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Bioaccumulation of heavy metals from aqueous solution using indigenous fungal isolates

Sadia Qayyum[1], Ibrar Khan [3], Ke Meng[1], Xiaolong Zang[1],Yangguo Zhao[1], Qingbao Gu & Changsheng Peng [1,2*]
[1] The Key Lab of Marine Environmental Science and Ecology, Ministry of Education, Ocean University of China, Qingdao 266100, China
[2] State Key Laboratory of Environmental Criteria and Risk Assessment, Chinese Research Academy of Environmental Sciences, Beijing 100012, China
[3] UNESCO Chinese Center of Marine Biotechnology, Ocean University of China, Qingdao 266003, China
*[E.Mail : pcs005@ouc.edu.cn]

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Twenty six fungal strains were isolated from heavy metals contaminated soil of which *A. flavus* (F4) and *R. pusillus* (F6) were the most dominant. Growth of isolates were noticed by Pb, Cr and Cd concentration in the growth medium in one- step and two- step process, thus about seven isolates can grow upto 10mg (Pb,Cr, Cd)/100ml medium. The formulation of Sabouraud dextrose broth (SDB) medium fortified the isolates by ingredients and favored the best growth yields that have the highest biosorption, compared to Chashi medium (CM) and Yeast peptone glucose (YPG) medium. Thus, 99% of Pb and Cr were absorbed in biosorption medium containing 10mg Pb and Cr in 100ml medium while 77% of Cd was absorbed in the biosorption medium containing 10mg Cd in 100ml medium. Uptake capacity of resistant fungal isolates against heavy metals was checked in different medium. With respect to Pb, Cd and Cr maximum uptake of 39.58, 68.02 and 68.87mg.g^{-1} was observed by fungi *Rhizomucor pusillus* (F6) and *Aspergillus flavus* (F4). This indicated the potential of these identified fungi as biosorbent for removal of high concentration metals from soil and industrial effluents and also it was observed that efficiency of two-step process is better than the one-step process.

[Key Words: Heavy metals, Fungi, media, bioaccumulation]

Introduction

Heavy metals such as iron (Fe), manganese (Mn), mercury (Hg), lead (Pb), zinc (Zn), cadmium (Cd), uranium (U), chromium (Cr) and several others are corner stone of human progress; they are quite literally the pillars of all the major civilizations, past and present because they are used widely as part of materials construction, agriculture, transportation, and in processing of many industrial materials and commercial products[1]. Mobilization of heavy metals in the environment due to industrial activities is a serious concern due to the toxicity of these metals in humans and other life forms. These metals enter into human beings and animals through food chain and cause many metabolic disorders[2,3]. Unlike organic chemicals, metals persist in environment indefinitely posing threats to all the organisms which are exposed to them. Chemical and metallurgical manufacturing are the main sources of metal ions in the environment[4]. Heavy metals present in contaminated soil may pose a threat to human health if these metals enter into the food chain.

By applying biotechnological tools like biosorption in managing and removal of metal ions pollution has been paid much consideration and gradually became important technique for the last few decade[5]. Using microorganisms as biosorbents for heavy metals is an attractive alternative to existing methods such as chemical precipitation, chemical oxidation or reduction, electrochemical treatment, filtration, ion exchange and membrane technologies for toxicity reduction and recovery of valuable metals from industrial effluents, because of good performance and low cost of biosorbent material[6]. These processes may be ineffective or expensive, especially when dissolved heavy metals concentration in the solution ranged from 1-100 mgL^{-1}.[7] Bioremediation of heavy metals involving microorganisms could be brought about by employing methods such as bio-accumulation, biosorption, bio-precipitation and uptake by purified biopolymers from microbial cells[8, 9]. Therefore, it is desirable to remove heavy metals from wastewater and soil through environment friendly low cost technology before its use in agriculture or discharge into water bodies[10, 11]. Heavy metal resistant

microbes might be present in heavy metal contaminated sites. The resistance and efficiency of microbes for removal of heavy metals vary greatly.

Therefore, there is need to isolate and screen heavy metal tolerant fungi from heavy metals contaminated sites. The Weifang red star chemical industry, Weifang, China is Smelting Industry mainly produces and sells lead-acid batteries and accessories. During the lead-acid battery production and assembly, activities such as transport of raw materials, the leaking of the production process, stacking behavior may cause the site soil lead contamination, mainly source are lead contaminated wastewater, lead dust and lead scrap ointment. Therefore, this study is an attempt to isolate heavy metal (Pb, Cr and Cd) tolerant fungi from this industrial effluent contaminated site and screening of their efficiency to remove heavy metals from liquid media is evaluated under laboratory conditions.

Materials and Methods

The contaminated soil used in this work was collected from the top soil (0−20 cm) of the sites under a slag heap at the Smelting Industry, which was built in 1954 and located in Weifang city, Shandong Province, China. These samples were brought to laboratory and kept in refrigerator at 4°Cfor further processing.

A stock Cadmium, Lead and Chromium ion solution (1000 mgL^{-1}) was prepared by dissolving cadmium chloride, lead nitrate and potassium dichromate (Fisher Scientific Ltd) in deionized distilled water, shaking for 15 min at 100 rpm and then left to stand for 24 h to obtain complete dissolution. Stock solution was diluted with deionized distilled water to obtain the necessary concentrations [12]. The metal concentration was determined with atomic absorption spectrophotometer (AAS) (M6 AAS). Fungal strains were isolated from soil samples by serial dilution method using Sabourad dextrose agar (SDA) containing Pb, Cd and Cr 100mgL^{-1} individually. A serial dilution of each sample was made up to 10^6 and 1ml of each 10^4 and 10^6 dilution were added in sterilized petri plates in duplicate. 20 mL SDA medium containing (100mgL^{-1}) Pb, Cd and Cr of one of these heavy metals was poured in the petri plates and incubated at 28°C for 96 h. The colonies of predominant genera of fungi were picked up and purified by streak plate method.

All the fungal isolates were further screened by streaking on SDA medium containing 50 and 100 mg of each of the three heavy metals separately. Streaking of fungal isolates on normal SDA medium reserved as control (normal growth) for comparison of growth of fungal isolates on SDA medium containing different concentration of heavy metals. Observations on growth of fungal isolates were made after 96 h of incubation.

Comparison between one and two-step process in bioaccumulation

Three types of medium containing Yeast peptone glucose (YPG), Chashi medium (CM) and Sabouraud dextrose broth (SDB) were used to check growth of resistant fungi and uptake of heavy metals.

Each 250mL flask containing 50mL of YPG medium (1% Yeast Extract, 2% Peptone and 2% Glucose) with 100mgL^{-1} heavy metals from stock solutions of $Pb(NO_3)_2$, $CdCl_2$ and $K_2Cr_2O_7$ salts individually. Medium was autoclaved at 121°C for 15 min. After autoclave 1mL of spore suspension (about 108 mL^{-1}) were inoculated in medium and then flasks were placed in incubator shaker (IS-RDS3C) at 25°C for 7 days at 150rpm. Room temperature was selected for growth of fungi because temperature is very important factor in biosorption.[13]

Similarly each 250mL flask containing 50mL of CM (30 g sucrose, 1 g K_2HPO4, 3 g $NaNO_3$, 0.5 g $MgSO_4$, 0.5 g KCl, 0.01 g $FeSO_4$ and 1 L H_2O) with 100mgL^{-1} heavy metals from stock solutions of $Pb(NO_3)_2$, $CdCl_2$ and $K_2Cr_2O_7$ salts individually. The pH value of CM was adjusted to 6.7 using 0.1 mol/L HCl. Flasks were placed in autoclave to sterilize medium. After autoclave 1mL of spore suspension (about 108 mL^{-1}) were inoculated in medium and then flasks were placed in incubator shaker at 25°C for 7 days at 150rpm.

Furthermore, each 250mL flask containing 50mL of SDB with 100mgL^{-1} heavy metals from stock solutions of $Pb(NO_3)_2$, $CdCl_2$ and $K_2Cr_2O_7$ salts individually. Flasks were placed in autoclave to sterilize medium. After autoclave 1ml of spore suspension (about 108 mL^{-1}) isolates were inoculated in medium and then flasks were placed in incubator shaker at 25°C for 7 days at 150rpm. Inoculated flasks

containing SDB broth with 100mgL^{-1} heavy metals served as control.

In the two-step process, 1 mL of spore suspension (about 108 mL^{-1}) was first inoculated in 50 mL of YPG, CM and SDB pre sterilized medium in 250 mL autoclaved conical flask without adding any metal solution (the first step). After two days incubation, 100mgL^{-1} heavy metal solution $Pb(NO_3)_2$, $K_2Cr_2O_7$ and $CdCl_2$ was added to the conical flask (the second step). Biosorption was carried out by tumbling the mixture in a rotary shaking incubator at 150rpm and 25°C for 5 days [7]. All the experiments were run in triplicate.

Uptake of heavy metals by fungal isolates from liquid medium

The highly heavy metal tolerant fungal isolates were evaluated for uptake of heavy metals in different medium (YPG, SDB and CM) containing 100mgL^{-1} concentration of different heavy metals Pb, Cd and Cr individually in triplicate. Medium in flask were added in 50 mL centrifuge tubes and centrifuged at 10,000rpm for 15min. Supernatant was stored for Atomic absorption spectrophotometer for estimation of heavy metal contents. The harvested fungal biomass was rinsed with double distilled water 3-4 times and dried in hot air oven at 80°C for 18 h and weighted. . All the experiments were conducted in triplicate and data were analyzed statistically.

The uptake of heavy metal by fungal biomass was calculated using the equation (1)

$$q_e = \frac{(c_i - c_f)V}{1000M} \qquad (1)$$

Where q_e is concentration of heavy metal accumulated by fungal biomass in (mg.g^{-1}), c_i is initial concentration of heavy metal (mg.L^{-1}), c_f is the final concentration of heavy metal (mg.L^{-1}); V (L) is the volume of the aqueous medium and M is the dry weight (g) of the fungal biomass.

Results

Twenty six fungal isolates tolerant to heavy metals were isolated from samples of Weifang Red star chemical industry Weifang city China contaminated with heavy metals such as Pb, Cr and Cd using standard methods.[14]This included ten isolates tolerant to Pb, nine isolates tolerant to Cr and seven isolates tolerant to Cd. Four fungi; *Aspergillus tubingensis* (F1), *Neosartorya hiratsukae* (F3), *Aspergillus flavus* (F4), *Rhizomucor pusillus* (F5), tolerant to Pb were further screened for their tolerance to Pb at 50 and 100mg of Pb. Out of fourteen fungal isolates tolerant to Pb at 10mg it was found that four isolates could tolerate Pb at 10mg. Among nine isolates tolerant to Cr only three isolates *Aspergillus. flavus* (F4), *Rhizomucor. pusillus* (F5) and *Aspergillus terreus* (F7) could tolerate Cr at 50mg while in case of Cd only three isolates *Rhizomucor pusillus* (F2), *Aspergillus flavus* (F4) and *Rhizomucor pusillus* (F6) could tolerate Cd at 10mg. This indicated inhibition of growth of the fungal isolates at higher concentration of heavy metals. Similar observations about toxic effect of higher concentration of heavy metals on growth of fungi have been reported [15, 16]. These fungi were then identified on the basis of morphological characteristics and biochemical tests for initial results and were named as series from F1-F7.

All fungal isolates showed different removal efficiencies for different heavy metals in different medium. Removal efficiency was compared in one-step with those in the two-step (Fig. 1-3). Three types of medium were used to compare heavy metal efficiencies. And it was found that two-step process is better than one-step because most of results showed good efficiency in two- step process.

Different medium showed different removal percentages. In YPG medium maximum removal efficiency of 99% by F4 in Pb in two-step process was observed, while in case of Cd fungal strain F6 showed maximum removal efficiency 77% in two-step process and in case of Cr maximum removal efficiency of 45% by F4 was observed (*Fig 1*). YPG medium showed maximum removal efficiency in two-step process in comparison with one-step process.

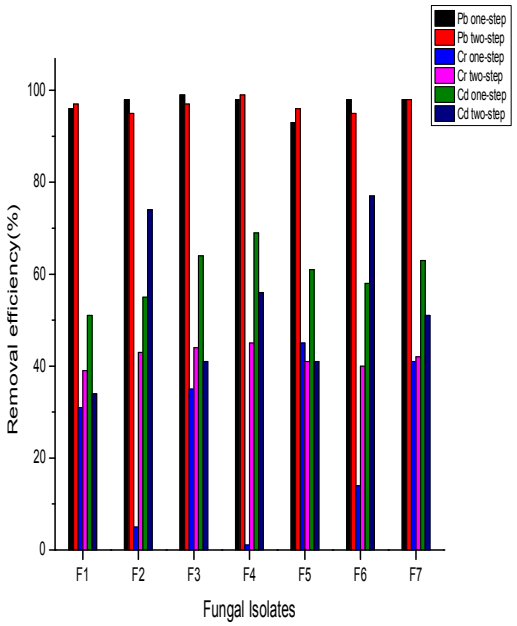

Fig 1. Different removal efficiencies of Pb,Cd & Cr in one and two-step process in YPG medium

In case of CM used for comparison of both steps process F2, F5 and F6 showed maximum removal efficiency of 98% in Pb in two-step process while minimum of 41% by F1 in one-step process. The fungal strain F4 and F7 showed maximum efficiency of 99% in Cr using one-step process while minimum of 67% by F1 and F5 in two-step while in case of Cd maximum removal efficiency of 63% by F4 and F2 in one-step and minimum of 41% by F1 was observed. When removal efficiency by different fungal isolates were observed in both steps in CM it was observed that by using this medium fungal isolates showed good efficiency in one-step process.

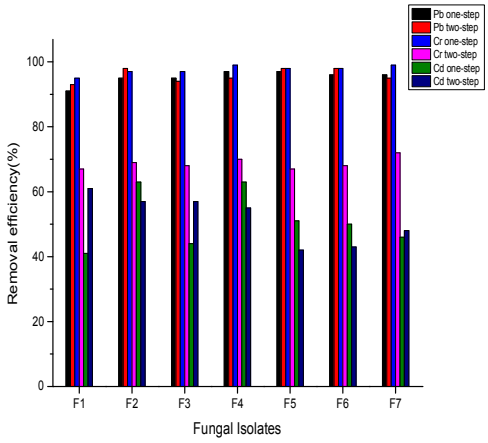

Fig 2. Different removal efficiencies of Pb,Cd & Cr in one and two step process in CM

The F5 and F6 showed maximum removal efficiency i.e. 87% in two-step process while minimum of 41% by F3 in two-steps process in SDB medium containing Pb. Maximum removal efficiency of 67% by F4 in two-step process was observed in same medium containing Cd while, medium containing Cr showed maximum efficiency of 72% by F7 in case of two-step process.

Among the seven fungal isolates used for comparison of both steps three fungal isolates F4, F2 and F6 showed approximate bio accumulating ratios and almost all results showed good efficiency in two-step process.

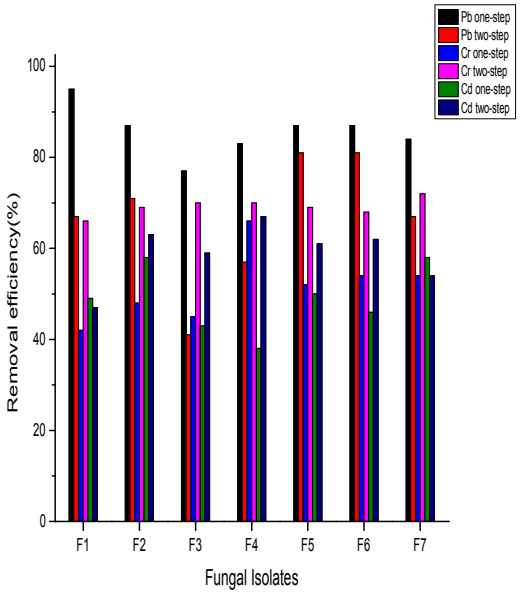

Fig 3. Different removal efficiencies of Pb,Cd & Cr in one and two step process in SDB medium

Table 1; Uptake of Pb in (mg.g^{-1})by different fungi in liquid media

Fungi Name	Uptake of Pb in YPG Media(mg.g^{-1})		Uptake of Pb in CM (mg.g^{-1})		Uptake of Pb in SDB Media(mg.g^{-1})	
	One Step	Two Step	One Step	Two Step	One Step	Two Step
F1	14.27	19.98	7.64	12.82	16.09	19.04
F2	13.21	15.01	12.34	35.74	22.29	25.26
F3	15.33	16.55	22.54	25.26	13.01	15.01
F4	22.98	29.02	13.68	13.87	19.98	20.72
F5	14.65	15.88	33.24	35.02	30.25	31.35
F6	12.78	16.57	11.56	12.78	30.24	39.58
F7	21.67	22.08	10.98	11.52	21.04	25.4

Table 2; Uptake of Cd in (mg.g^{-1}) by different fungi in liquid media

Fungi name	Uptake of Cd in YPG Media(mg.g^{-1})		Uptake of Cd in CM(mg.g^{-1})		Uptake of Cd in SDB Media(mg.g^{-1})	
	One Step	Two Step	One Step	Two Step	One Step	Two Step
F1	4.67	6.15	25.21	36.71	40.09	42.91
F2	10.65	14.83	42.56	43.71	18.05	19.06
F3	1.56	3.24	29.86	30.58	17.59	22.39
F4	12.65	13.21	39.95	60.2	29.92	68.87
F5	12.89	14.36	42.65	47.18	38.25	42.78
F6	13.86	29.64	48.43	50.37	32.29	37.13
F7	6.68	8	50.23	51.15	26.9	27.73

Uptake of Pb by fungal isolates from liquid medium

The maximum uptake i.e. (29.02mg.g^{-1}) of biomass in F4 in two-step process and minimum (12.78 mg.g^{-1}) in F6 in one-step process in Pb containing medium YPG while in case of CM in Pb (35.74 mg.g^{-1}) by the F2 in two-step and minimum of (7.64 mg.g^{-1}) by F1 in one-step process was observed while in medium containing SDB maximum uptake of (39.58 mg.g^{-1}) by F6 in two-step and minimum of (13.01 mg.g^{-1}) by F3 in one-step process in Pb was observed (Table1) which indicated more binding sites on cell wall of these fungi and their potential as bio sorbent to remove Pb from industrial wastewater containing higher concentration of Pb which correlates with Ahluwalia *et al.* [17] Removal of lead ions from aqueous solutions by non-living biomass of *Penicillium chrysogenum* was 116mg/g dry biomass [18.] Wherever there was less growth, there was higher uptake of Pb and vice versa. The results showed that F4 is suitable for using as Pb^{2+} accumulators in waste water and soil. Similar results with respect to differential Pb uptake by different fungi were reported by earlier workers [19,20.]

Uptake of Cd by fungal isolates from liquid medium

In two-step process the maximum uptake (29.64 mg.g^{-1}) of Cd was observed in F6 and minimum uptake of Cd (4.67mg.g^{-1}) was observed in F1 in one-step in YPG medium (Table 2). In case of CM maximum uptake of (60.2mg.g^{-1}) by F4 in two-step and minimum of (25.21 mg.g^{-1}) in one-step bioleaching by F1 was observed. In case of SDB medium maximum uptake by F4 i.e. (68.87 mg.g^{-1}) while minimum by F3 i.e (17.59mg.g^{-1}) (Table 2). The highest uptake of Cd by F4 indicated their potential as biosorbent and efficiency to remove Cd from aqueous solution, which relates with, Akar and Tunali, [21] who found that maximum biosorption capacities of Cd (II) and Cu (II) ions on *B. cinerea* were

Table 3; Uptake of Cr in ($mg.g^{-1}$) by different fungi in liquid media

Fungi Name	Uptake of Cr in YPG Media(mg/g)		Uptake of Cr in CM (mg/g)		Uptake of Cr in SDB Media(mg/g)	
	One Step	Two Step	One Step	Two Step	One Step	Two Step
F1	4.49	5.41	11.98	12.74	13.76	14.86
F2	5.76	6.74	32.22	32.66	16.65	17.9
F3	3.32	5.83	62.56	65.53	20.9	21.09
F4	8.54	10.66	28.93	68.02	14.89	25.82
F5	5.37	6.94	29.01	32.09	20.02	21.3
F6	3.98	6.76	20.65	27.92	25.56	29.9
F7	4.98	5.49	52.36	56.08	24.65	35.82

found to be 17.03 mg/g and 9.23 mg/g, respectively.

Uptake of Cr by fungal isolates from liquid medium

In case of two-step process the maximum uptake ($10.66mg.g^{-1}$) by F4 and minimum uptake of ($3.32\ mg.g^{-1}$) was observed in F3 in one-step process in YPG medium, while in case of CM maximum uptake of ($68.02mg.g^{-1}$) by F4 and minimum of ($11.98\ mg.g^{-1}$) by F1 was observed. In case of SDB medium maximum uptake of ($35.82mg.g^{-1}$) by F7 and minimum of ($13.76\ mg.g^{-1}$) by F1 was observed (Table 3). The highest uptake of Cr by F4 indicated their efficiency to remove Cr from aqueous solution containing higher concentration of Cr which correlates with other studies in which, Vasanthy, [22] reported that B. sp. was effective in Cr removal up to 83.4 per cent at 10 mgL^{-1} and 79.1 per cent at 50 mgL^{-1} concentration.

4. Discussion

A. flavus (F4) and R. pusillus (F5) have been shown in this study to be resistant to Pb, Cd and Cr when cultured with level upto $100mgL^{-1}$. This is in agreement with findings reported by other researchers in term of high tolerance level of Rhizomucor to HM and long chain hydrocarbons [23]. Different fungal strains were used to determine heavy metal biosorption with comparative analysis in different medium in one and two-step process. Fungal biomass were used to determine the percentage adsorption of heavy metals (Pb, Cd and Cr) from solution [24,25].

The results obtained affirmed that response of the isolates to heavy metals depends on the metal tested, its concentration in the medium and on the isolate considered. Numerous researchers have also reported similar findings, where aqueous solution with fungal biomass was observed to adsorb HM ions. *A. niger* capable of removing 34.4 mg/g lead (II) at 100 mgL^{-1} lead (II) concentration [26] and *Polyporous versicolor* and *Phanerochaete chrysosporium* were effective in removing Pb (II) from aqueous solutions with maximum absorptive capacity of 57.5 and 110 mg Pb (II)/g dry biomasses respectively [27]. Removal of lead ions from aqueous solutions by non-living biomass of *Penicillium chrysogenum* was 116mg/g dry biomass [18]. Akar and Tunali [21] who found that maximum biosorption capacities of Cd (II) and Cu (II) ions on *B. cinerea* were found to be 17.03 mg/g and 9.23 mg/g, respectively. *P. chrysosporium* was used to biosorp cadmium (II), lead (II), copper (II) and the adsorption capacities reached 23.04, 69.77 and 20.33mg/g dry biomass, respectively [28]. The basidiomycetes *Phanerochaete chrysosporium, Pycnoporus cinnabarinus and Pleurotus ostreatus* stopped growing when a concentration of 11.2 mgL^{-1} of cadmium was added to their culture medium [29]. *P. aeruginosa* was found to detoxify Cd^{2+} through production of intracellular cadmium-binding proteins [30]. Similar results with respect to bio sorption of Cd and other heavy metals by fungi have been reported earlier [19, 31-33, 34-38]. Nourbaksh *et al* investigated biosorption of chromium (VI) ions on the different filamentous funguses including *C. vulgaris, C. crispata, R. arrhizus, S. cerevisiae and Z. ramigera* and maximum adsorption capacity was found to be 4.5mg/g [38]. Variations with respect to tolerance to Cr by fungi and bacteria were also reported by earlier workers [39- 43].

However, although some authors found that microorganisms isolated from contaminated site were more tolerant than those isolated from

natural environment [44]. Some studies are not in this favor [45,46]. They reported very little differences in metal tolerance between strains from polluted and unpolluted sites. The resistance of isolates appeared could be correlated with the sites of their isolation. Various genera and also isolates of same genus did not necessarily have the same heavy metal tolerance. The variation in the heavy metal tolerance may be due to the presence of one or more strategies of tolerance and resistant mechanisms exhibited by fungi. It must also be taken into account that contamination at the polluted site is caused by the combination of metals and that the selection is probably driven by the most toxic element or by more different metals acting synergistically [47].

When comparing two biotreatement regiments in this portion of study, i.e. use of living fungal biomass versus the use of oven dried fungal biomass, there is overwhelming evidence to support the use of former to remove HMs from polluted sites, either it is wastewater or soil. One may argue that under optimum conditions which may be said are different for both regiments, there is not much difference between two types of biotreatements. But, upon closer examination, it may be arguable that amount of heavy metal ions removed within specific time frame and in higher concentrations of contamination the process that employs living biomass is more effective.

The use of living fungal biomass may therefore indeed be labelled as panacea for the removal of toxic substance from the environment. Not only it is cost effective, but intrinsic bioremediation principally relies on the indigenous microorganisms isolated from the contaminated site itself, to wield their natural power and remedy the situation. Such technologies although not new, but certainly are in their infancy in this part of world and must be encouraged and promoted by all concerned sectors.

As a result, improved industrial hygiene and increased use of new technologies to remove heavy metals from the environment have been adopted in the developed nations and must be strongly considered and implemented in developing nations as well [48].

Present study findings indicate that fungal population isolated from heavy metal contaminated site has ability to resist high concentration of heavy metals. Using fungal biomass particularly living biomass of *A. flavus* (F4) and *R. pusillus* (F5), ubiquitous microbes, may help to reduce heavy metal from our environment which makes them a promising candidate for future investigations regarding their ability to remove heavy metals from contaminated sites. Not only would this be saving our children and many generations to come from exposure to this poison, but we would also help to save the biodiversity within a defined biosphere.

Conclusion

In this study bioaccumulation of heavy metals in aqueous medium using indigenous fungal strains were studied. Pb, Cr and Cd resistant strains were identified from contaminated site. Results showed that fungi isolated from contaminated site have ability to resist heavy metals high concentration. Among different fungus isolated F4 and F6 *was* almost tolerant to these three heavy metals (Pb,Cd and Cr) level up to 100 mgL^{-1} medium. The bioaccumulation ratio of Pb, Cd and Cr by F4 and F6 was considerably influenced by the composition of the growth medium. Further analysis for removal of heavy metals in soil will be done.

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