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Bioaccumulation potential of *Aspergillus niger* and *Aspergillus flavus* for removal of heavy metals from paper mill effluent

Author Details

B. Thippeswamy (Corresponding author)
Dept. of Post Ggraduate Studies and Research in Microbiology, Kuvempu University, Shankaraghatta-577 451, India
e-mail : thippeswamyb205@gmail.com

C.K. Shivakumar
Dept. of Post Ggraduate Studies and Research in Microbiology, Kuvempu University, Shankaraghatta-577 451, India

M. Krishnappa
Dept. of Post Ggraduate Studies and Research in Applied Botany, Kuvempu University, Shankaraghatta-577 451, India

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Abstract

In the present study *Aspergillus niger* and *Aspergillus flavus* isolated from paper mill effluent showed tolerance and accumulation of toxic metals Ni, Zn, Cd, Pb, Cr and Cu from synthetic medium and paper mill effluent. Physico-chemical and heavy metals characterization of industrially treated paper mill effluent showed insignificant reduction in BOD, hardness, TDS and heavy metals as compared to permissible limits of BIS and WHO. *A.niger* and *A.flavus* were treated with synthetic medium containing 100-1000 mg l^{-1} of six heavy metals. *A.niger* was able to tolerate and grow in 1000 mg l^{-1} Pb, 500 mg l^{-1} Cu, 250 mg l^{-1} Zn and 100 mg l^{-1} Cr, Ni respectively. No growth of *A.niger* was observed in 100 mg l^{-1} of Cd. *A.flavus* was capable to tolerate and grow in 1000 mg l^{-1} Pb, Zn and Ni, 100mg l^{-1} Cu. *A.flavus* growth was completely inhibited in 100 mg l^{-1} of Cd and Cr. The Cd, Zn, Cu and Pb reduction were found significant ($p<0.05$) in the paper effluent inoculated with *A.niger* and *A.flavus* biomass compared to industrial treated effluent. *A.niger* and *A.flavus* accumulated maximum of Pb (75. 82%) followed by Zn(49. 40%) > Cu (45. 34%) > Ni (25. 20%), while only 41% Cr was accumulated by *A.niger* from 100 mg l^{-1} of Cr solution.

Key words

Bioaccumulation, Heavy metals, Paper mill effluent, *Aspergillus niger*, *Aspergillus flavus*.

Introduction

A serious problem of environmental pollution has arisen in recent years, due to heavy metals resulting from many industries such as metal plating, smelting, mining, pigment and metallurgical (Akar and Tunali, 2006). Pulp and paper mills are 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).Heavy metals are categorized based on their density (>5g cm^{-3}), atomic weight >sodium (>23), atomic number between scandium (21) and uranium (92). Electron acceptor property of metal ions results in covalent complex formation with different ligands (sulfur, nitrogen, oxygen) in humans and other life forms which makes metal nondegradable and toxic (Duffus, 2002). Hence, heavy metals even at low concentration can cause toxicity to humans and other living organisms (Rao *et al*., 2005). According to World Health Organization (2006) the metals of most immediate concern are cadmium, cobalt, copper, chromium, lead, nickel, mercury and zinc (Zaied *et al*., 2008). In most countries cadmium, mercury and lead are included in the "priority pollutants" due to their high toxicity and require suitable treatment prior to discharge into the environment. The removal and recovery of heavy metals from wastewater is necessary for the protection of environment and human health (Vimala and Das, 2009). Conventional methods

are ineffective in treatment of low concentration metal ions and also generate large quantity of toxic sludge which is to be disposed in further steps. Biological methods such as bioaccumulation and biosorption of heavy metals provide an attractive alternative to physical and chemical methods (Hussein *et al*., 2004). Many microorganisms including fungi have been identified as superior candidate for metal bioremediation (Bai and Abraham, 2001). Major advantages of fungi are its significant metal uptake ability at low anticipated price (Melgar *et al*., 2007). Microbial population present in metal polluted environment have adapted and become resistant to toxic heavy metals (Leung *et al*., 2000). Presence of heavy metals and their bioavailability exert main selective pressure on microbial community. A selective advantage is conferred to those organisms that have developed tolerant mechanisms for toxic heavy metals (Jaeckele *et al*., 2005). Heavy metals like Cu, Cd, Mg and Mn present in paper mill effluent can be removed by indigenous microbes isolated from effluent itself (Hakeem and Bhatnagar, 2010). *Aspergillus* sp accumulate micronutrients such as Cu, Zn, Mn and toxic metals like Ni, Cd, Sn, and Hg in amounts higher than the nutritional requirement. In light of the above, the present study aimed to investigate the metal removal and accumultion potential of *A.niger* and *A.flavus* from paper mill effluent.

Materials and Methods

Study area: Bhadra river originates from Western Ghats range and flows initially through city of Bhadravathi towards east across the Deccan plateau and empties into the Bay of Bengal. The present study was undertaken on effluent emanating from Mysore paper mill industry into Bhadra river in Karnataka state, Southern India. Bhadra River receives 75,000m^3 day^{-1} effluent from the paper industry.

Effluent sampling: Untreated and treated paper effluent samples were collected in a clean dry plastic can, labelled and stored in refrigerator (4^0C) for fungal isolation. The samples were also preserved for physico-chemical and heavy metal analysis by acidification with concentrated HNO_3.

Physico-chemical analysis of paper effluent: Temperature, pH, color and odor of paper mill effluent was recorded on the spot. While EC, TDS, BOD, COD, DO, total alkalinity, total acidity, oxidizable organic mater, chloride, sulphate and phosphate content were analyzed according to standard methods of APHA (2005).

Isolation and characterization of fungi: The *Asperfillus niger* and *Aspergillus flavus* strains were isolated from paper mill effluent 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 refrigerator. Sub culturing was carried once a month or when required.

Preparation of metal solutions: Stock solutions of 1000 mg l^{-1} Ni, Zn, Cd, Pb, Cr and Cu were prepared by dissolving analytical grade salts of $NiSO_4.7H_2O$, $ZnSO_4.6H_2O$, $CdCl_2$, $(CH_3COO)_2Pb.3H_2O$, $K_2Cr_2O_7$ and $CuSO_4.5H_2O$ separately in 1 l double distilled water. The desired (100, 250 and 500 mg l^{-1}) concentrations heavy metal solutions were prepared from stock solutions.

Bioaccumulation of metals from synthetic medium and paper mill effluent: Eight day old spore suspension of *Aspergillus niger* and *Aspergillus flavus* were inoculated at an inoculum level of 1.5 x 10^4 spores ml^{-1} of medium in 250 ml Erlenmeyer's flasks containing 100 ml specific growth medium (Czapek – Dox for *A.flavus* and minimal salt medium for *A.niger*) supplemented with 100, 250, 500 and 1000 mg l^{-1} concentration of each heavy metal. Inoculated flasks were incubated on reciprocating shaker (150 rpm) at 30^0C for 5 days with control containing spore inoculated medium without metal. Where as from accumulation of metal from effluent, 100 mg biomass of *Aspergillus flavus* and *Aspergillus niger* were inoculated separately into 100 ml untreated paper effluent enriched with 0.01% glucose in 250 m l Erlenmeyer's flasks. Inoculated samples were incubated with control containing 100 ml of treated effluent without fungal biomass. All the flasks were incubated at 37^0C for 72hr in a rotary shaker (150rpm) to check fungal growth and its metal uptake ability. After incubation, concentrations of heavy metals in fungal treated effluent and control was determined to check any significant heavy metals reduction by fungi compared to industrial conventional treatment methods.

Determination of dry weight of fungal biomass: After incubation the fungal matt was harvested from growth medium by sieving through Whatman's 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 80^0C for 12hr (over night) and constant dry weight was taken.

Analysis of heavy metals content: The metal content in the filtrate medium was estimated by acid digestion with HCl and HNO_3 (5:5). When brownish fumes were evident, the container was cooled, diluted with 100 ml double distilled water, filtered and analyzed for heavy metal content using atomic absorption spectrophotometer (AAS).

Results and Discussion

The physico-chemical properties of the untreated paper mill effluent showed yellow color, pinching odor, acidic nature with high COD (1488 mg l^{-1}), BOD (602 mg l^{-1}), TDS (765 mg l^{-1}) and total hardness (687mg l^{-1}). The COD (202 mg l^{-1}), total acidity (67 mg l^{-1}) and total alkalinity (63 mg l^{-1}) of treated effluent was within the permissible limits of BIS (1993). However, the BOD (60mg l^{-1}), total hardness (359mg l^{-1}) and TDS (803 mg l^{-1}) was found higher than the permissible limits of BIS (1993) and WHO (2006). Presence of lignin and its derivatives which are not readily biodegradable impart

dark yellow color to effluent. The untreated paper mill effluent contained high organic matter and chemical content compared to treated effluent. Lime and chemicals used to treat paper mill effluent color and reduce its toxic chemical content to safer level in turn increased heavy metal concentration in treated effluent (Sumithra and Narayana, 2004; Hakeem and Bhatnagar, 2010). The paper mill effluent is one of the highly polluting industries notified by Government of India (Nair, 1993). The concentration of Cu, Cr(VI) and Pb was significantly high in paper mill effluent and exceeded the permissible limit of BIS (1993) due to its application as catalyst, pigments, wood preservatives and corrosion inhibitors (Goel, 1996; Trivedy and Goel, 1984). Fungi have been proved to be more efficient and economical in removal of metals and organic toxicants from dilute aqueous solutions compared to conventional methods because of their filamentous morphology and high cell wall percentage (Lacina *et al.,* 2003). Leung *et al*. (2000) observed that under suitable growth medium, certain fungi including *Aspergillus* species produce spherical mycelia pellets which have been used for metal accumulation. Effect of Cu, Zn, Pb, Ni, Cd and Cr ions on fungal growth in terms of their biomass(dry weight) was determined at increasing concentrations of 100 to 1000 mg l^{-1} (Fig. 1a, b). *A.niger* showed tolerance to 1000 mg l^{-1} Pb followed by 500mg l^{-1} Cu, 250 mg l^{-1} Zn and 100 mg l^{-1} Ni, Cr. *A.flavus* tolerated 1000 mg l^{-1} of Pb, Zn, Ni and 100mg l^{-1} Cu. In all treated concentrations of Cd, the growth of *A.niger* was inhibited while *A.flavus* was inhibited at all treated concentrations of Cr and Cd. There was an increase in fungal growth in the media amended with Pb and Zn compared to control. The high biomass of *A.niger* and *A.flavus* was observed in 100 mg l^{-1} concentration of Pb followed by Zn> Cr> Ni> Cu inoculated with *A.niger* and Zn> Ni> Cu inoculated with *A.flavus*. The observed biomass of *A.niger* and *A.flavus* decreased with increasing metal concatenation. The vigorous fungal growth after increasing concentration of Pb and Zn indicate importance of these metals in fungal growth and utilization of there fungi in bioremediation of heavy metals contaminated waste water. The fungi capable of surviving in high metal concentration are due to formation of oxalate precipitation (Sintuprapa *et al*., 2000). In this study after 72 hr of *A.niger* growth in minimal salt medium, the solution changed from an initially translucent yellow to opaque brownish red due to formation of metal precipitate. No precipitate occurred in the fungal non-inoculated control flasks. Between 72 to 120 hr, the medium changed back to translucent yellow, whereas the biomass turned from white to brownish red. This indicated that previously formed precipitate present in the medium was absorbed by *A.niger* biomass. The presence of functional groups play major role in bio-sorption of heavy metals on fungal cell wall.

The result showed increasing amount of heavy metals had different influence on the fungal biomass and heavy metals accumulation. Both fungi showed resistant to Pb and Zn at high concentration but could not accumulate these metals at higher concentrations. Zetic *et al*. (2001) reported effect of heavy metals on fungal growth was variable and depends on the type of metal and its concentration in the medium. The observed toxicity of some heavy metals like Cr (VI) and Cd on fungi is due to their strong affinity complex with the cell membrane constituents causing loss of cell integrity and impairment of cell functions (Chen and Wang, 2007). The concentration of metal in medium after fungal growth indicates the metal removal efficiency of fungi. The isolated fungi indigenous to paper mill effluent were found superior removal of some heavy metals at a concentration of 1-60 mg l^{-1} present in the paper effluent. Table 2 shows significant ($p<0.05$) reduction of Cu(83%), Cd(96%) and Zn(66%) by *A.flavus* and Cu(96%), Cd(79%), Zn(70%), Pb(44%) by *A.niger* from the effluent. Srivastava *et al.* (2007) found that industrial effluent containing toxic organic compounds produce hindrance in the fungal growth and enhance heavy metals biosorption. Biomass of *A.niger* and *A.flavus* in treated effluent was found high (700 and 400mg) as compared to biomass (240 and 310mg) in untreated effluent. This indicates reduced pollution level in fungal treated effluent. Similar studies on biological treatment of paper effluent conducted by Verma *et al*. (2005) and Ragunathan and Swaminathan (2004) reported reduced physico-chemical and heavy metals content in paper effluent treated with *Pleurotus* sp.

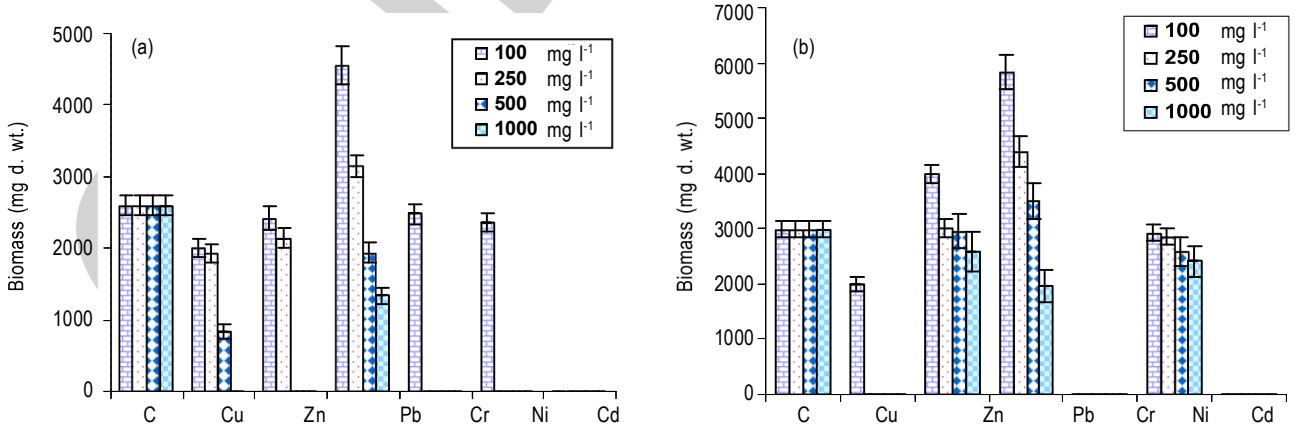

Fig. 1: Biomass of (a) *Aspergillus niger* and (b) *Aspergillus flavus* treated with different concentrations of heavy metals

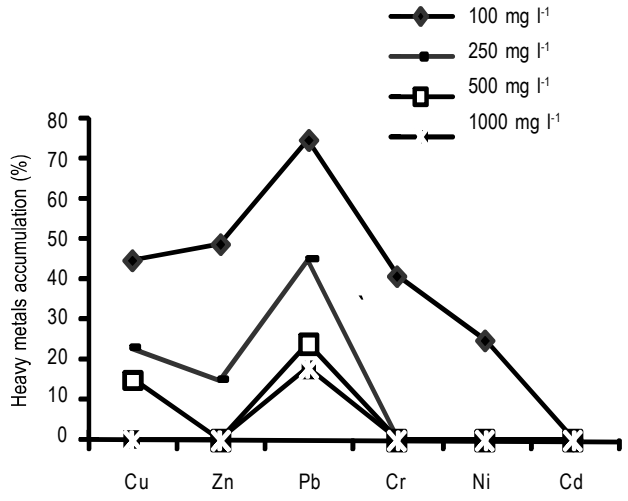

Fig. 2: Accumulation of heavy metals (%) by *A.niger*

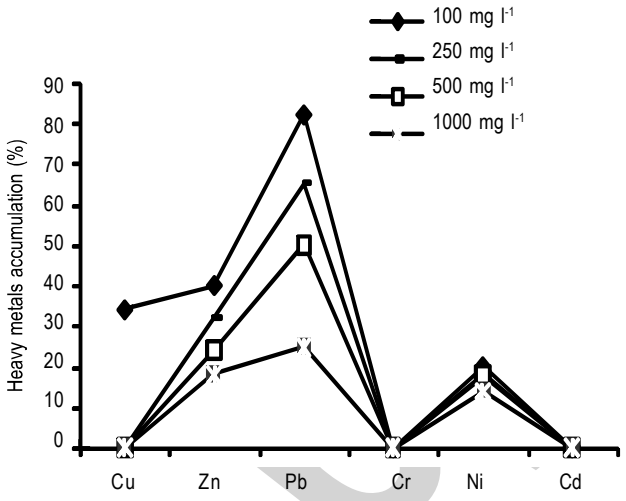

Fig. 3 Accumulation of heavy metals (%) by *A.flavus*

Table- 1: Physico-chemical parameters of untreated and treated paper mill effluent

		Observed value		Permissible limit*	
S. No.	**Parameter**	**Untreated effluent**	**Industrial treated effluent**	**(BIS,1993)**	**(WHO,2006)**
1	pH	3.5±0.03	7.8±0.08	5.5-9	-
2	Temperature	32±0.30	32.4±0.29	40	-
3	Color	Dark yellow	Light yellow	-	-
4	Odor	Pinching	Pinching	-	-
5	BOD (3days)	602 ±2.0	60 ±1.51	30	-
6	OD	0	4.3 ±0.28	-	-
7	COD	1488 ±2.1	202 ±2.26	250	-
8	Hardness	687 ±1.8	359 ±2.76	300	-
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	-
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

All the values are given in mg l^{-1} except pH, temperature and conductivity. Standard* for effluent discharge to inland surface water

Table- 2: Removal of heavy metals (mg l^{-1}) from paper mill effluent treated by *Aspergillus flavus* and *Aspergillus niger*

Heavy metal	*A.flavus* treated effluent	*A.niger* treated effluent
Cadmium	1.19±0.03*	1.14±0.06*
Chromium	59.69±0.60	59.82±0.66
Copper	7.13±0.10*	7.39±0.12*
Nickel	3.54±0.11	3.48±0.08
Lead	1.61±0.04	0.68±0.05*
Zinc	1.91±0.13*	2.03±0.15*

*Significant ($p<0.05$), values are mean of replicates ± SE

and *Eichhornia crassipes*. Rajendran *et al*. (2002) found that Ni content present in industrial effluent reduced after treatment with *A.niger*. The accumulation of heavy metals by *A.niger* and *A.flavus* is shown in Fig. 2, 3. *A.niger* accumulated high amount of Pb (75%) followed by Zn (49%) > Cu (45%) > Cr (41%) > Ni (25%) from 100 mg l^{-1} of metal solution. Where as metal accumulation from 250 and 500 mg l^{-1} of metal solution was found in the following order: Pb (45%) > Cu (23%) Zn (15%) and Pb (24%) > Cu (15%), respectively. However, *A.niger* accumulated only 18% Pb from 1000 mg l^{-1} metal solution. Kappor *et al*. (1999) reported Pb, Cu and Ni accumulation by *A.niger* from aqueous medium. *A.niger* exhibited high resistance, growth and uptake of Pb as compared to other metal ions (Dursun *et al.,* 2003). The order of metal accumulation by *A.flavus* (Fig. 3) from 100, 250, 500 and 1000 mg l^{-1} of metal solution are as follows Pb (82%) > Zn (40%) > Cu (34%), Ni (20%), Pb (65%) > Zn (32%) > Ni (18%); Pb (50%) > Zn (24%) > Ni (14%); Pb (25%) > Zn (18%) > Ni (10%), respectively. In earlier finding Akar and Tunail (2006) reported 22% Pb and 20% Cu biosorption by *A.flavus*. Rao *et al*. (2005) found that with increasing metal concentration heavy metal accumulation by fungi decreased due to saturation of biosorbent. As concentration of heavy metals increased more number of metal ions competing for the same binding sites cause in decreased metal removal, whereas in low metal concentration more number of binding sites are available for complexation of metal ions with fungal cell wall. High concentration of Pb enhanced the mass driving force with increased metal accumulation per unit weight of biomass. The Zn uptake enhanced with increasing metal ions concentration by increased number of collisions between metal ion and biomass (Kumar *et al.,* 2012). The fungal cell wall exhibit more covalent affinity to toxic transition metal ions such as Cu, Cd, Ni, Pb, Zn, and Cr compared to alkali metal ions like Na, K, Ca. Biosorption, adsorption, ion exchange and covalent binding contributed to fungal metal accumulation (Melgar *et al*., 2007). The observed percentage of metal accumulation was low in fungi treated with effluent compared to fungi inoculated in synthetic medium. Magyarosy *et al*. (2002) and Juwarkar (1988) reported that metal accumulation in the living fungi is high due to presence of essential energy metabolism and energy coupled transport system similar to metallothioneinin. In combination with extra cellular adsorption intracellular uptake of metal ions also occurs in metabolically active cells present in the synthetic medium (Sintuprapa *et al*., 2000). Hence, the living fungi can be used to remove heavy metals more efficiently from aqueous medium including industrial effluents (Srivastava and Thakur, 2006). In this study, Pb, Zn and Ni accumulation increased with increasing initial concentration due to increase electrostatic interaction of metal ions with the fungal cell wall (Yun-guo *et al*., 2006). Mukherjee *et al*. (2009) observed that metal detoxification and hyper tolerance in *Aspergillus* sp is attributed by conjugation of metal ions with thiol species and later stored in the vacuoles. The presence of toxic metals in the growth medium results in increased production of melanin and this in turn increased metal binding ability of fungi. Fungi are specific in heavy metal accumulation which is attributed by its genera, species variability, functional group, surface area, and cell division (Ahluwalia and Goyal, 2007). From over all study, it can be concluded that both *A.niger* an *A.flavus* are capable to remove Pb, Cu, Zn and Ni from paper mill effluent and synthetic medium. Both fungi displayed almost similar metal tolerance and accumulation ability whereas *A.niger* proved superior in removal of Cr as compared to *A.flavus*. Factors like pH, temperature, and contact time can be further optimized to enhance *A.niger* an *A.flavus* heavy metals bioaccumulation.

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