathogenic bacteria, viruses, pesticides, refractory organic compounds and heavy metals (Giller *et al.*, 2009). Sewage; on treatments and disposal in to open environment results in accumulation of large amount of sludge, which are disposed off worldwide through land disposal. One of the main concerns in the land disposal of sludge is presence of toxic heavy metals which gets magnified during various physicochemical and biological interactions occurring in sludge treatment.

Another major concern over the presence of these heavy metals in the environment is their non-biodegradability and consequent persistence. Since, the presence of heavy metals can pose a long term environmental hazard once accumulated in land with time (Mc-Bride, 1995), reduction or elimination of heavy metals from these sludge before land application is necessary (Mc-Bride, 1995). The commonest way of disposing waste water sludge and solid waste is incineration, which results in heat and electricity production, as well as a significant reduction in the waste volume. However, produced ash is a problem (Fedge *et al.*, 2010).

Accumulation of metals in living organisms is the cause of serious health problems, by interfering with, our normal body functions. Some of these metals are useful to the body in low concentrations like arsenic, copper, iron and nickel but are toxic at high concentrations. Other metals like aluminum, beryllium, cadmium, lead and mercury have no biological functions and are highly toxic disrupting bodily functions to a large extent. They disrupt bodily functions by accumulating in vital organs and glands in the human body such as in the heart, brain, kidney, bone and liver. They also displace vital nutritional minerals from their proper place in the body to provide biological functions e.g., lead or cadmium displaces calcium in an enzyme reaction disrupting the enzyme reaction to a large extent. As their impact in the body, is at such basic levels that they are the causal factors in multiple health problems. Metal causes genotoxicity as they affect the DNA and immunotoxicity as they are major irritants to the body. The genomic instability by these metals induces cancer (Leonard *et al.*, 2004).

Materials and Methods

Sampling and Sample Preparation

The effluent samples were collected from the contaminated sites of Sachin GIDC, Navsari GIDC, Navsari Udyognagar, Pandesara GIDC, Dabhoi khadi and Gabheni khadi in Gujarat, India. 1 ml of samples was mixed with 9 ml of diluents individually, centrifuged at 1200 rpm for 3 min, and supernatants were used for isolation of metal resistant fungi.

Isolation of Fungi from the Environmental Samples

The samples prepared from the effluents of contaminated sites were inoculated to Potato Dextrose Broth (PDB). PDB was prepared using 250 g of potato boiled in 100 ml of distilled water for 30 min and the filtrate was mixed with 20 g of dextrose and incubated at 30–35°C for a week. After incubation larger identical colonies from each flask were isolated. These isolates were further employed for heavy metal resistance studies (Shankar Congeevaram *et al.,* 2006).

Screening for Metal Resistance

All the fungal isolates were evaluated for their metal resistance against varying

concentrations of heavy metals that included copper as $CuSO_4*5\ H_2O$, zinc as $ZnSO_4$, manganese as $MnCl_2$, cobalt as $CoCl_2$, lead as $Pb(NO_3)_2$, nickel as $NiCl_2$ and chromium as $K_2Cr_2O_7$ (Nazareth and Marbaniang, 2008).

pH and Temperature Optimization

The fungal isolates were inoculated into a series of 250 ml conical flasks containing above described metals. The pH was varied from 3 to 8 (3, 4, 5, 6, 7 and 8). The pH of the medium was adjusted using 0.1N HCl or 0.1N NaOH. Studies on optimal temperature for the selected fungal isolates were carried out by growth at different temperatures (29, 30, 31, 32, 33, 34 and 35°C) in the presence of metal ions at threshold concentration. These were incubated on rotary shaker (120 rpm) in a temperature controlled water bath for 48 h. The optimum pH and temperature was evaluated based upon the biomass production by each selected fungal isolates (Shankar Congeevaram *et al;* 2006).

Resistance to Antibiotics by Metal Resistant Fungal Isolates

The isolates were evaluated for its resistance to antibiotics by disc diffusion method. The PDA (1.5%) was prepared. Then it was overlayed by PDA (0.8%) mixed with fungal isolates under examination. After solidification, antibiotic paper disc (Hexa-Disc HI-MEDIA) was placed over it. The plates were incubated at 4°C for 30 min for diffusion of respective antibiotics followed by the incubation at 30°C for 24 hrs. The zone of inhibition was measured for each plate.

UV Mutagenesis to Improve Threshold for Metal Resistance of Selected Fungal Isolates

Selected fungal isolates were exposed to UV radiation for 15 min 20 min 25 min and 30 min in UV chamber. The survival populations after each exposure were further screened for improved metal resistance. The mutants were inoculated in PDB with respective metal at concentration higher than that of threshold level for 3 days at 30°C in presence of the metal.

Characterization of Metal Resistant Fungal Isolates

Metal resistant fungal isolates were studied for its morphological features under the compound microscope. The isolates were mounted with the help of lactophenol blue (phenol 20 ml, lactic acid 20 ml, Glycerol 40 ml, Distilled water 20 ml, 0.05 % Methyl blue).

Results and Discussion

Isolation of Fungi

Eight fungi were isolated from the different sources (Sachin GIDC, Navsari GIDC, Navsari Udyognagar, Pandesara GIDC, Dabhoi khadi and Gabheni khadi). They were designated as SG1, SG2, NG1, NG2, PG1, DK1, GK1 and GK2. The results of screening for metal resistant fungi are summarized in Table 1.

Characteristics of Metal Resistant Fungal Isolates

Determination of MIC: Isolate SG1 was evaluated for the resistance against Mn, Cu, Co, Pb, Ni and Cr metal ions while isolate GK1 was evaluated for the resistance against Zn metal ion.

The threshold level of resistance against metal ions Mn, Cu, Co, Pb, Ni and Cr were 60 mg/ml, 5 mg/ml, 5 mg/ml, 4.5 mg/ml, 6 mg/ml and 3.9 mg/ml respectively for SG1 while resistance against Zn by GK1 was 80 mg/ml.

pH Optima for Metal Resistance

Studies in the past revealed that the fungal isolates shows higher resistance in acidic range of the pH (Juan Maldonado *et al*., 2010). Attempt was made to evaluate the optimum pH for the maximum resistance of metal ions by selected isolates SG1 and GK1. Optimum pH for maximum resistance against metal ions Mn, Cu, Co, Pb, Ni and Cr was found to be 5 except for Zn it was 4. The results are summarized in Fig 2.

Temperature Optima for Metal Resistance

Various studies in the past have shown that the resistances against the metal ions by isolates were closely related to the temperature of the environment (Juan Maldonado *et al*., 2010). In same context, attempt was made to investigate optimum temperature for resistance against metal ions. Optimum temperatures for maximum resistance against metal ions Mn, Zn, Co, Ni were found to be 30°C while for Cr, Cu and Pb they were 29°C, 32°C and 31°C respectively, as summarized in Fig 3 below.

Evaluation of Antibiotic Susceptibility

Studies revealed that metal resistance is correlated with antibiotic resistance (Zeng Xiao-xi *et al*., 2009). In same context, the attempt was made to investigate antibiotic resistance pattern by selected potential isolates against six different antifungal antibiotics (Plate 1).

GK1 isolate was sensitive to Nystatin, Amphotericin B and Ketoconazole antibiotics as there was no zone of inhibition. SG1 was found to show resistance against all of them as shown in Table 2.

UV Mutagenesis for Improvement of Threshold Limit of Metal Resistance

UV mutagenesis has been found to be productive in terms of the increase in resistance against the metal ions (Naveenkumar *et al*., 2010). Both isolates were treated with UV for mutagenesis and then the mutant population were subjected to assessment of whether the threshold limit for metal resistance was improved compared to wild type isolate or not. After UV treatment, no significant change in the resistance of metal ions was observed compared to wild type except for Zn metal ion. A mutant of GK1 was found to show improved resistance against Zn up to 82 mg/ml compared to 80 mg/ml of wild type.

Table.1 Isolation of Fungi from Different Sources

Sample site	Isolation method	Isolates
Sachin GIDC	Direct	2 (SG 1-2)
Navsari GIDC	Direct	2 (NG 1-2)
Navsari Udyognagar	Direct	Nil
Pandesara GIDC	Direct	1 (PG 1)
Dabhoi khadi	Direct	1 (DK 1)
Gabheni khadi	Direct	2 (GK 1-2)

Table.2 Antibiotic Resistance of Metal Resistant Fungal Isolates

ANTIBIOTICS	ZONE OF INHIBITION SG1(CM)	ZONE OF INHIBITION GK1(CM)
Nystatin (Ns)	-	1.9
Amphotericin B (Ap)	-	1.1
Ketoconazole (Kt)	-	1.4
Clotrimazole (Cc)	-	-
Fluconazole (Fu)	-	-
Itraconazole (It)	-	-

Fig.1 Threshold Level of the Metal Resistance by Isolates SG1 and GK1

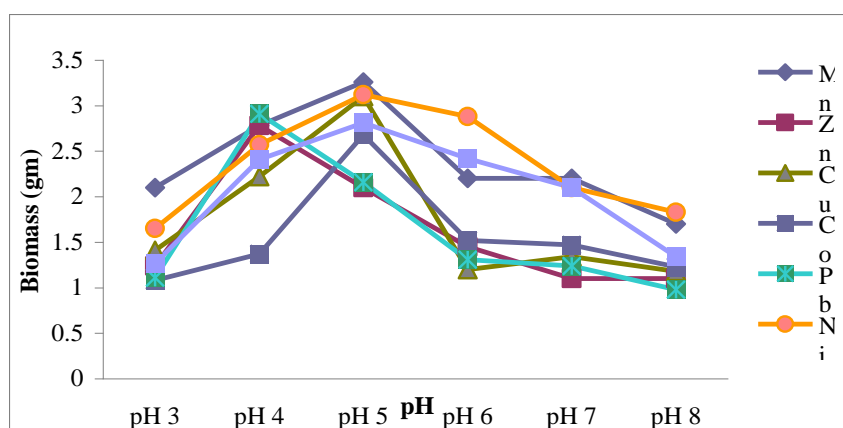|	Mn	Zn	Cu	Co	Pb	Ni	Cr
SG1	60		5	5	4.5	6	3.9
GK1		80					
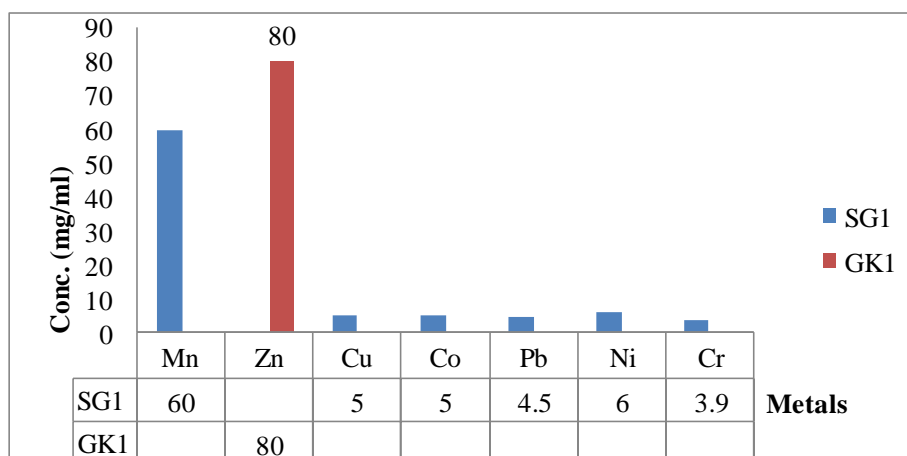
Fig.2 pH Optima for Different Metals

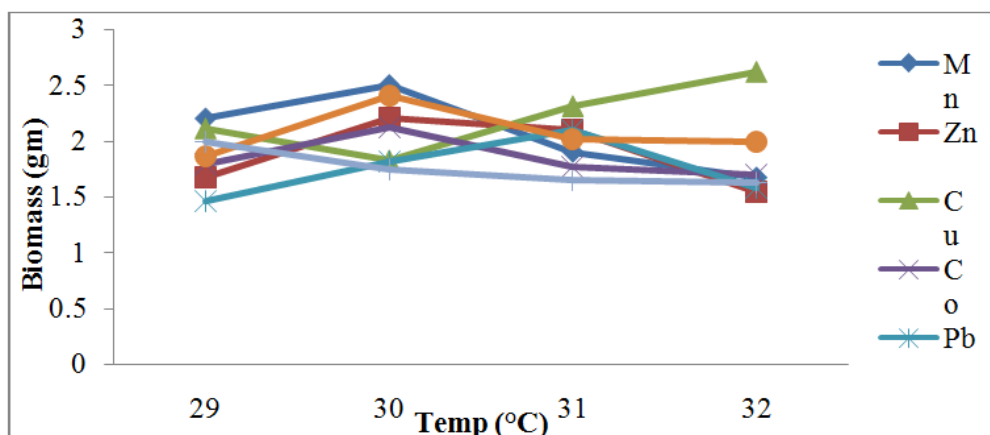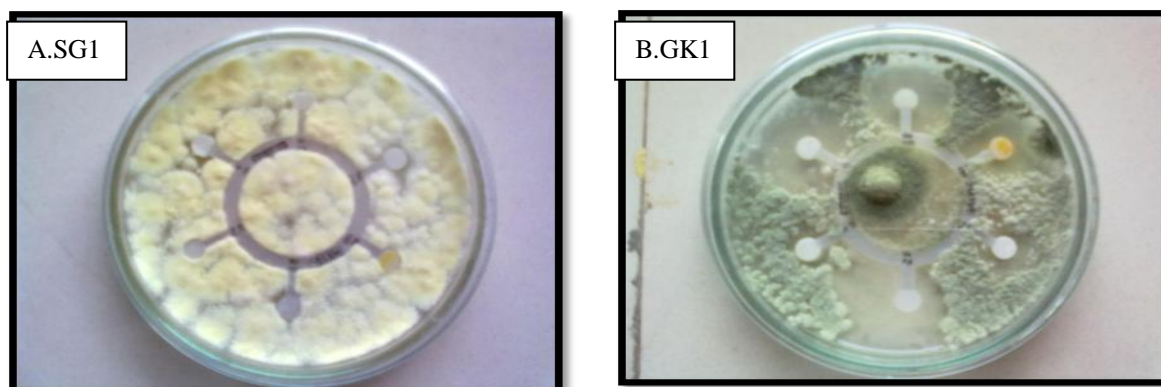**Fig.3** Temperature Optima for Different Metals

Plate.1 Antibiotic Resistance of Selected Fungal Isolates: A.SG1; B.GK1

Morphological Characteristics

Morphological characteristics of the isolates SG1 and GK1 were observed under the compound microscope. SG1 was found to be *Neurospora sp.* as it shown clear septate hyphae with large masses of ovate spores which are air-dispersed while GK1 resembled *Penicillium sp.* giving small, round spores borne in "brush-like" formations when mounted with lactophenol.

In conclusion, Species of *Neurospora* and *Penicillium* obtained during screening found to show significant resistance for metals. The threshold limit for metal resistance by *Neurospora sp.* is 60 mg/ml, 5 mg/ml, 5 mg/ml, 4.5 mg/ml, 6 mg/ml, 3.9 mg/ml to manganese, copper, cobalt, lead, nickel, chromium respectively. While *Penicillium sp.* was resistant to zinc up to 80 mg/ml concentration. Further UV mutagenesis yield a mutant of *Penicillium sp.* with improved resistance to zinc up to 82 mg/ml. These fungal isolates were also versatile as also showed the antibiotic resistance. These studies found to show their prospect for bioremediation of metal contaminated sites for cleanup of environment.

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