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METAL TOLERANCE ANALYSIS OF MICROFUNGI ISOLATED FROM METAL CONTAMINATED SOIL AND WASTE WATER

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ABSTRACT

The influence of Cr^{6+}, $Pb^{2+,}$ Cu^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+} on the development of 24 fungi was investigated for Metal Tolerance Index (MTI) at 1mg ml^{-1} Cr^{6+}, $Pb^{2+,}$ Cu^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+} concentrations and also for Minimum Inhibitory Concentration (MIC). The MIC ranged from 0.5 to 1.5 mg ml^{-1} depending on the isolate *Aspergillus, Fusarium* and *Penicillium* sp. were tested for their metal tolerance index. Out of these *Aspergillus flavus* (ED4) shows a better tolerance index of 0.80 Cr^{6+}, 0.72 for Pb^{2+} , 0.63 for Cu^{2+}, 0.58 for Ni^{2+}, 0.46 for Zn^{2+} and 0.60 Cd^{2+} for MIC value for the removal of heavy metals from contaminated soil and wastewaters.

Keywords: Bioremediation, Contaminated soil, Fungi, Metal resistance, Metal tolerance

INTRODUCTION

Heavy metal pollution is a major environmental concern which are continuously released into the environment due to various industrial developments (**Oztürk *et al*., 2004**). Several industries, particularly effluents from plating, metallurgical, electroplating, metal finishing, tanneries, chemical manufacturing, metal fabrication, battery, paints, fertilizers and steel industries are the potential sources of heavy metals poses a serious health hazards in contaminating the water resources (**Arora *et al*., 2008; Malik and Jaiswal, 2000**). Metals can variously influence soil fungi like diminish their population, improvise in diversity and to change fungal morphology and physiological activity and also to effect the growth rate, reproduction processes enzyme production etc. (**Gadd, 1992; Martino *et al*., 2000**). Polluted environments with higher concentration of heavy metals and their nature of medium may also play a vital role for microbial toxicity against metals (**Baldrian, 2003**). For this reason, extensive researches have been focused toward the search of potential methods and new technologies for the heavy metal pollution. Generally fungi and yeast biomasses are considered for metal tolerance when compare to other microorganisms (**Gavrilescu, 2004**). They have ability to grow under various extreme conditions such as high pH, temperature and high heavy metal concentrations which considered to be ideal choice for bioremediation work (**Anayurt *et al*., 2009**). In fungi, metal effects can vary not only among organisms, but also among different vegetative and reproductive form of the same organism (**Sabie *et al*., 1990**).
Metal resistance is a term denotes the ability of a microorganism to survive the heavy metal toxicity by means of the mechanism produced to the metal species concerned (**levinskaite, 2001**). Different physicochemical methods such as chemical precipitation, reduction, oxidation electrochemical process, filtration, ion exchange and resin technologies have been commonly used for the removal of heavy metal from the industrial effluents. But, these processes can be ineffective due to technical and economic difficulties such as cost expensive and some times, chemical methods may lead to further accumulation of metals in the environment (**Volesky, 1990**).
The application of microbial biosorbents for removal heavy metals offers a potential alternative to existing methods. The principle mechanisms behind the metal sorption using microbes involve extracellular and intracellular metal binding by physical adsorption, complexation or chemical adsorption (**Pethkar *et al*., 2001; Mao *et al*., 2008**). Microbial cellular surfaces consists of several cationic and anionic exchange sites such as amino, phosphate, carboxyl and sulfate groups which are considered to play major role in the heavy metal reduction. These are complex methods and the efficiency of metal removal depends on the metal ions and physicochemical properties of the biosorbent (**Hussein *et al*., 2004**).
The objective of the present study was to isolate various fungi from untreated effluents and to study the potential metal tolerance ability against selective heavy metals.

MATERIAL AND METHODS

Isolation of Metal tolerant fungi

Different untreated effluents samples were collected from Wise park industrial area, in and aound Kanjikode, Palakkad in the year 2011 in a sterile containers and analyzed for different physicochemical parameters, such as pH, Conductivity, Total hardness, Total Dissolved solids [TDS], Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD). The effluents samples collected from the above sites were serially diluted using sterile saline and plated onto the potato dextrose agar (PDA) using standard pour plate technique. The plates were incubated at 22°C to 25°C for 4 to 7 days for fungal growth, which was further sampled in a separate PDA plates for the isolation of pure cultures. On the basis of frequency of occurrence of fungal growth on selected plates the pure cultures isolated were maintained in a PDA slant for further studies. Different fungal isolated was tested for metal resistant ability against Cr^{6+}, $Pb^{2+,}$ Cu^{2+}, Ni^{2+}, Zn^{2+} and $Cd^{2+}$by amending the salts, Potassium dichromate, lead acetate, copper sulfate, nickel chloride, zinc chloride and cadmium chloride into the PDA plates at a concentration of 1 mg per ml. The isolated pure cultures of fungi were inoculated into the metal amended plates and observed for the growth after 4 to 7 days. The fungus which was able to grow in the plate amended with metal was selected for further study (**Iram *et al*., 2009**). The isolated fungal cultures were maintained using Potato dextrose agar (PDA) medium. The fungal cultures were identified based on the macroscopic characters such as colony morphology, texture, color, diameter of the colony and microscopic characteristics such as mycelium, reproductive structures, shape and structure of conidia (**Domsch *et al*., 1980; Barnett and Hunter 1999**).

Heavy Metals Analysis of Effluents

50ml untreated effluent samples was measured and filtered using Whatman No. 41 (0.45 µm pore size) filter paper for the estimation of dissolved metal content using acid digestion method . To the filtrate, 2 ml of concentrated nitric acid was added and subjected to digestion using microwave-assisted technique, with a pressure of 30 bars and power at 700 Watts (**Lee *et al*., 2000; Weston and**

Maraya, 2002). The preparation was appropriately diluted with distilled water with known volume and the estimation of metal ions was done by AAS 240. Standard solutions of different concentration of heavy metals were used for the preparation of calibration curves and three replicates were performed for each determination.

Determination of metal tolerance

To evaluate the metal tolerance potential among isolated fungal strains, PDA medium was prepared and amended with a concentrations 1mg $ml^{-1}$of heavy metal (Cr^{6+}, $Pb^{2+,}$ Cu^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+}). The pH of the medium was maintained at 5.2 ± 0.2 using 0.1 N NaOH and 0.1N HCl solution and control plates without metal solution were also kept. The isolated fungal strains were then spot inoculated (10^{-6} spores/ml) in the centre and the plates were incubated at 27 °C for 4-7 days. Fungal growth was monitored from the point of inoculation or centre of the colony in the metal solution amended plate. Metal tolerance index (Ti) was calculated as the ratio of the extended radius of the treated colony to that of the untreated colony (**Iram *et al*., 2009**).

$$Ti = Dt/Du$$

Where Dt is the radial extension (cm) of treated colony and Du is the radial extension (cm) of untreated colony.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of the fungal isolates was determined as the lowest concentrations of metals that inhibit the visible growth of the selected isolates. Eight different fungal strains were subjected to MIC test against various metals of (Cr^{6+}, $Pb^{2+,}$ Cu^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+}). The metal ions were separately added to PDA medium at a concentration ranging from 0.5 mg ml^{-1} to 5 mg ml^{-1}. The selected fungal strains were then inoculated (10^{-6} spores/ml) and the plates were incubated at 27 °C for 4-7 days. The experiments were done on triplicates and the minimum inhibitory concentration (MIC) was calculated based on the visible fungal growth in the metal solution amended plate (**Iram *et al*., 2009**).

Statistical analysis of data

A total eight different untreated effluent samples were collected and analyzed for different physicochemical characters such as pH, Total hardness, TDS, BOD and COD. The results were given in the table - 1, in which most of the sample's pH was in the range of 5.5 and 7.5.

Table 1 Physio-chemical parameters of untreated effluent samples

Analysis Parameter	Samples collected at Wise park Industrial Area. Palakkad, Kerala								
	Sample S-1	Sample S-2	Sample S-3	Sample S-4	Sample S-5	Sample S-6	Sample S-7	Sample S-7	Sample S-8
pH	7.5	5.6	5.5	6.2	5.8	6.3	7.1	7.3	6.5
Total Hardness, ppm	800	1000	260	540	35	522	79	53	300
TDS,mg L^{-1}	1800	2110	414	986	1985	5472	914	814	2100
BOD,mg L^{-1}	507	94	75	144	88	110	130	137	100
COD,mg L^{-1}	1425	528	410	488	170	676	442	518	250

RESULTS AND DISCUSSION

A total of 24 different fungi based on the colony morphology was isolated and screened for metal resistance against Cr^{6+}, $Pb^{2+,}$ Cu^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+} at a concentration of 1mg ml^{-1}. Among the 24 different fungi, eight were considered to be resistant against four or more metal ions, especially Cr^{6+}, $Pb^{2+,}$ Cu^{2+}, Ni^{2+} and Cd^{2+} were subjected for further study. Among various microbes, fungi are known to tolerate and detoxify metals using several mechanisms such as valence transformation, active uptake and extra and intracellular precipitation, biosorption using cell wall, decreased transport efflux, intracellular compartmentation, and sequestration (**Mala *et al*., 2006; Turnau *et al*., 2006; Balamarugan and Schaffner, 2006**). Considering the above mechanisms, fungal screening for metal resistant may lead to identify potential strains with higher metal tolerability and accumulation (**Zafar *et al*., 2007**). In present scenario, bioremediation treatments by exploiting microbial and associated biota for accumulating or removing the pollutants are increasingly alarming.

The metal resistant fungal strains isolated from various effluents belonged commonly to the genera *Aspergillus, Penicillium,* and *Fusarium*. Similar reports were also suggested by various researchers (**El-Morsy, 2004; Vadkertiova and Slavikova, 2006**) who have investigated the isolation of metal tolerance the fungi from various sources.The metal tolerance ability of eight different fungi shows that each fungal species appears different tolerance pattern against the heavy metals tested (Tab. - 2). The behavior of each fungal species were different when the metal ions source were changed. Among selected isolates, *Aspergillus flavus* (ED4) was more tolerant against most of the metals and *Penicillium sp* (EC2) was most sensitive (Fig. 1).

Generally, the heavy metal pollution in the environment may lead to a decrease in the microbial diversity due to the extinction of species which were seem to be sensitive due to the stress imposed. **Levinskaite (2002)** investigated the metal tolerant of various fungi isolated from different soil.The MIC was also calculated for the eight different fungi against metal ions, which shows a moderate activity against most of the metals. At lower metal concentrations, the fungal isolates showed resistance and exhibited good growth compare to higher metal concentrations in which the growth decreased compared to the control. Among the isolates *Aspergillus flavus* (ED4) shows higher MIC values to all the metals tested when compared to other isolates, followed by *Aspergillus niger* (EF1). The isolate *Aspergillus flavus* (ED4) shows MIC value of 3, 1.5, 2, 1.5, 1.5, 1.5 for Cr^{6+}, $Pb^{2+,}$ Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} respectively (Tab. 3; Fig. 2).

Baldrian and Gabriel (2002) have also discussed that various strains of fungi, originating from metal-contaminated sites did not have the same level of tolerance against heavy metals and this may due to the difference in level of resistance mechanism (**Ezzouhri *et al*., 2009; Sani *et al*., 2003**). Similar reports were also published by **Zafar *et al*., (2007)** who have isolated filamentous fungi from metal contaminated soils.

The results obtained from the above study affirmed that the response of the fungal isolates to heavy metals depends on the metal tested, concentration and its isolation source. The present study also reveals the fungi isolated from heavy metal-contaminated sites have the potential to resist higher concentrations of metals which can be used for bioremediation agents for the removal of heavy metals from the contaminated environment.

Table 2 Heavy Metal Tolerance Index

Heavy Metals	Heavy metal tolerance of isolated fungal strains (mg/ml)								
	Aspergillus niger EF1	*Aspergillus flavus* ED4	*Fusarium* sp. EG2	*Penicillium* sp. ED1	*Aspergillus* sp. EA2	*Fusarium* sp. EH3	*Aspergillus niger* EC1	*Penicillium* sp. EC2	Control
Cr6+	0.62	0.8	0.52	0.39	0.53	0.47	0.6	0.48	1
Pb2+	0.55	0.72	0.51	0.34	0.37	0.52	0.47	0.28	1
Cu2+	0.32	0.63	0.15	0.5	0.16	0.29	0.28	0.37	1
Ni2+	0.45	0.58	0.25	0.2	0.4	0.19	0.38	0.16	1
Zn2+	0.29	0.46	0.36	0.3	0.33	0.21	0.38	0.19	1
Cd2+	0.28	0.6	0.4	0.33	0.46	0.18	0.31	0.33	1

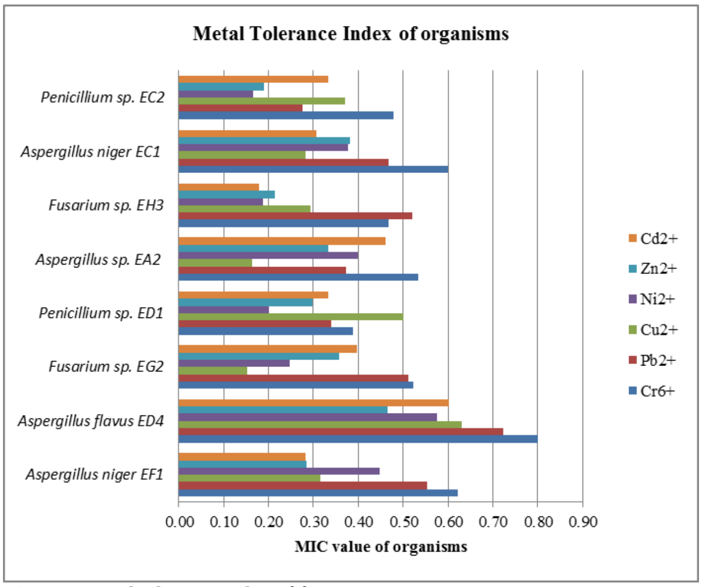

Figure 1 Metal tolerance index of fungi strains

Table 3 Minimum inhibitory concentration (MIC) of fungi against heavy metals

Test organisms	MIC (mg ml^{-1})					
	Cr	**Pb**	**Cu**	**Ni**	**Zn**	**Cd**
Aspergillus niger EF1	2	1	1	1	0.5	0.5
Aspergillus flavus ED4	3	1.5	2	1.5	1.5	1.5
Fusarium sp. EG2	1.5	1	0.5	0.5	1	0.5
Penicillium sp. ED1	1	0.5	1.5	0.5	1	0.5
Aspergillus sp. EA2	1.5	0.5	0.5	1	1	1
Fusarium sp. EH3	1.5	1	1	0.5	0.5	0.5
Aspergillus niger EC1	1.5	1	1.5	1	1	1
Penicillium sp. EC2	1	0.5	0.5	0.5	0.5	0.5

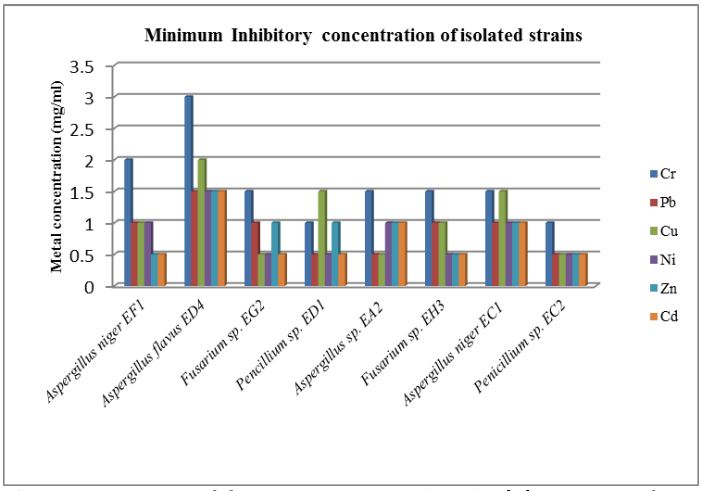

Figure 2 Minimum Inhibitory Concentration (MIC) of fungi against heavy metals

CONCLUSION

Findings of the present study reveals that the fungal microorganisms isolated from effluents contaminated with heavy metal have the ability to resist higher concentrations of metals. The resistance and tolerance ability of the isolates depends on the fungus and its habitat. This may be due to adaptation and tolerance of the fungi towards heavy metals. The present investigation shows that *Aspergillus flavus* was the most resistant fungi tested against various metals, which are considered as promising candidates for further investigations for heavy metal removal.

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