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Isolation of heavy metal (W, Hg, Pb, Sn, Cu, Fe) tolerant fungi from different polluted sites of Pune and Karad region in Maharashtra, India

Shikha Jaiprakash Dixit and Kiran Singh

Department of Biological Science and Engineering, Maulana Azad National Institute of Technology, Bhopal, M.P., India

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ABSTRACT

The environmental risk of heavy metal pollution is pronounced in soil adjacent to large industrial complexes and spreading throughout the world along with industrial process. Microbial technologies may provide an alternative to conventional method of metal removal and recovery. The present study deals with isolation and characterization of heavy metal tolerant fungi from polluted sites of Pune and Karad, Maharashtra, India. These areas are known to be polluted with waste from electronic manufacturing industry, Pharmaceutical industry, Hospitals and Residential area. Five fungal isolates were found tolerant for the different concentrations of heavy metals (W, Hg, Pb, Sn, Cu and Fe). On the basis of morphological and Microscopic characteristics fungal isolates were identified as *Aspergiilus niger, Penicillium sp., Aspergiilus flavus, Aspergiilus sydowi* and *Mucor globossus.* The identified heavy metal tolerant fungi could be useful for bioremediation of heavy metal contaminated waste.

Key words : *Heavy Metal, Tolerance, Fungi*

Introduction

Heavy metal occur naturally in rocks and soils but concentrations are frequently elevated as a result of pollution through metal mining, metal smelting, activities of metallurgical industries, waste disposals, corrosion of metals, use of pesticides in agricultural land and petroleum exploration among others (Joshi *et al.*, 2013). The discharge of effluents containing heavy metals mounts pressures on the ecosystem and consequently causing health hazards to plants, animals, aquatic life and humans (Robin *et al.*, 2012), as a consequence of surface contamination which leads to bioaccumulation of the toxic metals to groundwater (Mohiuddin *et al.*, 2011; Santhaveerna *et al.*, 2010). Heavy metals may exert an inhibitory action on microorganisms by blocking essential functional groups, displacing essential metal ions or modifying the active confirmations of biological molecules (Tejirian *et al.*, 2010), but some microorganisms have been reported to evolve mechanisms to detoxify heavy metals and some even use them for respiration thereby becoming resistant to such metals (Ezaka *et al.*, 2011). Thus, the bioprospection of these naturally selected organisms represents an important strategy in order to obtain agents for bioremediation processes (Ledin *et al.*, 1996). The present work describes the isolation and characterization of the heavy metal tolerant fungi from soil samples collected from agricultural soil of Karad and waste disposal area of Sangvi Pune, Maharashtra, India. The waste disposal area of Sangvi Pune is known to have waste from Hospitals, Pharmaceutical industry, Electronic industry, Main-

***Corresponding author's email:** Researchnm.kiran@gmail.com; Researchnm.kiran@gmail.com

tenance garage and house waste, thus rich in heavy metals. Agricultural land of Karad was contaminated with industrial effluent and pesticides rich in heavy metals.

Materials and Methods

Heavy Metal Analysis of Soil

The determination of heavy metals was performed using the Atomic Absorption Spectrophotometry.

Isolation of Fungal Strains from Polluted Sites

Samples of soil from two contaminated sites of Maharashtra (waste disposal area Sangvi, Pune, agricultural soil, Karad) were collected. The upper layer of soil was removed and samples were collected from deep inside (about 10 g) in sterilized polythene bags and was kept on ice. Bag was properly sealed and brought to the laboratory for analysis. The samples were suspended in sterile enrichment solution (1g/L glucose and 0.5g/L yeast extract) 1:10 w/v supplemented with antibiotics. Suspended medium was incubated on shaker (200 rpm) for 24-72 hours at R.T. and then diluted (10- to 10,000-fold). 100 µl of different dilutions were plated onto Sabouraud's agar medium supplemented with antibiotics. After at least 3 days of incubation at 30^0 C, developed colonies were randomly picked and isolated. Purified colony were obtained by streaking repeatedly colonies on Sabouraud's agar medium and observed under light microscopy.

Pure cultures of isolated fungal strains were identified using the keys of Pitt (1979) and Domsch *et al.* (1980). The cultures were characterized to the genus level on the basis of macroscopic characteristics (colonial morphology, colour and appearance of colony, shape) and microscopic characteristics (septation of mycelium, shape, diameter and texture of conidia).

Screening and Selection of Heavy Metal-Resistant Fungi

Purified isolates were screened on the basis of their tolerance to tungsten (W), stannous (Sn), mercury (Hg), iron (Fe) and lead (Pb). A disk of mycelium was inoculated aseptically on Sabouraud's plates supplemented individually with 1 mM of heavy metal. The metal salts used were Sodium tungstate, stannous chloride, mercuric chloride, ferrous chloride and Lead acetate. The inoculated plates were incubated at 25°C for at least 7 days. The effect of the heavy metal on the growth of the isolates tested was estimated by measuring the radius of the colony extension (mm) against the control (medium without metal) and the determination of the index of tolerance. The index is defined as the ratio of the extension radius of the treated colony to that of the untreated colony. Isolates showing resistance to W, Sn, Hg, Fe and Pb were selected for the following experiments.

Determination of Minimum Inhibitory Concentrations (MICs)

The resistance of the selected isolates to W, Sn, Hg, Fe and Pb was determined by serial dilution method. Metal ions were added separately to sabouraud's medium at concentration of 0.01 to 1mM. Three replicates of each concentration and controls without metals were used. The inoculated plates were incubated at 25^0C for at least 7 days. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of metal that inhibit visible growth of the isolate.

Results and Discussion

In the present study various fungal strains were isolated (Table 1) from the site, which was exposed to heavy metals and other pollutants for several years. The heavy metals content of soil samples is listed in Table 2. The concentration of W, Hg, Pb, Sn, Cu and Fe in the soil samples was found to be above the permissible limits. The long time exposure to industrial effluent, chemical fertilizers, pesticides and domestic waste water are the main reason for the high heavy metal content of the selected sites.

Fungi isolated belong to genera *Aspergillus, Penicillium* and *Mucor* (Fig. 1-4). Species of the genus *Aspergillus* was the most abundant at all the sites, followed by *Penicillium* species. The difference between the sampling sites regarding their richness on

Table 1. List of fungi isolated from the contaminated soil

Fungal isolate	Code no.
Aspergillus niger	F_1S_k, F_2S_k, F_3S_p
Penicillium sp.	F_4S_k, F_5S_p
Aspergillus flavus	F_6S_k, F_7S_p
Aspergillus sydowi	F_8S_p
Mucor globosus	F_9S_k, $F_{10}S_p$, $F_{11}S_p$

Table 2. Heavy metal contamination of the sampled sites

Sampling sites and description	Heavy metal content (mg/Kg)					
	W	Hg	Pb	Sn	Cu	Fe
S_k(Agricultural soil contaminated with chemical fertilizers and industrial water, Karad, Maharashtra)	0.24	0.18	1.03	0.60	1.05	2.7
S_p (Waste disposal area, Sangvi, Pune, Maharashtra)	0.42	0.07	2.10	1.50	0.80	2.9

microbial isolates appear to be closely linked to the degree of heavy metal pollution. Generally, pollution of soil and water by heavy metals may lead to a decrease in microbial diversity. This is due to extinction of species sensitive to the stress imposed and enhanced growth of other resistant species. Here in present study few isolates of *Aspergillus niger* (F_1S_k and F_3S_p) was more tolerant in comparison to F_2S_p for W, Pb, Cu and Fe. While in case of Hg, F_3S_p was more tolerant than other two isolates of *A.niger*. At the same time for Sn there was no tolerance shown by all the three isolates of *A.niger*. Isolates of *Aspergillus flavus* (F_6S_k) shows more tolerance to W, Pb, Cu and Fe than F_7S_p. Both F_6S_k and F_7S_p was sensitive to Hg and Sn. *Aspergillus sydowi* (F_8S_p) was moderately tolerant for W, Pb and Fe but sensitive to Hg, Sn and Cu. Isolates of *Penicillium* sp. F_4S_k and F_5S_p was more tolerant to Cu but was moderate tolerant to W, Hg, Pb and Fe. There was sensitivity for Sn Shown by F_4S_k and F_5S_p isolates of *Penicillium* Sp. The three isolates F_9S_k, $F_{10}S_p$ and $F_{11}S_p$ of the *Mucor globossus* shows variability in tolerance level with the type of metal. There was high tolerance for W

Fig. 1. Microscopic photograph of *Aspergillus flavus* (100×)

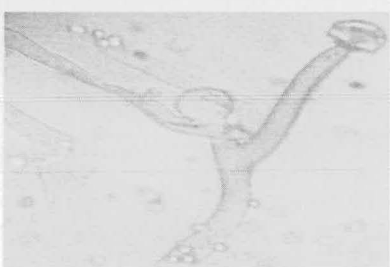

Fig. 2. Microscopic photograph of *Mucor globossus* (100×)

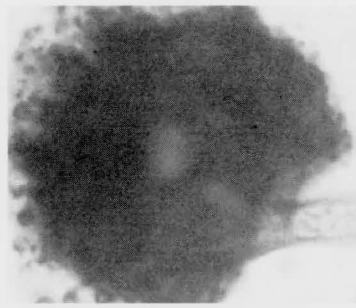

Fig. 3. Microscopic photograph of *Aspergillus flavus* (100×)

Fig. 4. Microscopic photograph of *Penicillium species* (100 ×)

and Pb, moderate tolerance for Sn and Sensitivity for Hg, Cu and Fe. (Table 3)

Determination of MIC revealed that strain of *Aspergillus niger* (F_1S_k), *Penicillium* (F_4S_k), *Aspergillus flavus* (F_7S_p) and *Mucor globosus* (F_9S_k) was tolerant up to higher concentration of heavy metals even though tolerance level changes with the type of metal (Table 4). Sn was shown to be more inhibitory toward isolates only *Mucor globosus* was able to grow. The screening test shows heterogeneity in the heavy metal tolerance of isolates. The resistance against individual metals was much more dependent on the isolate than on the sites of its isolation. Jones and Hutchinson (1988) demonstrated that whatever the concentration in the medium comparable tolerance rates were observed for isolates originating from metal contaminated sites.

Table 3. Heavy metal tolerant index of fungal strains isolated from the polluted soil

Isolates	W	Hg	Pb	Sn	Cu	Fe
F1Sk	0.98	0.26	1.07	0	1.09	0.86
F2Sk	0.72	0.42	1.00	0	0.93	0.77
F3Sp	1.02	0.57	0.92	0	0.88	0.90
F4Sk	0.32	0.66	0.88	0	0.72	0.92
F5Sp	0.29	0.48	0.71	0	1.01	0.63
F6Sk	0.68	0	0.96	0	0.66	1.01
F7Sp	0.72	0	0.73	0	0.82	0.91
F8Sp	0.56	0	0.98	0	0	1.01
F9Sk	0.87	0	0.95	0.13	0	0
F10Sp	0.82	0	0.94	0.21	0	0
F11Sp	0.56	0	0.78	0.12	0	0

Table 4. Determination of Minimum Inhibitory Concentration

Fungi Strains	MIC(mM)					
	W	Hg	Pb	Sn	Cu	Fe
F_1S_k	20<MIC<25	15<MIC<20	20<MIC<25	0	15<MIC<20	20<MIC<25
F_4S_k	15<MIC<20	10<MIC<12.5	20<MIC<25	0	12.5<MIC<15	15<MIC<20
F_7S_p	20<MIC<25	0	15<MIC<20	0	10<MIC<12.5	15<MIC<20
F_9S_k	12.5<MIC<15	0	10<MIC<12.5	5.5<MIC<10	0	0

The isolates of same genus could present a marked difference in the level of metal resistance (Ezzouhri *et al.*, 2009). Major differences in W, Hg, and Fe tolerance have been found among isolates. The variation in the metal tolerance may be due to the presence of different types of tolerance processes or resistance mechanisms exhibited by different isolates. One of the demonstrations is that both intracellular bioaccumulation and extracellular bio sorption contributed to the high resistance of *Penicillium* sp. Psf-2 to Pb (Sun *et al.*, 2007). Copper is a co-factor in numerous enzymatic processes and represents the third most abundant transition metal found in living organisms (Brandolini *et al.*, 2002). The growth of all fungi tested was decreased after addition of copper in comparison of Fe. The most tolerant isolate belonged to the genus *Aspergillus* with a MIC upto 25 mM. Tolerances for Sn was exhibited by Single isolate i.e. *Mucor globossus*. 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. It must also be taken into account that the contamination at the polluted sites is usually not caused by a single metal and that the selection is probably driven either by the most toxic element or by more different metals acting synergistically (Baldrian *et al.*, 2002). According to report the micro biota isolated from co-contaminated environments could exhibit resistance to more than one ion and consequently, co-tolerance may be a common natural response (Gadd *et al.*, 2000).

Conclusion

Our study demonstrated fungal population isolated from heavy metal contaminated sites has the ability to tolerate higher concentrations of heavy metals. The set of methods applied in this study were sensitive to site differences and contributed to a better understanding of heavy metals effects on structure, size and activity of microbial community in soil.The use of "omics" such as Genomics, Proteomics, Metabolomics, Transcriptomics and System Biology can provide insights in to some metabolic pathways in order to identify genes, proteins, metabolites that could be involved in heavy metal tolerance and makes them promising candidates for further investigations regarding their role in Bioremediation.

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