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HEAVY METALS ACCUMULATION POTENCY OF *ASPERGILLUS NIGER* AND *ASPERGILLUS FLAVUS* INDIGENOUS TO PAPER MILL EFFLUENT

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ABSTRACT
This study aimed to reduce Cu^{2+}, Zn^{2+}, Cr^{6+}, Cd^{2+}, Ni^{2+} and Pb^{2} heavy metals present in effluent and aqueous medium using *Aspergillus niger* and *Aspergillus flavus* isolated from paper mill effluent. Physicochemical content for untreated and treated paper mill effluent was analyzed. 6 heavy metals on fungal growth, morphology and uptake were studied. *A.niger* tolerated and accumulated up to 1000ppm Pb followed by Cu (500ppm), Zn (250ppm) and Cr, Ni (100ppm). *Aspergillus flavus* tolerated and accumulated up to 1000ppm of Pb, Zn, Ni and 100ppm of Cu. At 100ppm concentration *A.niger* showed high Pb (75%) uptake followed by Zn (49%), Cu (45%), Cr (41%) and Ni (25%). *A.flavus* accumulated metals in the order of Pb (82%), Zn (40%), Cu (34%), and Ni (18%). Significant reduction in Cd, Zn, and Pb heavy metals was reported in untreated industrial effluent inoculated with *A.niger* and *A.flavus* biomass with respect to industrial treated effluent.

INTRODUCTION

Heavy metals are categorized based on their high density, atomic weight, atomic number and molecular weight. Lewis acids property of metal ions results in covalent complex formation with different ligands in humans and other living forms which make metals nondegradable and cause toxicity (Duffus, 2002). Pulp and paper mill are categorized as one of the 12 most polluting industries in India which release environmentally hazardous effluent containing heavy metals and other organic toxicants (Verma *et al*., 2005). According to World Health Organization cadmium, cobalt, copper, chromium, lead, nickel and zinc are the metals of most immediate concern. Because of high toxicity they require suitable treatment prior to discharge into the environment (Zaied *et al*., 2008; Rao *et al*., 2005). The conventional processes used for metal removal are sometimes restricted because of technical or economical constraints (Akar and Tunali, 2006). Biological methods through bioaccumulation and biosorption for heavy metal removal from industrial wasted streams provide an attractive alternative to conventional process (Hussein *et al*., 2004; Kiran *et al.,* 2005). Microorganisms including fungi can be explored for heavy metals removal because of their potential application in protection of environment and recovery of precious metals (Vimala and Das, 2009; Sag and Kutsal, 2000). Metal polluted environment contains fungi which have adapted to toxic concentration of heavy metals have become metal resistant (Jaeckel *et al*., 2005). Metals uptake by living fungi depends on species, culture conditions, metal concentration and cells in the solution (Melgar *et al*., 2007). Little is known about the removal of lead, cadmium, copper and nickel from aqueous solution using *Aspergillus* sp. (Srivastava and Thakur, 2006). The paper deals with heavy metals accumulation potency of two specis of *Aspergillus*.

MATERIALS AND METHODS

Study area and sample collection

Present study was undertaken on the paper mill effluent discharged into the Bhadra River located in the city of Bhadravathi. Bhadra River originates in Western Ghats range and flows initially through 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.

Untreated and treated effluent samples were collected in clean container and stored in refrigerator (4°C) for fungal isolation.The samples were preserved by acidification with concentrated HNO_3 for physicochemical analysis.

Analysis of physicochemical parameters

Temperature, pH, color and odor were recorded on the spot. Electrical conductivity, TDS, BOD, COD, DO, total alkalinity, total acidity, oxidizable organic mater, chloride, sulphate and

phosphate analysis for effluent was carried out according to standard methods (APHA, 2005). Cu, Zn, Cr. Cd, Ni and Pb content were analyzed using Atomic Absorption Spectrophotometer (AAS), after acid digestion of the effluent sample.

Isolation and characterization of fungi

Fungi were isolated from the effluent sample by serial dilution method on Potato Dextrose Agar (PDA) medium. 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.

Preparation of heavy metal solutions

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 were prepared from stock solution.

Heavy metals tolerance and accumulation potency of isolated fungi

Aspegillus niger was grown in Minimal salt medium and *Aspergillu flavus* in Czapek-Dox medium. 8 days old culture spores of *A.niger*. and *A.flavus* were inoculated into aliquots of 100mL specific growth medium supplemented with 100ppm, 250ppm, 500ppm and 1000ppm concentration of each heavy metal in 250mL Erlenmeyer's flask. Inoculated flasks were incubated with control containing spore inoculated medium without metal on rotary shaker (150rpm) at 30ºC for 7 days. After incubation period fungal matt was harvested from the growth medium by sieving through Whatman No.1 filter paper and filtrate medium was collected. Fungal Matt was washed with distilled water to remove non-biomass ash

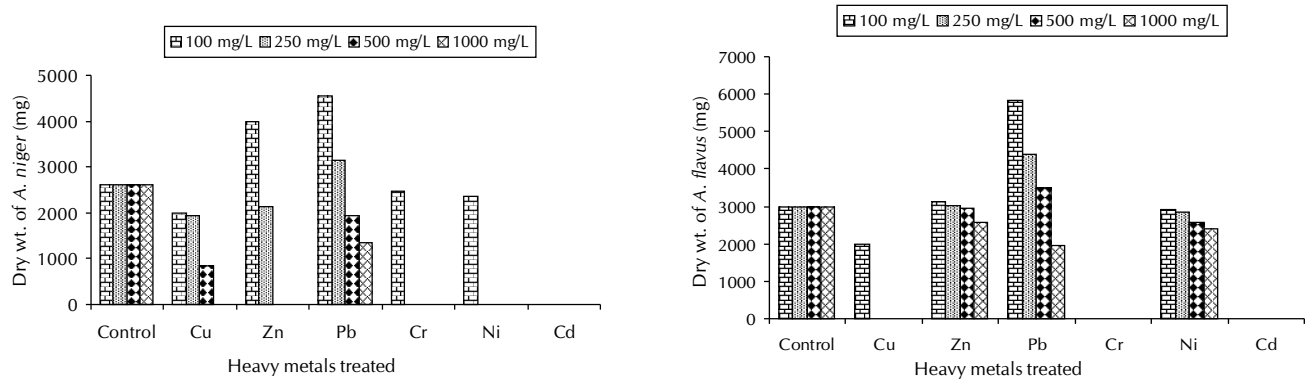

Figure 1: Dry weight of *Aspergillus niger* and *Aspergillus flavus* treated with different heavy metals concentrations

Table 1: Physicochemical parameters for untreated and treated industrial effluent

S. No.	Parameter	Observed values Untreated effluent	Treated effluent	Tolerance limit (IS 10500)	Tolerance limit (WHO 2006)
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 IS 10500(1998); Bureau of Indian Standard for effluent discharge to inland surface water

and dried in an oven at 80ºC for 12h (over night) and constant fungal biomass dry weight of was taken (Jaeckel *et al.,* 2005).

Determination of heavy metals

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, diluted with 100mL double distilled water and filtered through Whatman No.1 filter paper. The concentration of heavy metals 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 the container was cooled, diluted to 100mL with double distilled water and filtered through Whatman No.1 filter paper to analyze heavy metals by AAS.

Uptake of heavy metals from effluent using fungal biomass

A.niger and *A. flavus* biomass of 100mg was inoculated each separately into 100mL untreated effluent sample enriched with 0.01% glucose as carbon source. Inoculated samples were incubated at 37ºC for 72h in a rotary shaker at 150rpm (Zaied *et al*., 2008).

RESULTS

The physicochemical and heavy metal characterization for untreated and treated effluent samples was recorded (Table1). The untreated effluent had dark yellow colour, 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. *A.niger* and *A.flavus* were isolated from the effluent sample. Critical metal concentration limit was identified at 100, 250, 500 and 1000ppm beyond which uptake decreased with increase in metal concentration. *A.niger* tolerated up to1000ppm of Pb, 500ppm of Cu, 250ppm of Zn and 100 ppm of Cr, Ni. No growth observed with Cd at 100ppm concentration. *A.flavus*. tolerated 1000ppm of Pb, Zn, Ni and 100 ppm of Cu. No growth observed with Cr and Cd at 100 ppm concentration (Fig. 1).

In the present investigation accumulation potential of heavy metals by *A.niger* at 100ppm aqueous medium was in the order Pb (75%)> Zn(49%)> Cu(45%)> Cr(41%)>Ni(25%) followed by Pb(45%)> Cu(23%)> Zn(15%) at 500ppm, Pb(24%)> Cu(15%) at 500ppm and Pb(18%) accumulation at 1000 ppm was observed (Fig. 2) *A. flavus* accumulated

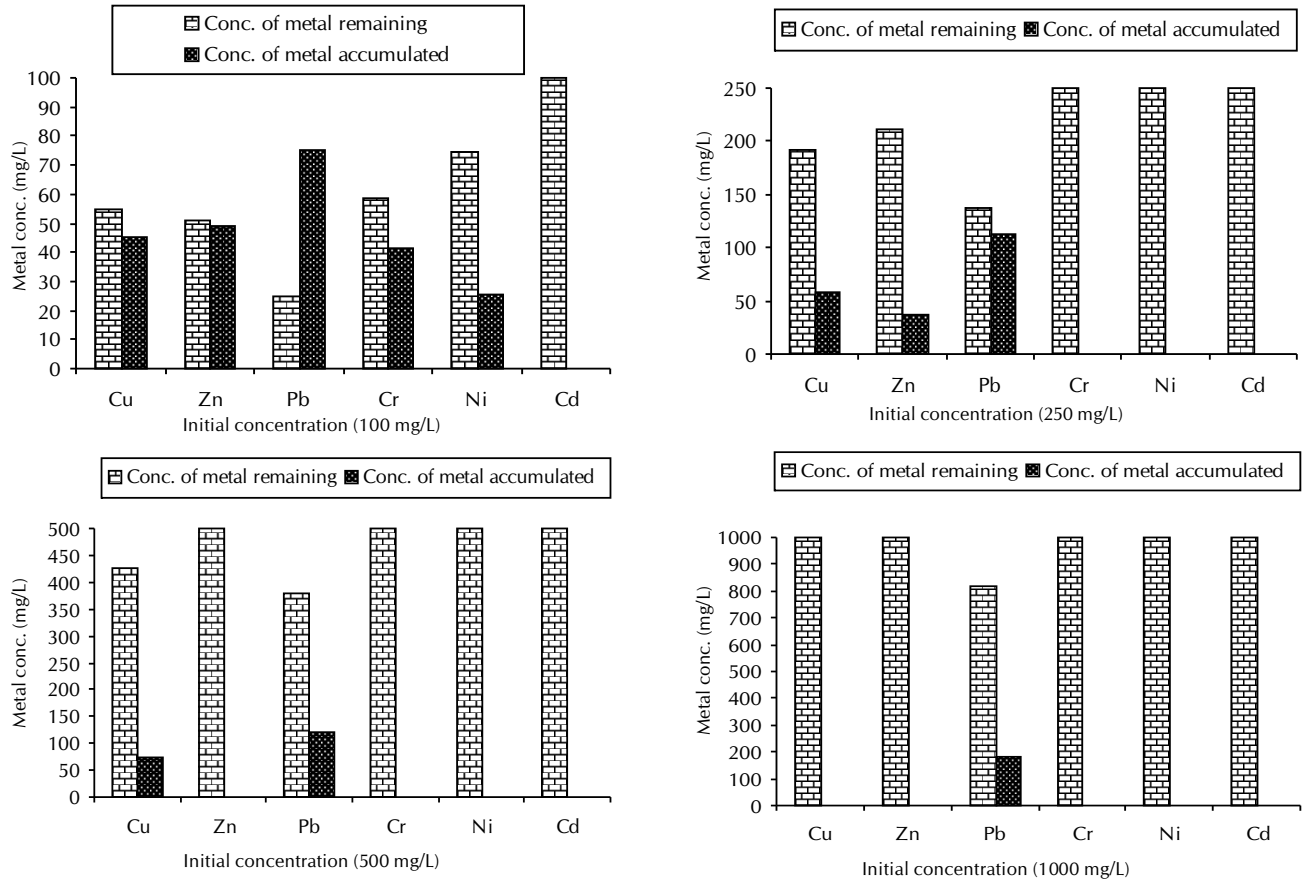

Figure 2: Heavy metals accumulation by *Aspergillus niger* at 100, 250, 500 and 1000 mg/L metal concentration

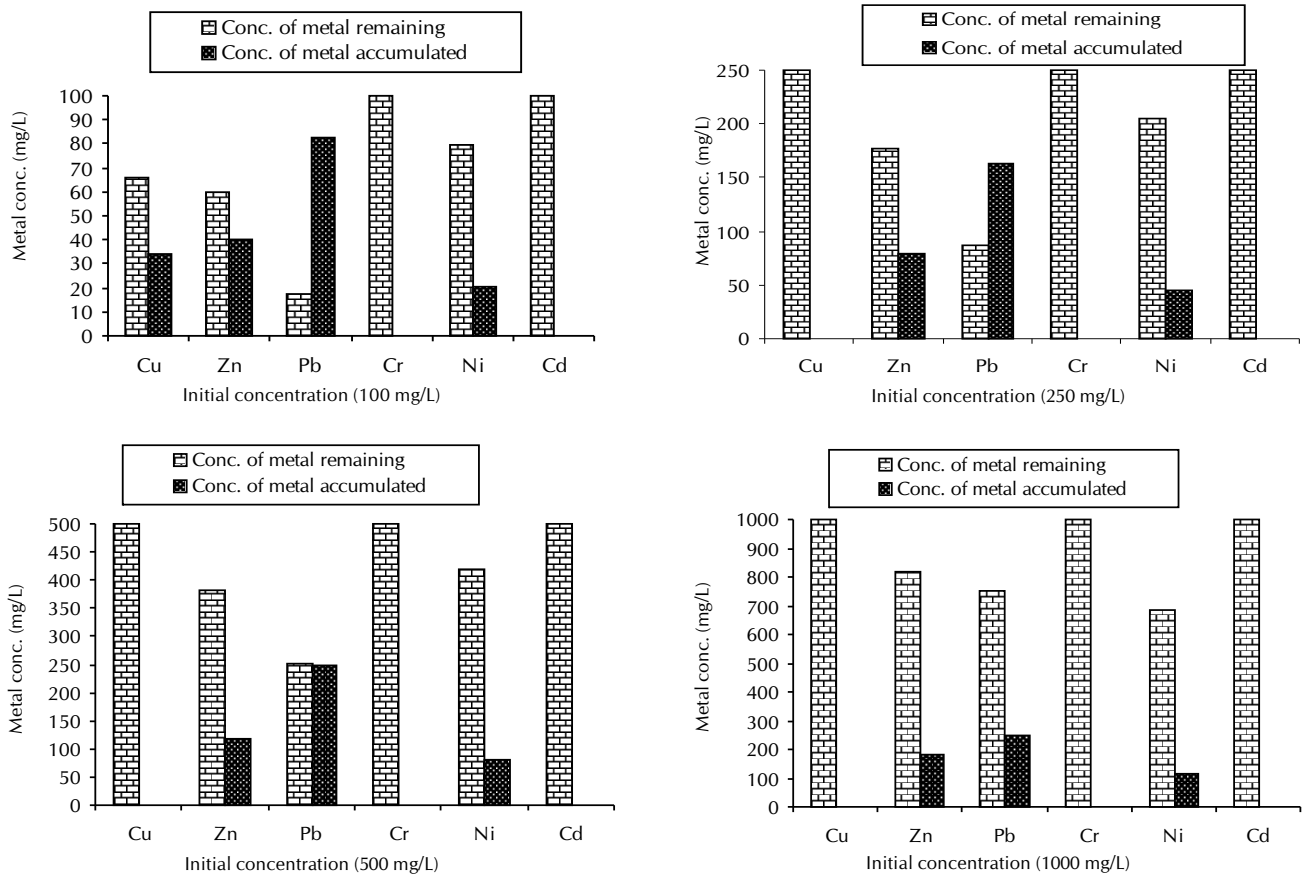

Figure 3: Heavy metals accumulation by *Aspergillus flavus* at 100, 250, 500 and 1000mg/L metal concentration

Table 2: Heavy metals concentration in effluent treated with *Aspergillus niger*

Heavy Metal	Untreated effluent (Industry)	Treated effluent (Industry)	*A.niger* treated effluent (Lab)
Cadmium	1.71 ± 0.01	1.44 ± 0.01	1.14 ± 0.06*
Chromium	63.15 ± 0.29	59.82 ± 0.66	59.02 ± 0.43
Copper	8.01 ± 0.08	7.72 ± 0.11	7.39 ± 0.12*
Nickel	3.76 ± 0.14	3.49 ± 0.13	3.48 ± 0.08
Lead	1.92 ± 0.10	1.54 ± 0.06	0.68 ± 0.05*
Zinc	3.64 ± 0.13	2.90 ± 0.15	2.03 ± 0.15*

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

Table 3: Heavy metals concentration in effluent treated with *Aspergillus flavus*

Heavy Metal	Untreatedeffluent(Industry)	Treated effluent (Industry)	*A.flavus* treated effluent (Lab)
Cadmium	1.71 ± 0.01	1.44 ± 0.01	1.19 ± 0.03*
Chromium	63.15 ± 0.29	59.02 ± 0.43	59.69 ± 0.60
Copper	8.01 ± 0.08	7.72 ± 0.11	7.13 ± 0.10*
Nickel	3.76 ± 0.14	3.49 ± 0.13	3.54 ± 0.11
Lead	1.92 ± 0.10	1.54 ± 0.06	1.61 ± 0.04
Zinc	3.64 ± 0.13	2.90 ± 0.15	1.91 ± 0.13*

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

Table 4: Weight of fungal biomass in untreated and treated industrial effluent

Fungal isolate	Weight of biomass in untreated effluent (mg)	Weight of biomass in treated effluent (mg)
Aspergillus niger	240	700
Aspergillus flavus	310	400

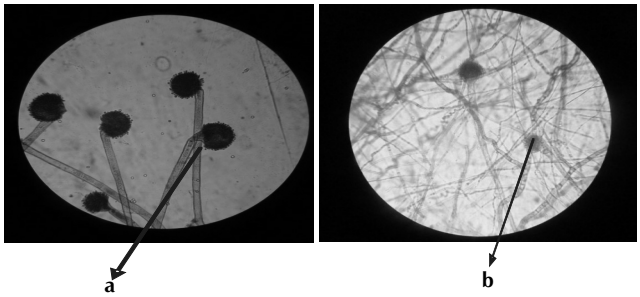

Figure 4: The effect of heavy metals on *Aspergillus* sp. (a) Metal untreatred Sporangium (b) Metal treated sporangium

metals in the order of Pb(82%)> Zn(40%)> Cu(34%)> Ni(20%) at 100 ppm followed by Pb(65%)> Zn(32%)> Ni(18%) at 500 ppm, Pb(50%)> Zn(24%)> Ni(18%) at 250 ppm and Pb(25%)> Zn(18%)> Ni(14%) accumulation observed at 1000 ppm concentration (Fig. 3).

The concentration of metal removed from untreated industrial effluent was calculated by the difference between initial concentration and the concentration of metal after fungal growth. A significant reduction in Cd, Cu, Pb and Zn by *A.niger* and Cd, Cu and Zn by *A.flavus* was observed when compared with treated effluent (Table 2 and 3).

Effect of heavy metals on fungal morphology was observed. Sporangium and sporangiophore showed shrinkage at higher metal concentrations (Fig. 4).

Increased fungal biomass weight observed in treated effluent compare to biomass in untreated effluent (Table 4).

DISCUSSION

The presence of lignin and its derivatives impart color to the effluent. Untreated paper mill effluent has high COD, BOD, TDS, hardness and heavy metals content when compared with treated effluent. Due to the presence of lignin and fibbers which are not readily biodegradable, lime and chemicals is used to treat effluent color and reduce its content to the safer level. These treatments in turn increase the heavy metals concentration in treated effluent. Cd and Cr toxicity to *Aspergillus* sp. is due to their strong affinity with the cell membrane constituents causing loss of integrity and impairment of cell functions. This study is in confirmation with the observation of Chen and Wang, (2007) and Zetic *et al*., (2001) fungal growth is dependent on the type of metal and its concentration in the medium. Metal accumulation by fungi increases with increasing in initial metal ion concentration is due to increase electrostatic interactions of metal ions on the cell surface (Yun-guo *et al*., 2006). As observed by Mukherjee *et al*. (2009) metals detoxification hyper tolerance and accumulation by *Aspergillus* sp. is due to conjugation with thiol species and stored in vacuoles. Energy metabolism is essential for metal removal. Metal accumulation by living fungi is higher due to an intracellular metal uptake take place by metabolically active cells in combination with extra cellular adsorption, which is in agreement with findings of Sintuprapa *et al*. (2000) and Magyarosy *et al*. (2002). Fungi in their native state and after processing in culture media can be used for metal accumulation, is in conformation with the observation of Ahluwalia and Goyal, (2007). Chemical composition of the fungal wall is mainly dependent on the culture conditions and this in turn affects metal accumulation. Addition of toxic metals in to the growth medium, alters the cell wall composition and increases the fungal metal binding efficiency. Under suitable growth medium *Aspergillus* sp. produce spherical mycelia which help in metal accumulation, this is similar to the findings of Leung *et al*. (2000). Fungi have higher and more covalent affinity towards toxic transition metal ions (Cu, Cd, and Ni). As noted by Rao *et al*.(2005) metal accumulation efficiency decreased with increasing in metal concentration is due to the saturation of biosorbent**.** Fungal biomass in treated effluent was high when compared with the biomass in untreated effluent. This indicates reduced pollution level in fungal treated effluent. A differential metal uptake property of *A.niger* and *A. flavus* has been attributed by its functional group, surface area and cell division. Fungal cell walls mainly consisting of polysaccharides, proteins, lipids and many functional groups that are responsible for the binding of metals (Melgar *et al*., 2007). Toxic heavy metals present in paper mill effluent can be removed by indigenous *Aspergillus* sp. isolated from the effluent itself, which is confirmed with the previous work of Hakeem and Bhatnagar (2010). As observed by Srivastava and Thakur (2006), *Aspergillus* sp. is able to accumulate toxic metals higher than the nutritional requirement. Hence *A.niger* and *A.flavus* can remove toxic Zn^{2+}, Cu^{2+}, Ni^{2+}, $Pb^{2+}$and Cr^{6+} metal ions from aqueous solution including industrial effluents.

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