

Isolation, Screening and Identification of Cadmium Tolerant Fungi and Their Removal Potential



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Abstract

Cadmium tolerance and bioremediation capacity of isolates including *Aspergillus versicolor*, *Aspergillus fumigatus*, *Paecilomyces* sp.9, *Paecilomyces* sp.G, *Terichoderma* sp, *Microsporum* sp, *Cladosporium* sp were determined. Minimum inhibitory concentration values among 2,000-6,000 mg l⁻¹ proved great ability of isolated strains to survive in cadmium polluted environments. The most tolerant fungi, *Aspergillus versicolor*, showed tolerance index of 0.85 in 100 mg l⁻¹ cadmium agar media. Fungal resistance against cadmium is depended directly on strain's biological function. *A. versicolor* was found to bioaccumulation over 7.67mg of cadmium per 1 g of mycelium, followed by 6.546, 6.354, and 6.286, 6.134 by *Paecilomyces* sp, *Aspergillus fumigatus*, *Microsporum* sp and *Terichoderma* sp, respectively. It can be noted that tolerance of the strains appears to be independent from bioaccumulation capacity. Finally, the results indicated that *A. versicolor* could be a prospective candidate for bioremediation processes.

Keywords: Cadmium tolerance; Bioremediation; Fungi

Abbreviations: PDA: Potato Dextrose Agar; MEA: Malt Extract Agar; PDB: Potato Dextrose Broth; TI: Tolerance Index; MIC: Minimum Inhibitory Concentration

Introduction

The environment is polluted by heavy metals from industrial wastewaters during steel processing as well as other pollutant routes. Virtually, any industrial activity using metals has a metal disposal problem [1]. Nature of heavy metals is non-biodegradable and persistent; therefore environmental compartments (soil and water body) are not able to purify themselves from these toxic pollutants. Heavy metals can be divided into essential metals such as copper, manganese, zinc, and iron, and nonessential metals such as cadmium, lead, mercury, and nickel [2]. Cadmium and lead are included among the major pollutants because of their high toxicity [3-6]. Cadmium is released to ecosystem by mine tailing, effluents from textile, leather, tannery, electroplating and galvanizing industries, as well as cadmium batteries.

Biomagnifications of cadmium in nature and migration through drinking water, food and air to human body cause severe health effects like kidney damage, bronchitis and cancer [3]. As industrial development train is not stoppable, struggle with heavy metal pollution requires novel remediation methods. Conventional treatment systems have failures which include

insufficient metal sequestration, high costs, high reagents and/or energy requirements, and generation of toxic sludge or other waste products that require disposal. Restoring metals in an efficient and economical procedure has necessitated the use of different options in metal-separating methods. Research shows that bioaccumulation of metals by organisms has been successful to some extent [3]. Bioremediation of heavy metals from aqueous solutions is a relatively new process that has been confirmed as a promising process in the removal of heavy metal pollution. The major advantages of biosorption are its high effectiveness in reducing the heavy metal ions, and the use of inexpensive biosorbents. Biosorption processes are particularly suitable to treat dilute heavy metal wastewater [7].

Biomass obtained from different sources is used in biotreatment and its key feature and potential controlled for the process. Rather than searching thousands of microbial species for particular metal sequestering features, it is beneficial to look for biomasses that are readily available in large quantities to support potential demand. While choosing of the biomaterial for metal sorption, its origin is a major factor to be taken into

account [8]. Among biological sources, fungi possess a number of advantages including producing great biomass, rapid growth, availability, and flexibility to rough circumstances. The uptake of metals by fungal biomass appears to involve a combination of two processes: bioaccumulation (i.e. active metabolism-dependent processes, which may include both transport into the cell and partitioning into intracellular components) and biosorption (i.e. the binding of metals to the biomass by processes that do not require metabolic energy) [9].

Fungi are ubiquitous members of subaerial and subsoil environments, and often become a dominant grouping metal-rich or metal-polluted habitats [10]. Recent studies have shown that the strains isolated from contaminated areas have remarkable potential to tolerate such toxic conditions. Microorganisms have been shown to possess ability to survive by adapting or mutating at high concentrations of heavy metals [11,12]. This could play a key role to explore newcomer resistant isolates from pool of biomasses. Exhibiting high tolerances to heavy metals, these isolates were selected for bioremediation studies. Generally, fungi have often been proposed as bioagents for metal recovery processes [13]. This study was carried out to isolate fungi from cadmium- contaminated sites Textile industry for the first time and evaluate their resistance level toward cadmium as well as assessing their bioaccumulation capacity in order to expand knowledge about bioremediation science.

Materials and Methods

The aqueous solutions of cadmium were prepared by diluting Cd (II) standard stock solution (concentration 1000 mg L⁻¹) obtained by dissolving Cd (NO₃)₂·4H₂O in deionized water. Fresh dilutions were prepared for each experiment. Potato dextrose agar (PDA), malt extract agar (MEA) and potato dextrose broth (PDB) were used as solid and liquid medium, respectively. Deionized water was used in all experiments.

Sampling and Experimental Sites

The research areas characterized in this study were steelplants contaminated sites. These locations were selected based on their cadmium pollution densities. Waste water samples were taken from six different spots to a depth of 20 cm from waste dumping areas. Effluent samples of industries were collected from wastewater discharge of plants. Control sample was obtained from aeration tank of municipal wastewater treatment plant. Samples were placed in sterilized glass bottles, transported on ice (4°C), taken to the laboratory and analyzed within 8 h. The wastewater and soil samples were analyzed for total content of Cd. The soil samples were dried at 105°C manually ground and sieved (500 µm pore size). 1g of soil samples were digested with 70% HNO₃ (1 M) and 30% H₂O₂ using microwave digestion (Sineo MDS-10, China). Cadmium concentrations were analyzed by Atomic Absorption Spectrophotometer [14].

Isolation of Strains

Fungal strains were isolated on PDA and MEA by serial dilution method in order to avoid overlapping colonies. Streptomycin (15 mg l⁻¹) and chloramphenicol (50 mg l⁻¹) were added to mediums after autoclaving at 15 psi for 15 min and 121°C to arrest bacterial growth. The soil samples (1 g) were suspended in 100 ml of sterilized water. The mixture was shaken (200 rpm) for 30 min at room temperature. All samples were diluted up to (10⁻³). 0.1 ml of different dilutions were spread on Petri plates (diameter 10 cm) containing 20 ml media. Plates were incubated at 28°C in dark condition and monitored every day up to 10 days and each developed colonies were sub-cultured and isolated into fresh PDA plates. Purified isolates were kept on slants at 4°C and sub-cultured every 4 weeks [15].

Screening for Cd- Tolerant Fungi

In order to select Cd- tolerant strains, 100 mg l⁻¹ Cd stress was added to PDA medium. The pH of the solid growth medium was adjusted to 6 with 1 M sodium hydroxide solutions before autoclaving in all experiments in this study. The small agar plugs with young mycelium from the edge of the stock cultures were cut and transferred to surface of solid medium. Plates were incubated at 28°C for at least 10 days and visually inspected for microbial growth every day. Cadmium tolerant strains were subjected to resistance study.

Cadmium Tolerance Index Of Fungi

Five mm disks from 10 day old pure cultures of each fungal isolates were inoculated into PDA (three replicates) supplemented with 200 mg l⁻¹ Cd [4]. The inoculated plates were incubated at 28°C for 10 days. In parallel, cultures without cadmium were performed as a control. The radial growth was evaluated from four measurements (in millimeters) that passed through the center of the inoculated portion. The initial diameter of the portion was subtracted from the growth diameter [5]. The mean of perpendicular diameter measurements was recorded for each plate on the 10th day. The tolerance index (TI), an indication of the organism response to metal stress was calculated from the growth of strain exposed to the metals divided by the growth in the control plate [16]. The higher the TI, the greater the resistance.

Determination of Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) was defined as the minimum inhibitory concentration of the heavy metal that inhibited visible growth of test fungi [17]. PDA medium was enriched with increasing concentration of Cd (200, 400, 600, 800, 2,000, 4,000, 6000 mg l⁻¹Cd). Plates were inoculated with agar plugs from the edge of the 10 day old growing cultures. If no apparent growth of fungi was observed after ten days on the plates, the metal concentration was considered as the highest metal concentration tolerated by the tested fungus.

Identification of Selected Fungus

All the resistant fungal isolates were initially identified by colony characteristics on PDA characterized to the genus level on the basis of macroscopic characteristics (colonial morphology, color and appearance of colony, and shape), microscopic characteristics (septation of mycelium, shape, diameter and texture of conidia) and the help of the Principles and practice of clinical parasitology [18].

Cadmium Bioaccumulation by Active Fungus

To determine the bioaccumulation ability of the 7 fungal isolates, inoculums (six 5 mm disks of mycelia strain) were prepared from 10-day-old pure fungal culture and inoculated into 250 ml Erlenmeyer flask containing 100 ml potato dextrose broth (PDB) plus 100 mg l⁻¹ cadmium. Initial concentrations of Cd (II) in each conical flask were checked by AAS before fungal inoculation. pH was adjusted to 6 [19]. Un-inoculated controls (PDB medium with 100 mg l⁻¹ of Cd and without any fungal inoculums) were served to detect any possible abiotic Cd (II) reduction brought about by media components. All flasks were incubated at 28°C on a rotary shaker at 120 rpm in dark conditions.

After 10 days of incubation (logphase) [19-21], flasks containing fungal biomass were harvested and filtered through Whatman No.42 filter paper. Filtered PDB medium was used for determining total Cd concentration.

Biomass samples were rinsed three times with distilled water and dried in hot air oven at 80°C until a constant weight (24 h) was achieved. The dried fungal biomass was weighed and defined as dry biomass (g) [21]. The amount of heavy metal uptake (q, mg/g) was calculated by using the following equation [22]:

$$q = [C_i - C_f/m] V$$

In above equation, q (mg/g) is mg of metal ions uptake per gram biomass; C_i (mgL⁻¹) is the initial metal concentration of liquid phase; C_f (mgL⁻¹) is the final metal concentration; m (g) is the amount of dry biomass; and V (L) is the volume of the medium.

Data Analysis

All experiments were carried out by triplicate sample. Values reported in this paper are the means ± S.D. The difference in TI and uptake capacity of each isolate was studied by one-way ANOVA followed by post-Hoc multiple comparisons by Duncan's method using SPSS16 (USA, IL, Chicago, SPSS Inc.). The difference was considered as significant when P < 0.05.

Results and Discussion

Mean ± SD Cd concentrations of soil samples used in this study were 545.15 (mg kg⁻¹soil) for vicinity of steel plant. Since steel plant dumping waste areas were selected for soil samples,

Cd content was too high. The Cd concentration in effluent of mentioned plant was 250.86(mg l⁻¹), whereas Cd in aeration tank of municipal wastewater treatment plant was 1.78 (mg l⁻¹). Clearly these values are higher than Industry Effluent Guidelines regulated by EPA [23].

Number of Cadmium-Resistant Isolates and Their Origin

Table 1: Cadmium concentration of samples and number of resistant isolates.

	Sites	Cadmium concentration	Number of resistant isolates
Soil(mg kg ⁻¹ of soil)	Industrial dumping area	545.15 ± 2.47	8
Wastewater(mg /l)	Industrial effluent	150.86 ± 3.45	7
	Municipal wastewater treatment plant (aeration tank)	1.78 ± 0.09	1

As shown in Table 1. Sixteen strains could tolerate 100 mg l⁻¹ Cd toxicity. These strains were from different sampling sites. It is known that microorganisms isolated from natural environments contaminated with heavy metals often exhibit tolerance to heavy metal pollutants [24]. Cadmium stress exerted in this study to isolated fungus from municipal wastewater treatment site, made non-favorable lethal growth medium. Discrepancy in conditions the fungi were adapted to resulted in extinction of isolated fungal population from municipal wastewater. Only one resistant isolate was obtained from low-polluted area. In contrast, the number of resistant isolates from heavy metal industrial sites was significant. It is well known that a long-time exposure of water and sediment to heavy metals can produce considerable modification of their microbial populations, reducing their activity and their number. Generally, pollution of soil and water by heavy metals may lead to a decrease in microbial diversity. This is due to the extinction of species sensitive to the stress imposed, and enhanced growth of other resistant species [25].

Cadmium-Resistant Assays Minimum Inhibitory Concentration

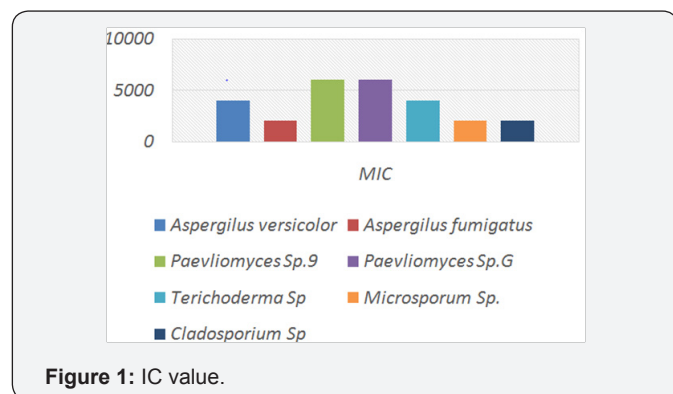
MIC border line of 2000 (mg l⁻¹) was chosen. Strains with MIC value over 2000 (mg l⁻¹) were subjected to molecular identification. Data of top seven MIC values and their relevant isolate identification are shown in Results from Table 2. Show that soil natural sources trigger microorganisms with higher tolerance ability than aquatic sources. Except *Terichoderma* sp, there was no strain from industrial effluent which could survive in over 2000 (mg l⁻¹) Cd. Evolutionary adaptation to metal-contaminated soils is a well-documented phenomenon, particularly because it is one of the most striking examples

of microevolution driven by edaphic factors [26]. Cadmium concentration in sites and the levels of survival against Cd toxicity were not related to each other, as *Terichoderma* sp was able to survive up to 4000 (mg l⁻¹) Cd toxicity which is 10000 orders of magnitude than Cd concentration in urban wastewater. Other examples are about *Paecilomyces* sp.9 and *Paecilomyces* sp. G from steel plant were able to grow up to 6000 (mg l⁻¹) Cd stress,

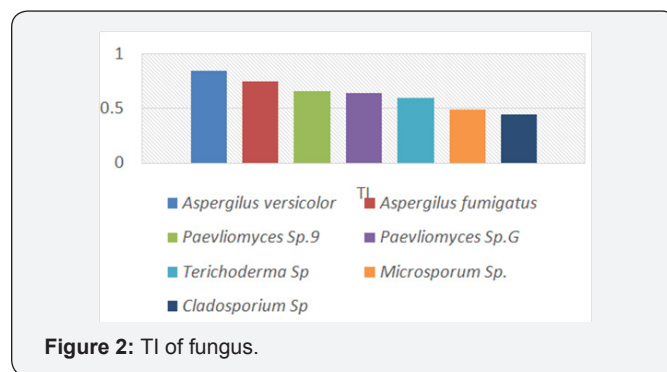
whereas strains from steel plant effluent with Cd concentration over eight order of magnitude in soil than industry effluent only could tolerate up to 2000 and 4000 (mg l⁻¹) Cd. It implies that some microorganisms without any consideration about their native root could develop ability to keep up living in severe toxic environments. The MIC values suggested that the resistance level against individual metals was dependent on the isolates [27].

Table 2: Fungi isolated from different sites and their MIC values.

Origin	strain	MIC(mg l ⁻¹)
Industrial dumping area	<i>Paecilomyces</i> sp.9	6000
	<i>Paecilomyces</i> sp.G	6000
	<i>Microsporium</i> sp	2000
	<i>Aspergillus fumigatus</i>	2000
	<i>Cladosporium</i> sp	2000
	<i>Aspergillus versicolor</i>	4000
municipal waste water treatment plant (aeration tank)	<i>Terichoderma</i> sp	4000



The cadmium concentration of 6000 (mg l⁻¹) was the highest MIC value in this study that *Paecilomyces* sp. G and *Paecilomyces* sp.9 could tolerate. To a lesser degree were *Aspergillus*, *versicolor* and *Terichoderma* sp with MIC value of 4000 (mg l⁻¹) and *Microsporium* sp, *Cladosporium* sp, *Aspergillus fumigatus* with MIC of 2000 (mg l⁻¹). There are some studies supporting the idea that there is a very little difference in metal tolerance between strains from polluted and unpolluted sites [28]. Indeed, presence of metal may act as a fatal toxicity on microorganism population, but does not have any influence on microbial tolerance ability. MIC values of 0.328 mM for filamentous fungi and 1(mg l⁻¹) for *Aspergillus*, *Penicillium* and *Fusarium* were reported [25- 29]. In another study no determinations were made for cadmium since the majority of the tested fungi were unable to grow in the presence of this metal [30]. In a study conducted in 2007, MIC values of 3,000, 4,000 and 5,000 (mg l⁻¹) Cd were reported for *Rhizopus* sp., *Terichoderma* and *Aspergillus*, respectively. These reported MIC values are relatively similar to the values we observed [27]. Considering previous studies, *Paecilomyces* sp. G and *Paecilomyces* sp.9 are newly introduced fungus with remarkable tolerance potential (Figures 1 & 2).



The reduced tolerance index reflects the inhibitory growth function of heavy metal [31]. To select the most tolerant fungi, the actual resistant potential of fungi must be tested. Tolerance level of fungi can be revealed through both TI and MIC assays. Although it is crucial for scientists to discover a fungus with great ability to survive in extremely high heavy metal concentration, from an environmental engineer view, that fungus is salient and applicable for purification systems which in response to metal toxicity could grow and multiply faster. It implies that the more rapidly the fungi can adapt to polluted environment and develop its colonies, the more beneficial it is for treatment process. The term adaptation speed is an important armor that prompts one fungus more powerful than other fungi with higher MIC property.

TI of each fungus in this study demonstrated different orders of tolerance, as follows: *Aspergillus versicolor* and *Terichoderma* sp have shifted up to first level of tolerance with TI of 0.85 and 0.75 respectively, followed by *Paecilomyces* sp.G, *Paecilomyces* sp.9 and *Aspergillus fumigatus* with TI of 0.66, 0.64 and 0.6 respectively which were fairly tolerant fungi and finally at end of the list were *Cladosporium* sp (0.486) and *Microsporium* sp (0.454). Cadmium tolerance index of different isolates is significantly different (P < 0.05). *Aspergillus versicolor* from

highly polluted steel industrial site posed minimal reduction in growth (15%). It might be due to the high concentration of cadmium in soil of strain's original environment that induced resistance strategy on fungal metabolism; therefore *Aspergillus versicolor* was able to adjust rapidly to polluted culture media. Based on the result, *Aspergillus versicolor* was a highly adaptable fungus in response to cadmium stress. Excessive Cd in soil could trigger the evolution for higher Cd tolerance in *Suillus luteus* [32]. An exceptional fungus was *Terichoderma* sp again. Despite the fact that this fungus was from least polluted area with 31% reductions in growth, it adapted better than five remaining fungi. Trying to find rigid general regulation between the microorganism's origin and fungal resistance to heavy metal is cumbersome effort.

The trustworthy theory is: fungal resistance to heavy metal is depended directly on biological function of the strain. The Cd-resistance was found to be independent from the pollution level at the site of origin [25,28,33]. TI results in this study illustrated that *Aspergillus versicolor* were the most tolerant and *Microsporom* sp and *Cladosporium* sp with 65% suppression of mycelia growth were the most sensitive fungus. Results from relative study showed that Cadmium at concentration of 1 mM posed the strongest inhibition toward isolates from the genera *Aspergillus*, *Fusarium*, *Alternaria* and *Geotrichum*. Only *Penicillium* isolates expressed tolerance index of 0.8 [30]. In another study, growth of *Aspergillus flavus* was inhibited by 40% at 1 mM Cd concentration [34]. Copious heterogeneity in TI of the isolates, especially in strains of the same genus (*Aspergillus versicolor*, *Aspergillus fumigatus*) and (*Paecilomyces* sp.9, *Paecilomyces* sp.G) proved the theory that various genera and also isolates of the same genus do not necessarily have the same heavy metal tolerance [22,27,30,35]. It comes into mind that tolerance skill is not inherited among microorganisms, in other word it is acquired from ecosystem.

Various Tolerance Strategies Related to Different Morphological Alteration

On exposure to cadmium, morphological changes were observed in all isolated fungi. Several authors have reported the formation of colorful mycelia in the presence of heavy metals on agar media [24]. Other than *Aspergillus versicolor* and *Cladosporium* spgenera, decoloration of fungus occurred by increasing the cadmium concentration in medial growth. Pink color changed to white in *Paecilomyces* sp. and *Paecilomyces* sp.9 genera. In *Terichoderma* sp and *Aspergillus fumigatus*, green color changed to white. Red color appeared in cracked mycelium of *Aspergillus versicolor* and sides of its colonies in media were polluted with Cd. Red pigmentation in *Aspergillus versicolor* was probably due to the binding of Cd to the protein in the cell wall of mycelium. It has been previously suggested that production of pigments in fungal cell and cell free media is accompanied with precipitation of metal ions on the call wall [20]. Consistent

with this study, *Sills hirsutum* produced a yellow-orange pigment both extracellular and in the mycelium, when cultivated in the presence of 0.25 mM or more Cd [36]. Jarosz-Wilkołazka et al. reported that the presence of Cd induced formation of orange–brown pigment which colored the fragile mycelium of *Abortiporus biennis*, as well as the cell-free culture medium [37].

The appearance of condensed, frizzy mycelium was clearly visible in *Aspergillus fumigatus*, *Cladosporium* sp, *Microsporom* sp, *Paecilomyces* sp.G, and *Paecilomyces* sp.9 generals. In the case of a toxic metal-containing domain, aggregated mycelia could produce high local concentrations of extracellular products such as complexing agents, precipitating agents, polysaccharides and pigments with metal-binding abilities [38]. In *Terichoderma* sp, isolated in this study, aerial mycelium vanished. On Cd-containing agar most of the mycelium of *Paxillus involutes* grew submerged rather than on the surface as occurs on Cd-free agar [36]. Possible explanation for these different morphological changes among isolates may be due to the vast detoxification/tolerance mechanisms that each strain applies. The variation in the metal tolerance might be due to the presence of one or more types of tolerance strategies or resistance mechanisms exhibited by different fungi [27] (Figure 3).

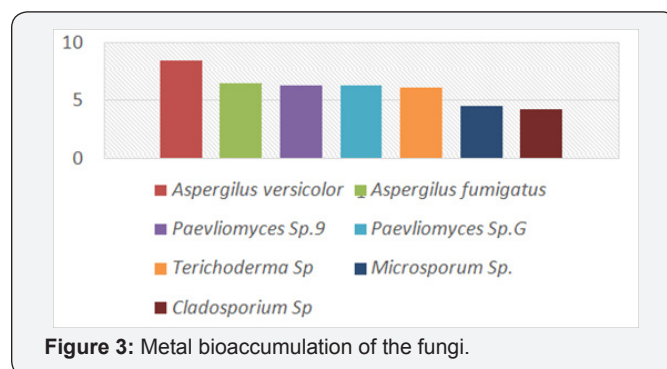


Figure 3: Metal bioaccumulation of the fungi.

There has been steady progress in studying the biosorption of heavy metals, resulting in the identification of some biomass types that show very promising uptake of metallic ions [39]. The biosorbents used in heavy metal biosorption are usually obtained after screening the heavy metal resistant/tolerant microorganisms from polluted environments [14]. Adaptation of fungal isolates to heavy metal successfully created organisms with greater efficiency in bioaccumulation [31]. Because the putative specific resistance mechanism(s) could have a potential for biomitigation of contaminated sites, the metal sequestration capacity of the fungus was evaluated [40]. Cadmium bioaccumulation (mg of cadmium uptake per g of dry biomass) of all the tested fungal isolates from liquid media containing 100 mg l⁻¹ of Cd is presented.

Metal bioaccumulation of the fungi under 100 mg l⁻¹ of Cadmium, the uptake of cadmium by different isolates is significantly different. (P < 0.05). Bars represent the standard deviation (n = 3) The genera, *Aspergillus versicolor*, *Paecilomyces*

sp.G, *Aspergillus fumigatus*, *Microsporium* sp, *Trichoderma* sp, *Paecilomyces* sp.9 and *Cladosporium* sp showed the bioaccumulation capacities of 7.67, 6.546, 6.354, 6.286, 6.134, 4.537 and 4.252 mg g⁻¹ in sequence of decreasing the potential. Surprisingly the best accumulator fungus was the most tolerant strain too.

However, for the rest of the fungus this trend was not continued. Except *Paecilomyces* sp.G that appeared semi accumulator and semi tolerant, *Aspergillus fumigatus* and *Microsporium* sp was among the sensitive isolates; however, they were moderately accumulator fungi. These results suggest that removal capacity is not proportional to level of tolerance. Similar observations regarding the lack of correlation between metal tolerance and removal capacity have been reported earlier [22,27,28]. Indeed; uptake capacity was related to the type of tolerance mechanism of fungi. In bio-treatment criteria, the resistant mechanism and remediation strategies of microorganism should be distinguished and the parts that these topics have in common be selected. Going through mechanisms of tolerance that finally leads to discovering new bio-uptake activities (bioaccumulation and biosorption) is essential in this field.

A diversity of specific metal accumulation strategies has been known. The physicochemical properties of metals and the physiology of the organism both influence metal uptake [41]. It can be hypothesized that diminished uptake in *Cladosporium* and *Paecilomyces* sp.9 genera contributed to cadmium rejection mechanism of tolerance utilized by this fungus. Fungi are able to restrict entry of toxic metal species into cells by reduced metal uptake and/or increased metal efflux [38]. These microorganisms are known as metal excluders [42]. In contrast, higher Cd removal in *Aspergillus versicolor* may relate to accumulation of cadmium in cell structures. Fungal biomass can act as a metal sink, either by: (1) metal biosorption to biomass (cell walls, pigments and extracellular polysaccharides) or (2) intracellular accumulation and sequestration; or (3) precipitation of metal compounds onto and/or around hyphae [38,43]. Fungi were known to accumulate significant amount of cadmium, for example uptake concentrations of 6.46 mg g⁻¹ by *Aspergillus niger* and 16.25 mg g⁻¹ by *Trichoderma viride* have been reported earlier [44]. *Pisolithus tinctorius* presented maximal uptake of 600 mg kg⁻¹ dry weight at 10 mg l⁻¹ cadmium concentration [4].

Conclusion

The present study declared seven highly tolerant fungi. These fungi exhibited various resistance strategies towards cadmium and they had an ability to sequester cadmium from liquid media. *Aspergillus versicolor* remarkably differed in detoxification behavior from other isolated fungi in this study. The fungus showed a remarkable potential to actively grow in presence of Cd and reduce cadmium concentration to less toxic

levels. Introducing *Aspergillus versicolor* as scavenger biota is the first step of emerging this fungus in bioremediation science.

Efforts are being made to make bioremediation technically/economically feasible; therefore, we should direct our attention to exploit whole potential of microorganism. Understanding metal uptake process genetically, manipulation of cell structure such as autoclaving or drying biomass, and using combo strains are innovative technologies in biotreatment studies.

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