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Bioaccumulation Potency of Ecotoxic Heavy Metals by Indigenous *Rhizopus* and *Penicillium* sp: A Comparative Study

C.K. Shivakumar[1], B. Thippeswamy[1], M. Krishnappa[2] and K.S. Ananthamurthy[3]

[1]Department of P.G. Studies and Research in Microbiology,
[2]Department of P.G. Studies and Research in Applied Botany, [3]Department of P.G. Studies and Research in Applied Geology, Kuvempu University, Bio-Science Complex, Jnanasahyadri, Shankaraghatta-577 451, India.

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Tolerance and accumulation ability for Cu^{2+}, Zn^{2+}, Cr^{6+}, Cd^{2+}, Ni^{2+} and Pb^{2+} were investigated in aqueous medium and untreated effluent by *Rhizopus* sp and *Penicillium* sp isolated from paper mill effluent. Physicochemical content for untreated and treated paper mill effluent was analyzed as per standard method. Effect of heavy metals on fungal growth, morphology and metal accumulation was observed with control. *Rhizopus* sp tolerated 1000ppm of Pb followed by Cu, Zn (500ppm), Cd (250ppm). *Penicillium* sp tolerated metals in the order of Pb (1000ppm), Zn (500ppm), Cr (250ppm) and Cd (100ppm). *Rhizopus* sp showed high Pb (69%) uptake followed by Cu (48%), Cd (47%) and Zn (44%). In *Penicillium* sp Zn (47%) tolerance was high followed by Pb (46%), Cd (26%), and Cr (18%). Growth with significant reduction in heavy metals (Cd, Zn, and Pb) observed in untreated industrial effluent inoculated with fungal biomass when compared with treated industrial effluent.

Keywords: Bioaccumulation, Heavy metals, Industrial Effluent, *Rhizopus* sp., *Penicillium* sp.

Heavy metals are categorized based on their density (>5g/cm^3), atomic weight >sodium (>23), atomic number between 21 (scandium) & 92 (uranium) and high molecular weight. Electron acceptors (Lewis acids) property of metal ions results in covalent complex formation with different ligands (sulfur, nitrogen, oxygen) in humans and other forms of life which makes metals none degraded able and cause toxicity (Duffus, 2002). Pulp and paper mill categorized as one of the 12 most polluting industries in India which release environmentally hazardous liquid effluent containing heavy metals and other organic toxicants (Verma *et al*., 2005). According to the World Health Organization (1984) the metals of most immediate concern are cadmium, cobalt, copper, chromium, lead, nickel, mercury and zinc (Zaied *et al*., 2008). By most of the countries cadmium, mercury and lead are included among the "priority pollutants" because of their high toxicity requiring suitable treatment prior to discharge into the environment**.** Heavy metals cannot be degraded and even at low concentration can cause toxicity to humans and other forms of life (Rao *et al*., 2005). The chemical precipitation, ion-exchange, adsorption and reverse osmosis are commonly used processes for metal removal. However, these methods are sometimes restricted because of technical or economical constraints (Akar and Tunali, 2006). Biological methods for heavy metals removal may provide an attractive

* To whom all correspondence should be addressed.
E-mail: thippeswamyb205@gmail.com

alternative to physicochemical methods (Hussein *et al*., 2004). Bioaccumulation and biosorption have attracted as an alternative method to conventional process involved in toxic metal removal from industrial wasted streams (Kiran *et al*., 2005). Accumulation of metal by microorganisms has been known for few decades because of its potential application in environment protection or recovery of precious metals. The active mode of metal accumulation by living cells is designated as bioaccumulation (Sag and Kutsal, 2000). A variety of microorganisms have been explored for heavy metal biosorption and bioaccumulation including fungi (Vimala and Das, 2009). Fungi affect the mobility and environmental fate of metals. The ability of some fungi to survive in environments with extremely high levels of heavy metals depends on a range of tolerance mechanisms (Jaeckel *et al*., 2005). The uptake of metals by living cells depends on species, contact time, pH of the metal solution, culture conditions, initial concentration of metal ion and cells in the solution (Melgar *et al*., 2007). The microbial populations in metal polluted environments contain microorganisms which have adapted to toxic concentration of heavy metals and become metal resistant (Leung *et al*., 2000). The heavy metals like Cu, Cd, Mg and Mn present in low concentration of paper mill effluent can be removed biologically by indigenous microbes isolated from the effluent itself (Hakeem and Bhatnagar, 2010). Fungi (*Aspergillus* sp, *Mucor* sp, *Rhizopus* sp, *Penicillium* sp and *Saccharomyces* sp) accumulates micronutrients such as Cu, Zn, Mn and non-nutrient metals like Ni, Cd, Sn, Hg in amounts higher than the nutritional requirement. Fungi belonging to the genera *Rhizopus* and *Penicillium* have already been studied as a potential biomass for the removal of heavy metals from aqueous solution (Srivastava and Thakur, 2006). But little is known about the removal of heavy metals such as lead, cadmium, copper and nickel from aqueous solution using the *Rhizopus* sp and *Penicillium* sp.

MATERIAL AND METHODS

Study area

The present study was undertaken using the industrial effluent resulted from paper mill factory to Bhadra River in Karnataka state in southern India. The Bhadra River originates in the Western Ghats range and flows initially through the city of Bhadravathi towards east across the Deccan plateau which empties into the Bay of Bengal. Bhadra River receives 75,000m^3/day waste water from paper mill factory.

Effluent sampling

Untreated and treated effluent samples were collected in clean, dry plastic cans, labeled properly and stored in the refrigerator (4^0C) till the isolation of fungi. The samples were preserved for physico-chemical and heavy metals analysis by acidification with concentrated HNO_3 (1.5ml/ltr) and stored at 4^0C till analysis. To ensure accuracy and precision triplicate effluent samples were drawn from the sampling point.

Analysis of Physicochemical characters

Temperature, pH, color and odor of samples were recorded on the spot. Electrical conductivity, TDS, BOD, COD, DO, total alkalinity, total acidity, oxidizable organic mater, chloride, sulphate and phosphate analysis was carried out according to standard methods (APHA, 2005). Heavy metals (Cu, Zn, Cr. Cd, Ni and Pb) were analyzed after acid digestion of effluent by Atomic Absorption Spectrophotometer (AAS).

Isolation and Characterization of Fungi

Fungi were isolated from the collected effluent sample by serial dilution method. 1ml effluent sample was serially diluted to 10^{-6} dilution in sterile distilled water and 0.1ml of each dilution was spread on Potato Dextrose Agar (PDA) plates containing Streptomycin. Inoculated plates were incubated at room temperature for 5-7 days. After incubation fungal colonies on PDA plates were identified based on their morphology and reproductive structural characteristics (Nagamani *et al*., 2006). Isolated pure cultures were maintained and stored in a refrigerator. Subculture was carried on once a month or when required.

Preparation of heavy metal solution

Stock metal solutions of 1000mg/L of Ni(II), Zn(II), Cd(II), Pb(II), Cr(VI) and Cu(II) were prepared by dissolving AR grade salts of $NiSO_4.7H_2O$, $ZnSO_4.6H_2O$, $CdCl_2$, $(CH_3COO)_2Pb.3H_2O$, $K_2Cr_2O_7$, $CuSO_4.5H_2O$ in double distilled water. The working metal solutions (100, 250, 500 and 1000ppm) were prepared from stock solution. Before mixing the media and microorganisms pH of each test metal solution were

adjusted to desirable value with that of media using 1N HCl and 1N NaOH.

To check tolerance and bioaccumulation of heavy metals by fungal isolates

Microorganisms and growth medium used for heavy metals bioaccumulation are,

Rhizopus- Potato Dextrose Broth, *Penicillium*-Glucose Yeast Extract Medium.

Media composition- Potato Dextrose Broth (PDB) (g/L): Potato infusion, 200; Dextrose, 20. pH was adjusted to 3.5 subsequent to sterilization. Glucose Yeast Extract Medium (g/L): Glucose, 20; $NaNO_3$, 2;K_2HPO_4, 1;$MgSO_4.7\ H_2O$, 0.5; KCl, 0.5; $FeSO_4$ 0.01;Yeast extract, 1. pH was adjusted to 5.0 before sterilization. The fresh cultured fungal spores of *Penicillium* sp. and *Rhizopus* sp. were inoculated in to aliquots of 100ml specific growth medium supplemented with 100ppm, 250ppm, 500ppm and 1000ppm of each heavy metals in 250ml Erlenmeyer's flask. Inoculated flasks were incubated with positive control containing spore inoculated medium without metal in rotary shaker (150rpm) at 30°C for 7 days.

To analyze the dry weight of fungal biomass treated with heavy metals

After incubation period the fungal matt were harvested from the growth medium by sieving through Whatman No.1 filter paper and filtrate medium was collected. Fungal Matt was washed twice with distilled water to remove non-biomass ash and dried in an oven at 80°C for 12h (over night) and constant dry weight was taken (Jaeckel *et al.,* 2005).

Determination of heavy metals

100ppm metal treated filtrate medium was digested using concentrated HNO_3 (5ml) and boiling chips. The content was boiled and evaporated to 16-20ml on hot plate. Concentrated HCl (5ml) was added and boiled till sample become clear and brownish fumes were evident. Then dried container was cooled and diluted to 100ml by double distilled water and filtered through Whatman No.1 filter paper. The concentration of heavy metal in the filtered solution was determined using AAS. The dried fungal matt was crushed in a pestle and mortar. The ground material was placed in a conical flask and 5:1 (nitric / perchloric acid) mixture was added (Juwarkar, 1988)**.** The content of the flask was placed on a hot plate until the production of red nitrous fumes ceased and liquid becomes colorless**.** Finally container was cooled, diluted to 100ml with double distilled water and filtered through Whatman No.1 filter paper to analyze heavy metals by AAS. Heavy metals under investigation in this study included Cu^{2+}, Zn^{2+}, Cr^{6+}, Cd^{2+}, Ni^{2+} and Pb^{2+}

Heavy metals uptake in effluent samples using fungal biomass

This study aimed to reduce heavy metals pollution in factory effluents by fungal biomass and lower addition of carbon source to effluent samples. *Penicillium* sp. and *Rhizopus* sp. (100mg) biomass were inoculated each separately into untreated industrial effluent (100ml) sample enriched with 0.01% glucose. Inoculated samples were incubated at 37^0C for 72hrs in a rotary shaker (150rpm) to check their ability to grow and remove heavy metals from effluent (Zaied *et al*., 2008).

RESULTS

The physicochemical and heavy metal characterization for untreated and treated effluent samples was recorded in Table1**.** Obtained results were compared with the standard values (IS, WHO).The untreated effluent had dark yellow colored, pinching odor, acidic nature with high COD (1488 mgL^{-1}), BOD (602 mgL^{-1}), TDS (765 mgL^{-1}) and total hardness (687 mgL^{-1}). In treated effluent COD (202 mgL^{-1}), total acidity (67), total alkalinity (63) were within the desirable limit. However, the level of BOD (60mgL^{-1}), total hardness (359 mgL^{-1}) and TDS (803 mgL^{-1}) was higher than tolerance limit. The heavy metals content was higher than the permissible limit in treated industrial effluent. *Rhizopus* sp and *Penicillium* sp were isolated from the effluent. Metal tolerance of *Rhizopus* sp were in the order of Pb (1000ppm) > Cu, Zn, Cd (500ppm) and no growth observed with Cr, Ni (100ppm). Metal tolerance of *Penicillium* sp were in the order of Pb (1000ppm) > Zn, Cr (500ppm) > Cd (250ppm) and no growth observed with Cu, Ni (100ppm). The potential of these isolated fungi able to grow and remove the lead, chromium, copper, nickel, zinc and cadmium from the aqueous solution and effluent was investigated. In the present investigation accumulation ability for heavy metals at 100ppm in aqueous medium was observed using living fungal isolates. The obtained results

for metal accumulation by *Rhizopus* sp was in the order of Pb (69%)> Cu (48%)> Cd(47%)> Zn(44%) at 100ppm, followed by Pb (45%)> Cu (27%)> Cd(25%)> Zn(20%) at 250ppm, Pb (23%)> Cu (19%)> Zn(18%) Cd(11%) at 500ppm and only Pb (10%) accumulation at 1000ppm concentration was observed. For *Penicillium* sp Zn (47%)> Pb (46%)> Cd(26%)> Cr(18%) at 100ppm, Zn (36%)>

Table 1. Physicochemical parameters for untreated and treated industrial effluent

S. No.	Parameter	Observed values		Tolerance limit (IS 10500)	Tolerance limit (WHO 2006)
		Untreated effluent	Treated effluent		
1	pH	3.5±0.03	7.8±0.08	5.5-9	No guideline
2	Temperature	32±0.30	32.4±0.29	40	No guideline
3	Color	Dark yellow	Light yellow	-	-
4	Odor	Pinching	Pinching	-	-
5	BOD (3days)	602 ±2.0	60 ±1.51	30	No guideline
6	OD	0	4.3 ±0.28		No guideline
7	COD	1488 ±2.1	202 ±2.26	250	No guideline
8	Hardness	687 ±1.8	359 ±2.76	300	No guideline
9	Total acidity	157± 1.9	67 ±1.24	-	-
10	Total alkalinity	164 ±1.2	63± 0.96	-	-
11	Free CO_2	81 ±0.9	53 ±1	-	-
12	TDS	765 ±1.3	803± 2.1	500	No guideline
13	Conductivity	1528± 2.4	1598± 3.1	-	-
14	Chlorine	101 ±0.7	75 ±1.29	-	-
15	Calcium	95 ±0.8	79 ±0.86	-	-
16	Magnesium	110 ±0.8	45 ±1.23	-	-
17	Chloride	1768± 1.8	686± 2.64	-	-
18	Sulfate	446 ±1.3	192 ±2.64	-	-
19	Copper	8.01± 0.08	7.72± 0.11	3.0	2.0
20	Chromium	63.15 ±0.23	59.02± 0.43	0.1	0.05
21	Nickel	3.76 ±0.14	3.49± 0.13	3.0	0.07
22	Zinc	3.64 ±0.13	2.90 ±0.15	5.0	2.0
22	Lead	1.92 ±0.10	1.54 ±0.06	0.1	0.01
23	Cadmium	1.71 ±0.01	1.44± 0.01	2.0	0.03

Parameter unit -mgL^{-1}, except pH, temperature and conductivity,
± -Standard Error
WHO (2006)-World Health Organization
BIS (1993)-Bureau of Indian Standard for effluent discharge to inland surface water

Table 2. Heavy metals concentration in effluent treated with *Penicillium* sp

Heavy Metal	Untreated effluent (Industry)	Treated effluent (Industry)	*Penicillium* treated effluent (Lab)
Cadmium	1.71±0.01	1.44±0.01	1.07±0.02*
Chromium	63.15±0.29	59.02±0.43	60.30±0.63
Copper	8.01±0.08	7.72±0.11	7.76±0.17
Nickel	3.76±0.14	3.49±0.13	3.36±0.14*
Lead	1.92±0.10	1.54±0.06	1.48±0.06
Zinc	3.64±0.13	2.90±0.15	2.41±0.12*

* Significant level: $p<0.05$, ± Standard Error

Pb (25%)> Cd(18%)> Cr(16%) at 250ppm, Zn (25%)> Pb (20%)> Cd(17%)> Cr(14%) at 500ppm and Pb(18%) accumulation observed at 1000ppm. The amount of metal removed from untreated industrial effluent was calculated by the difference between the initial concentration and the concentration of metals after fungal growth. The test fungal biomass accumulates metal ions in untreated industrial effluent showed variable removal capacity. A significant reduction in Zn, Ni and Cd by *Penicillium* sp and Cd, Zn and Pb for *Rhizopus* sp has observed when compared with treated effluent. Obtained results were reported in the Table 2 and Table 3. Increased fungal biomass weight observed in treated effluent compare to biomass in untreated effluent. Results were reported in the Table 4. The effect of heavy metals on fungi in terms of their dry biomass (Fig. 1 and Fig. 2), metal accumulation (Fig. 3 and Fig. 4) and morphology (Fig. 5) is reported. All values reported in this paper are the average of three independent experiments each in triplicate. The concentration of heavy metal was determined by AAS.

Table 3. Heavy metals concentration in effluent treated with *Rhizopus* sp

Heavy Metal	Untreated effluent (Industry)	Treated effluent (Industry)	*Rhizopus* treated effluent (Lab)
Cadmium	1.71±0.01	1.44±0.01	1.04±0.03*
Chromium	63.15±0.29	59.02±0.43	60.26±0.4
Copper	8.01±0.08	7.72±0.11	7.72±0.13
Nickel	3.76±0.14	3.49±0.13	3.53±0.11
Lead	1.92±0.10	1.54±0.06	0.91±0.03*
Zinc	3.64±0.13	2.90±0.15	2.24±0.13*

* Significant level: $p<0.05$, ± Standard Error

Table 4. Fungal biomass weight in untreated and treated industrial effluent

Fungal isolate	Weight of biomass in Untreated effluent (mg)	Weight of biomass in Treated effluent (mg)
Penicillium sp	130	120
Rhizopus sp	170	418

DISCUSSION

The presence of lignin and its derivatives impart color to the effluent. Untreated paper mill effluent contain higher load of organic matter and chemical content when compared with treated effluent. Presence of lignin and fibbers are not readily biodegradable. Hence lime treatment and chemicals were used to treat effluent color and reduce its content to the safer level which in turn increases the heavy metal concentration. Toxicity of some heavy metals to fungi is due to their strong affinity complex with the cell membrane constituents causing loss of integrity and impairment of their functions (Chen and Wang, 2007). Effect of heavy metals observed on fungal inhibition was variable and depends on the metal and its concentrations in the medium (Zetic *et al*., 2001). Living fungal biomass used in this study able to remove the heavy metals from aqueous medium and industrial effluent (Srivastava and Thakur, 2006). In this study accumulation by living cells was higher due to intracellular metal ion uptake by metabolically active cells in combination with extra cellular adsorption (Sintuprapa *et al*., 2000). The energy metabolism is essential for metal removal (Magyarosy *et al*., 2002). The chemical composition of the fungal wall is mainly dependent on the culture conditions and this may in turn affect metal accumulative properties. The addition of toxic

metals in to the growth medium can alter the cell wall composition resulting in production of melanin and increased metal binding capacity. Under suitable growth medium certain fungi including *Aspergillus*, *Rhizopus* and *Penicillium* species, produce spherical mycelia pellets which helps in metal accumulation (Leung *et al*., 2000). Carboxyl, phosphate and hydroxyl functional groups involved in the binding of heavy metals to microbial cells. These sites have higher and more covalent

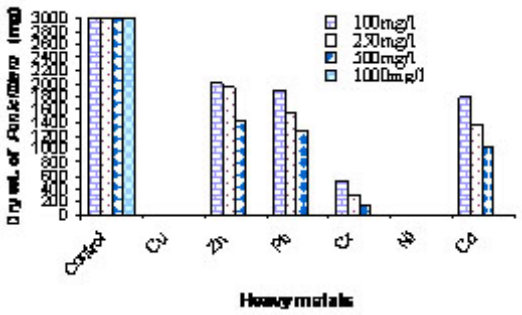

Fig. 1. Dry weight of *Penicillium* treated with heavy metals

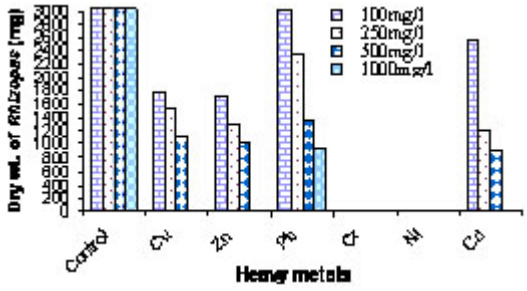

Fig. 2. Dry weight of *Rhizopus* treated with heavy metals

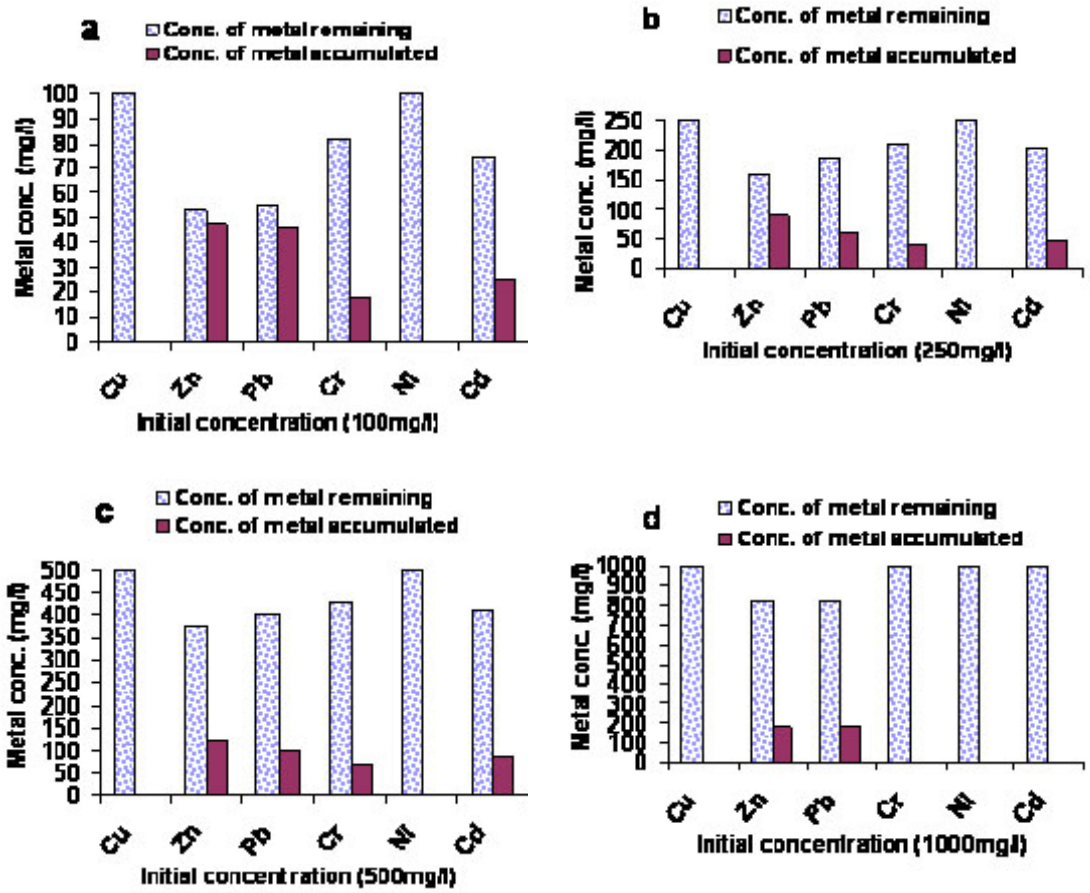

Fig. 3. Heavy metals accumulation by *Penicillium*

affinity towards toxic transition metal ions(Cu, Cd, Co, Ni etc) compare to alkali earth metal ions(Na, K, Ca) With increase in the concentration metals bioaccumulation efficiency decreased due to saturation of the biosorbent (Rao *et al*., 2005). Growth of fungal biomass in treated effluent was high compared with biomass in untreated effluent. This indicates reduction of pollution level in treated effluent. The differential innate and inherent properties of genera and species specific variability for metal ions uptake have been attributed by its functional group, surface area, cell division. Fungal

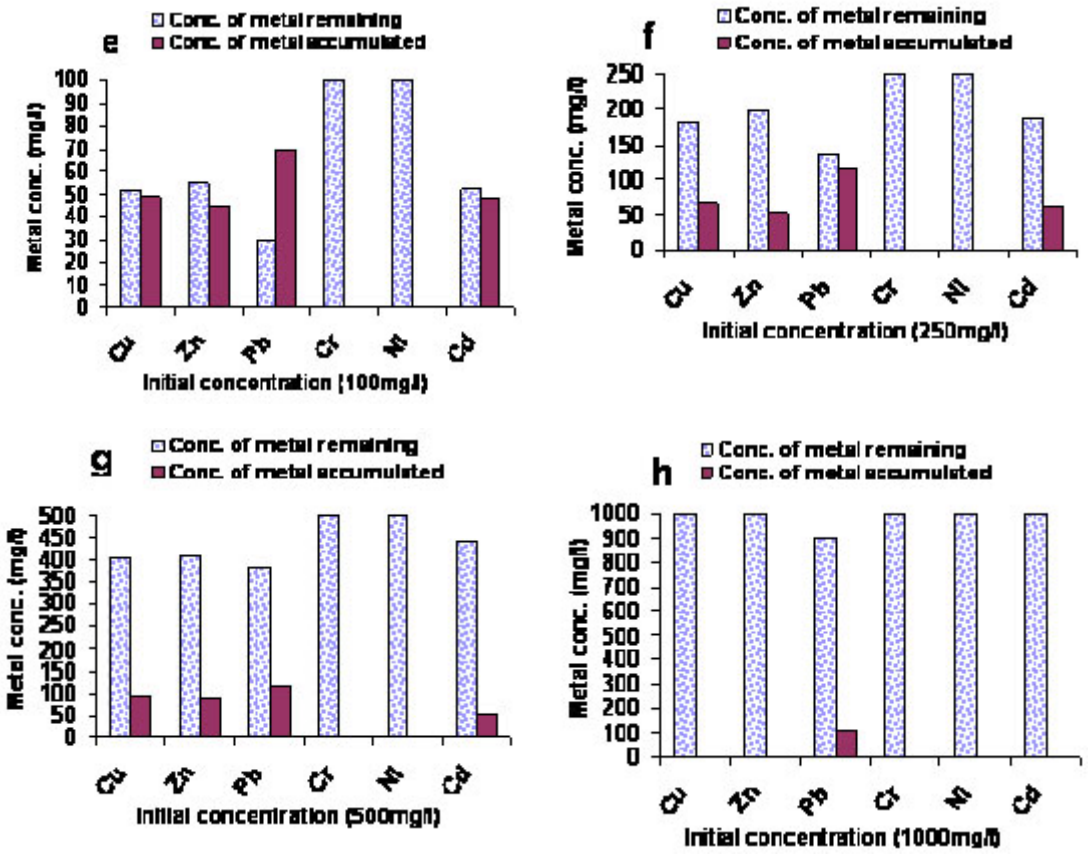

Fig. 4. Heavy metals accumulation by *Rhizopus*

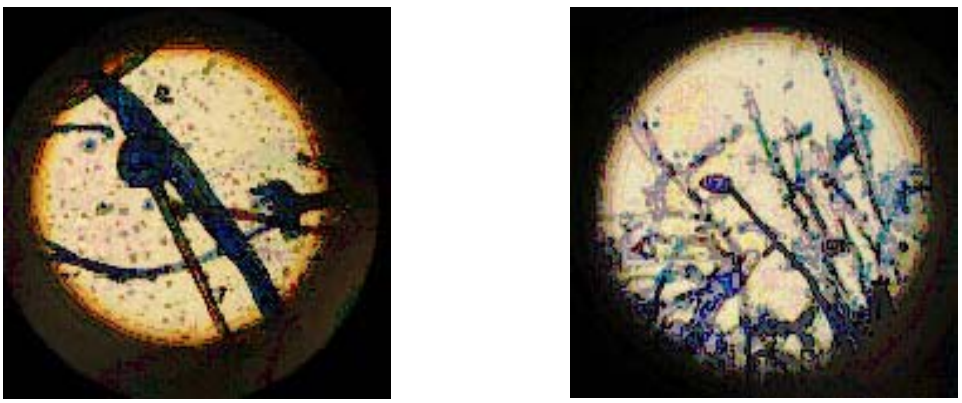

Fig. 5. The effect of heavy metals on fungal morphology **(a)** Metal untreated Sporangium **(b)** Metal treated sporangium

cell walls mainly consisting of polysaccharides, proteins and lipids have many functional groups that are responsible for the binding of metals (Akar and Tunali, 2006) (Melgar *et al*., 2007). Accumulation of metals by *Penicillium* sp. which binds to the cell is due to energy coupled transport system similar to metallothionein (Juwarkar, 1988). The amount metal ions accumulation increases with increasing initial metal ion concentration due to an increase in electrostatic interactions of metal ions on the cell surface (Yun-guo *et al*., 2006). Under suitable growth conditions certain fungi including *Aspergillus*, *Rhizopus* and *Penicillium* species produce spherical mycelia pellets which have been used in metal accumulation (Leung *et al*., 2000).

Fungi used in this have unique metal accumulation and are ubiquitous with easy to cultivate. They can successfully remove toxic metal ions Zn^{2+}, Cu^{2+}, Cd^{2+}, Pb^{2+}, $Ni^{2+}$and Cu^{6+} from aqueous solution. Metal accumulation in this has been carried out using easily available biomass in their native state or after simple processing in culture media (Ahluwalia and Goyal, 2006).

CONCLUSION

Thus the present study suggested that proper installation of effluent treatment with natural ubiquitous fungi can remove toxic metals rendering the effluent more eco-friendly by control pollution and health hazards.

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