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Application of Live, Dead, and Dried Biomasses of Aspergillus Versicolor for Cadmium Biotreatment

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ABSTRACT

Background: Various industries produce and discharge wastes containing different heavy metals into the environment. Apart from using living biomass, dead and dried biomasses have been introduced as a new field of biotreatment technology.
Method: The cadmium (Cd) (II) removal characteristics of live (growing), dead (autoclaved), and oven-dried biomasses of *Aspergillus versicolor* were examined as a function of initial pH, contact time, and initial Cd concentration.
Result: Maximum bioaccumulation of Cd for live biomass [11.63 (mg g$^{\−1}$)] occurred at an optimal pH of 4 and incubation time of 4 days. Themaximum biosorption of 27.56 (mg g$^{\−1}$) for dead biomass occurred at 1.5 h and at a pH of 4. The maximum biosorption [18.08 (mg g$^{\−1}$)] with dried biomass was reached at an equilibrium time of 3 h at a pH of 6.
Conclusion: The present study confirmed that heat treatment promoted the removal capacity of fungi. Cd removal was increased by decreasing the pH in live and dead-mode experiments. Inversely, Cd removal was increased with increasing pH for the dried biomass of *A. versicolor*. Varying responses to environmental conditions (pH and contact time) clearly proved the different removal mechanisms used by three biomasses of *A. versicolor*. Higher Cd concentration increased the removal ability of three types of biomasses. The results indicated that all biomasses of *A. versicolor* used in this study, particularly dead biomass, are a suitable biosorbent for the removal of Cd (II) ions from aqueous solution.

1. Introduction

In many ways, industrialization is drastically impacting our world today. With increasing industrialization and urbanization, nature cannot cope with

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pollution and clean the environment naturally.

Various industries produce and discharge wastes containing different heavy metals into the environment, such as mining and smelting of metalliferous ores, surface finishing industry, energy and fuel production, fertilizer and pesticide industry and application, metallurgy, electroplating, leatherworking, photography, electric appliance manufacturing, metal surface treating, and atomic energy installation [1].

Once toxic metals are present in the environment, they are cycled between its abiotic and biotic elements, posing toxicity in the latter group. The mostly dangerous metals include the so-called "toxic trio" i.e., cadmium (Cd), lead, and mercury, for which no biological function has been found [2-4]. Minor Cd exposure causes cancer, kidney damage, and bone deterioration. Itai itai is a fatal disease brought about by Cd. To cut the harmful Cd magnification chain, which finally comes to an end in the human body as a final consumer, cleaning this chain is a vital activity. Typical wastewater treatment techniques such as chemical precipitation, filtration, ion exchange, electrochemical treatment, membrane technologies, adsorption on activated carbon, and evaporation suffer from deficiencies in treatment plants.

An environmental engineer's practical approaches through economic, flexible, efficient, and less residual purification systems have led to the discovery of biotreatment technology.

Biotic methods exploit natural biological processes that allow certain plants and micro-organisms to help in the remediation of metals in soil and water [5]. Bioremediation is gaining importance in recent times as an alternate technology for the removal of elemental pollutants in soil and water, which require effective methods of decontamination [6].

Biosorption and bioaccumulation are two processes involved in biotreatment studies. Heavy-metal bioaccumulation is an active process including metabolic activity within living organisms [7]. Biosorption is a term that usually describes the removal of heavy metals from an aqueous solution through their passive binding to a biomass [8]. In bioaccumulation, the first stage is biosorption and then, subsequent stages, related to the transport of pollutant (mainly via energy-consuming active transport systems) into the inside of cells, occur [2].

Apart from using living biomass, dead and dried biomasses have been introduced as a new field of biotreatment technology. Many studies have revealed that inactive/dead microbial biomass can passively bind metal ions via various physicochemical mechanisms [1]. It has been suggested that the pretreatment modifies the surface characteristics/groups by removing or masking the groups or by exposing more metal-binding sites [9].

Here, we attempted to optimize the performance of the laboratory scale bioremoval experiments. The effect of operational conditions (pH, contact time, and concentration of Cd) on removal capacity (expressed in mg/g) of different biomasses of *Aspergillus versicolor* were investigated in this study. Comparative investigation was also conducted to weigh the pros and cons of different studied biomasses in real wastewater treatment plants.

2. Materials and methods

Fungi that are applied in heavy metal removal experiments first must be proven to be heavy metal resistant. It is essential to initially check the tolerance levels of the candidate species [10]. Our earlier study concerning the tolerance of *A. versicolor* indicated the possible existence of Cd tolerance in this fungus. The *A. versicolor used* in the study was obtained from dumping area of plant. Three biomasses of *A. versicolor* were used: live growing, autoclaved dead, and oven dried biomasses.

2.1. Cd solutions and Media

The 1000-ppm Cd stock solutions were made by dissolving Cd $(NO_3)_2 4H_2O$ in deionized water. Fresh dilutions were utilized for each test. Cd $(NO_3)_2.4H_2O$ was acquired from Merck, Germany. Potato dextrose broth (Scharlau, European Union) and potato dextrose agar (Merck, Germany) were used as liquid and solid culture medias, respectively. All laboratory glassware and plastic ware were rinsed with a solution of 2-M HNO_3 technical grade to remove any metal contamination. Deionized water used in this study was obtained from the TKA Smsrt2Pure, Germany water purification system.

2.2. Biomass preparation

2.2.1. Live biomass

The pure cultures of *A. versicolor* were maintained on potato dextrose agar(PDA) slants at 4°C.

To produce mycelium pellets, 6 agar plugs (5 mm) originating from actively growing seven-day old PDA solid cultures(log phase) [11], were collected and inoculated in 500 ml conical flasks containing 250 ml autoclaved (121°C, 15 min and 15 psi) potato dextrose broth (PDB) medium. Flasks were incubated in a rotary shaker (120 rpm) at 28°C for 7 days in dark conditions. A 7-day-old mycelium was used as the inoculum in the bioaccumulation experiments [12, 13].

2.2.2 Dead biomass

Mycelial pellets obtained from section 2.2.1 were harvested through Whatman No.42 filter paper and washed three times with deionized water to remove any residual growth media from biomass. Pellets were heat inactivated by autoclaving and dead biomass was used immediately thereafter [14].

2.2.3 Dried biomass

An appropriate amount of washed live biomass was dried in oven at 80°C overnight. The dried mycelia were grinded using a mortar to obtain powder in the smallest particle size and subsequently used as a biosorbent. The smaller the particle resulted in a larger surface area [15]. Biomass has been crushed to prevent particle aggregation for enhancing the biosorption capacity. The dry biomass was stored at room temperature in polyethylene tubes in a vacuum desiccator until use [16].

2.3. Bioaccumulation tests by growing fungus

The effects of contact time (1–7 days) [13], pH (4, 5, and 6) [17], and initial Cd concentrations (25, 50, and 100 ppm) [16] on removal capacity of active mycelia were determined respectively. Time course experiments, were conducted in 250 mL Erlenmeyer flasks with a working PDB volume of 100 mL contaminated with 100 ppm Cd concentration at pH 6. The initial pH of solutions was adjusted by adding 0.1 M HCL and 0.1 M NaOH. The investigated pH values were less than 7.0 since insoluble Cd hydroxide starts to precipitate from the solution at higher pH values, making true sorption studies impossible [18, 19]. The pH was maintained unless the second set of assays (effect of pH on Cd removal) was studied. Due to the negative impact of pH control on the whole sorption performance, experiments have been performed with adjusting the pH at the beginning of the tests (uncontrolled) [20].

The experimental procedure of bioaccumulation was adapted from Yuan et al. with some modifications [21]. A total of 10 ml mycelium suspension: 10% (v/v) (equal volume for each test) were acclimated to 250 ml Erlenmeyer flasks containing the 100 ppm Cd solution supplemented with 100 ml PDB media. Samples were withdrawn every day up to seven days. The Cd concentration was consistent for all tests until the third set of tests (influence of initial Cd concentration on removal) were conducted. Optimal contact time and pH values were used for third set of experiments. Flasks were incubated at 28°C in an agitated condition (120 rpm).

Control experiments without biomass were also run in parallel. Control tests showed that there was no sorption on the

filter papers or by the media organic matter.

Flasks containing fungal biomass were harvested by Whatman No.42 filter paper and washed three times with deionized water and left in oven at 80°C until a constant weight was achieved (in this study 24 h), and this weight was defined as dry biomass.

2.4. Biosorption tests by dead and dried biomass

The selection of biosorption method was selected on the basis of a study conducted by Zafar et al. with minor modifications [22].

4 g wet weight of dead biomass were added in to each of 250 ml flasks containing 100 ml Cd solution and 100 ppm Cd ions in deionized water (without any media). The flasks were incubated as described in section 2.3. The samples were removed at time intervals of 15, 30, 60, 90, 120, 150, and 180 min [23].

The parameters (initial metal concentration, pH, and contact time), which were considered in a Cd biosorption assay by dried mycelia, were the same as those for biosorption by dead mycelia, except that 0.2 g of dried biomass powder was placed in each Erlenmeyer flask.

2.6. Cd determination

The residual Cd concentrations in the liquid solution were measured using a Varian Atomic Absorption Spectrophotometer, AA 240, Australia [18]. The Cd removal capacity is calculated as follows [24]:

$q = [(C_i - C_f)/m]\ V$

where:

q is the removal capacity of Cd (mg g^{-1}),

Ci is the initial concentration of metal (mg L^{-1}),

C_f is the final concentration of Cd in the PDB (mg L^{-1}), V is the volume of the liquid medium (L), W is the biomass of mycelia (g).

2.7. Statistical analyses

Each determination was repeated three times and the results provided are the mean values with standard deviation (represent as error bars). Data were analyzed by using SPSS16 (USA, I1, Chicago, SPSS Inc.) statistical software. The effects of initial metal ion, initial pH, and contact time on *A. versicolor* were examined using one way ANOVA followed by post-Hoc multiple comparisons by Duncan's method. The difference was considered significant when $P < 0.05$.

3. Result

3.1. Optimization of process parameters

Analysis of factors influencing biosorption is important for evaluation of the full biosorption potential of any biomaterial. The important factors include: type and nature of biomass, initial solute concentration, and physicochemical factors such as PH [9].

3*.1.1Effect of contact time*

In field application studies from an engineering perspective view, it is logical to determine sludge retention time based on the optimal time at which the highest removal rate will occur in that time.

The uptake rate of Cd ions by live biomass is exemplified in Fig. 1. Equilibrium in bioaccumulation did not happen. Therefore, live microorganisms do not behave like ordinary sorbents. Rapid and considerable bioremoval rate were performed on the first day and it reached the highest on the fourth day, and then an efflux reaction occurred by growing cells. These results are consistent with previous reports where the level of metal accumulation by *A. foetidus* appeared to decrease with time and it was assumed that fungi rejected copper with the passing of time. It is proposed that generating complexing agents, which promote heavy metal dissolution, may significantly reduce the extracellular uptake of the metals by precipitation and adsorption on the biomass [25]. Growing fungus *Phoma sp.* reached the maximum Cd removal within 4 days, which is similar to our results [21]. The observed rapid kinetics during the first day has also significant practical importance as it will facilitate the scale-up

of the process to smaller reactor volumes ensuring efficiency and economy [26].

In time course data analysis, it was observed that Cr (VI) accumulation by *Paecilomyces lilacinus* increased when cells wereincubated between 96 h to 120 h, which is close to optimal contact time in this study [27].

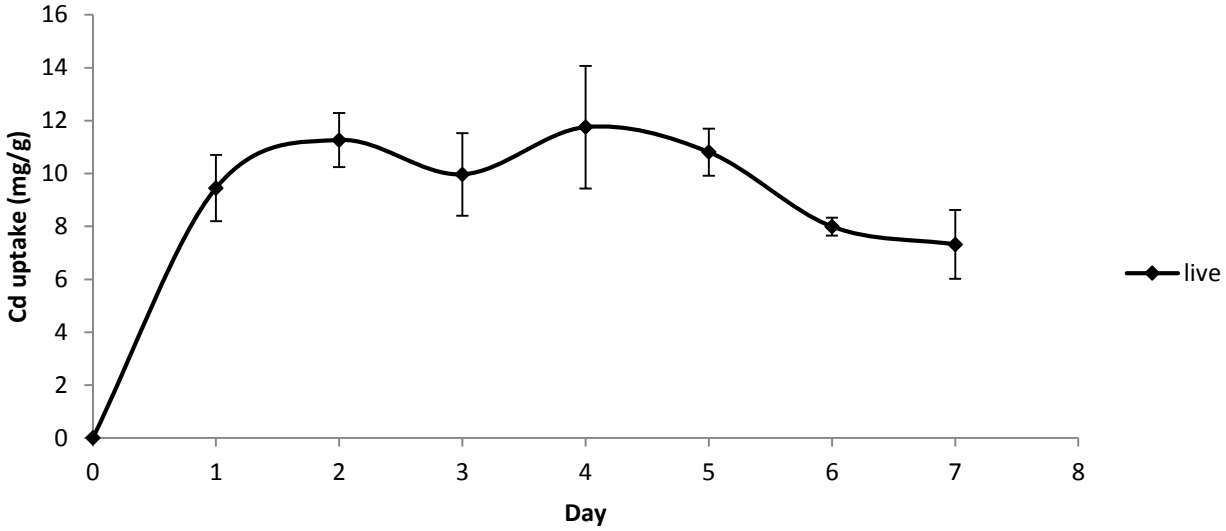

Fig.1: Effect of contact time on the Cd removal by growing biomass. Data was found to be significant at $P < 0.05$.The vertical line on each bar shows the standard deviation.

In equilibrium sorption studies, enough time has to be afforded for the contact before sorption equilibrium is reached between the sorbate sequestered on the solid sorbent and the sorbate concentrate in the liquid phase [3].

Fig. 2. Shows that the biosorption pattern of the dead and dried fungus was observed to be similar during the first thirty minute interval. In the next period, Cd sorption of dead biomass increased steeply and the best sorption appeared after 1.30 h exposure followed by a gradually decreased sorption.

In contrast to the dead mode, equilibrium stabilized within 3 h of contact time by dried fungi. Despite the rapid biosorption time, if dried fungi were applied *in situ*, the biomass would need to be exchanged after a maximum of 5–10 sorption–desorption cycles. Consequently, it seems to be impossible to develop a continuous system based only on a biosorptive removal of metals using a microbial biomass [28]. If adsorption had been the ultimate process involved in removing Cd by dead biomass, saturation time would have been visible by viewer. Nevertheless, dead fungus was not as metabolically active as live biomass; however, similar to the bioaccumulation experiment, the Cd excretion process also occurred by the dead fungus, which confirms the semi-active mechanism. These variations in uptake pattern are possibly due to different removal strategies employed by biomasses. This three-phase curve with an initially rapid uptake of metals (5 min) followed by slower uptake and desorption phase, has been described previously for dead and live biomass of *Agaricus macrosporus* [29].

In accordance with this study, Velmurugan et al., reported that after 3 h of exposure between the metal ion and the fungal biomass of *Penicillium* sp., the metal removal equilibrium was reached [30]. Biosorption process is rapid and takes place between a few minutes to a few hours [31]. A possible explanation for quick Cd sequestration during the first period of experiments via each three types of biomass is that biosorption was

dominating and controlling the uptake process during this period.

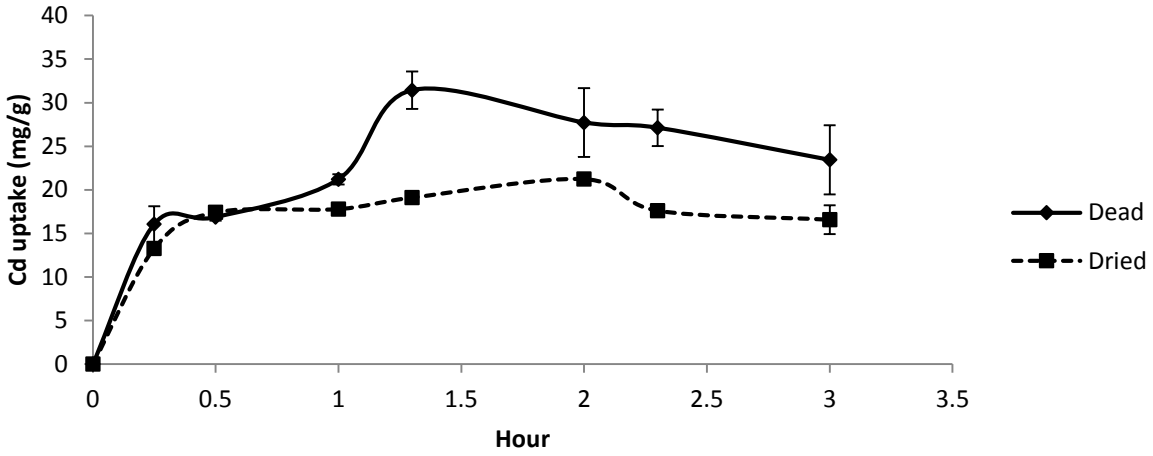

Fig. 2: Effect of contact time on the Cd removal by dead and dried biomass. Data were found to be significant at $P < 0.05$.The vertical line on each bar shows the standard deviation.

3.1.2Effect of pH

The most important parameter influencing the sorption capacity is the pH of the adsorption medium [9, 32].

The pH control in the system is important because it affects both the configuration of the active ion-exchange sites as well as the ionic state of the sorbate in the solution. At low pH, the concentration of protons is high and the ion-exchange sites become solidly protonated [3]. Precipitation, hydrolysis, complexation, and redox reactions are among the changes in the solutions with PH [33].

The highest potential of Cd uptake was found at a pH of 4 for live and dead biomasses and pH of 6 for dried biomass (Fig. 3).The biosorption of dried biomass increased with the solution pH. At pH of 6, due to the more amounts of OH^- ions within the solution, the binding sites on the fungal cell wall are negatively charged. This exerted an influential attraction between active sites and positively charged Cd^+ ions. Such behavior have also been observed when dried biomass of *Microsphaeropsis* sp. was employed as biosorbent [34].

Vice versa, Cd removal for dead and live biomasses was increased by decreasing the pH. The data agreed with bioaccumulation tests performed on *Paxillus involutus*. A pH of 4.5 was found to be optimal pH for sorption of Cd, and the sorption decreased when the pH was raised to 7 [35].This is explained by interaction between the accumulated/adsorbed metal ions with the surrounding liquid solution and strengthening the fungus's cell wall in the lower pH, and decreasing the cell wall porosity and restricting the Cd ion transportation through the cell-membrane.

During the strain adaptation to heavy metal toxicity, fungus synthesizes intracellular/extracellular chelating compounds and renders the metal removal phenomenon. Lowering pH may hamper this type of defense mechanism; the result is slows the motion of the Cd chelating compound. Increasing the oxygen content of the fungi biomass would confer a more acidic surface as the fungi becomes more tolerant to heavy metals. This would promote the adsorption of the more positively charged metal ions and complexes onto its surface [25].The obtained data shows that pH has a great impact on metabolic activity, and on the biomass detoxification mechanism. These results along with the removal pattern in section 3.1.1, confirm that the sorption

behavior of dead and live biomasses were approximately similar.

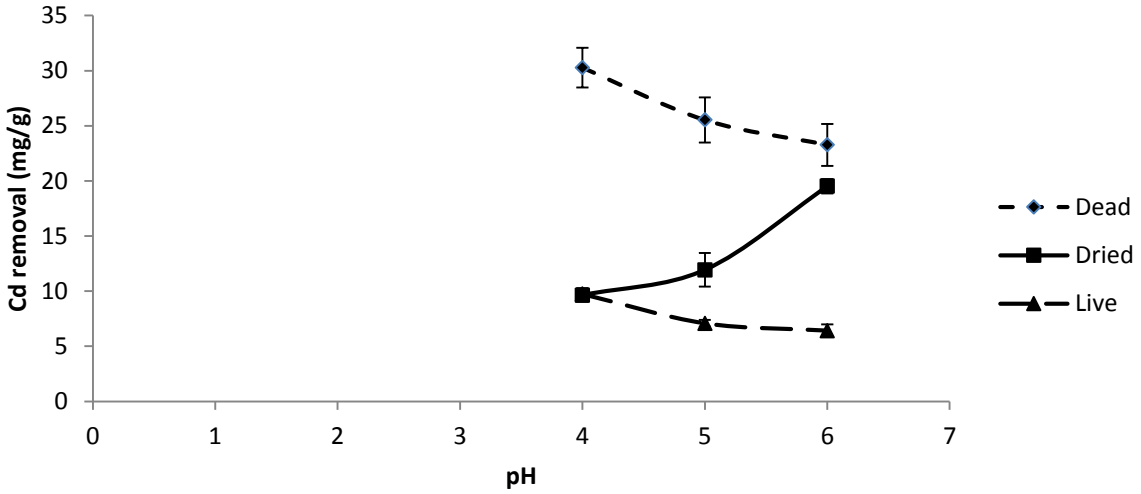

Fig. 3: Effect of initial pH on the Cd removal. Contact times of 4 days, 1.30 h, and 2 h were performed for live, dead, and dried biomass respectively as optimal values. Data were found to be significant at $P < 0.05$.The vertical line on each bar shows the standard deviation.

Similar observations were found in the study on bioaccumulation of multiple metal using *A. lentulus*. They observed declining in metal bioaccumulation as the pH value increased from 4 to 7 [36].

3.1.3 Effect of initial Cd concentration

The data given in Fig. 4 shows that, the uptake yield of Cd enhanced with an increased initial Cd concentration.

At lower initial solute concentrations, the ratio of the initial molecules of solute to the available surface area is low; subsequently, the sorption becomes independent of the initial concentration. However, at higher concentrations, the sites available for sorption become fewer compared with the molecules of solute present. Hence, the removal of solute is strongly dependent upon the initial solute concentration [9]. An increased metal uptake by increasing the initial metal ion concentration is a result of the increased driving force of the concentration gradient, rather than the increased initial metal ion concentration [19]. Several researches have also found similar results as this study [34, 35, 37].

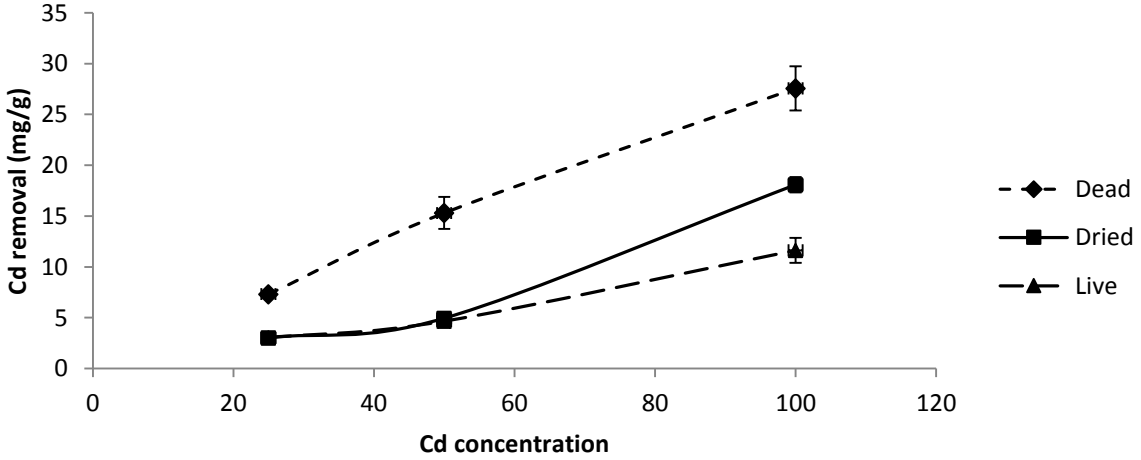

Fig. 4: Effect of initial Cd concentration on the Cd removal. Contact times of 4 days, 1.30 h and 2 h were performed for live, dead and dried biomass respectively as optimal values. Optimum pH of 4 in case of live and dead biomass and pH of 6 in case of dried biomass were used. Data were found to be significant at $P < 0.05$.The vertical line on each bar shows the standard deviation.

3.2. Effect of biomass pretreatment on Cd removal

Taking in to consideration the maximum uptake capacity of dead biomass (27.56 mg g^{-1}) compared with dried biomass (18.08 mg g^{-1}); it seems that the cell wall mechanical disruption by wet thermal treatment improved the removal capability of fungus more than the dry thermal treatment. If the inactivated microorganism is used in the treating heavy metals, the most important mechanism that governs the whole removal process is biosorption. Biosorption is a simple physicochemical process resembling conventional adsorption or ion exchange [2].

Recently the dominating role of the ion exchange process during biosorption was confirmed [2, 7, 38]. Metal biosorption by biomass mainly depends on the component on the cell, especially through cell surface and the spatial structure of the cell wall. Various polysaccharides, including cellulose, chitin, alginate, and glycan exist in fungi cell walls and have been proven to play an important role in metal binding [1]. Therefore, few simple mechanisms contribute in the removal of Cd ions by dried biomass (ion exchange and physical adsorption).

The different behavior of autoclaved fungi against Cd toxicity from dried fungi, the resemblance of removal patterns between autoclaved biomass and live biomass, and that passive biosorption contributed in the Cd removal process show that heterogeneous activities including partially metabolic dependent mechanisms were involved in the uptake procedure of the autoclaved fungi.

Heavy metal tolerant microorganisms such as *A. versicolor* instinctively use defense mechanisms on exposure to metal stress. The loss of cellular water (dehydration in oven) led to deactivating the natural detoxification/defenses strategies of dried fungi, whereas autoclaved fungi may preserve its natural cell structure.

The uptake increase in autoclaved biomass, may often be associated with enhancing permeabilization of cell membrane on account of further binding of metal to exposed intracellular sites.

In general view, heat treatment methods mechanically disrupt the tough fungi cell wall and uncover the metal binding sites. Consequently, fungal mycelia become porous and flexible, and more available adsorbing area will be accessible for metal ions.

It is important to note that during bioaccumulation experiments in the present study, the growing fungal mycelia gave a red appearance. Valix et al. has shown that the detoxification of metals by *A. foetidus* occurred by two overall mechanisms. The first mechanism was production of extracellular metabolites that is capable of adsorbing and precipitating the metal ions on the cell surface. The second mechanism was intracellular binding of heavy metals to thiol containing compounds such as GSH and sequestering these metal–thiol complexes into sub-cellular compartments or vacuoles [25]. Metallothioneins are metal binding proteins, which have been postulated as responsible for the detoxification of various class (II) metals in many different species [39]. Using compartmentation experiments on *Paxillus involutus,* Blaudez et al. has declared binding of Cd onto cell walls and accumulation of Cd into the vacuolar compartment as two essential detoxification mechanisms [35]. It is clear that tolerant strain such as *A. versicolor,* which is considered in this study releases metabolites to get an insoluble form of Cd ions and finally convert Cd to a less toxic form. The changing color in response to Cd in the present study was probably due to the reduction of Cd ions by chelating enzymes excreted via the *A. versicolor* mycelia.

In conclusion, naturally occurring biosorbent and/or alternative adsorbents with good adsorption properties are always preferred over activated ones, unless some exceptional value exists to justify its use

[7]. There are many advantages in bioaccumulation process that an environmental engineer prefers applying live microorganism rather than dead one *in situ* application even if the bioremoval capacity of live biomass (11.63 mg g^{-1}) was the lowest among the all biomasses tested.

Applications of active and growing cells should be a better choice because of their abilities of self-replenishment and continuous metabolic uptake of metals after physical adsorption. Moreover, growing cells have unlimited capacities to cleave organic–metallic complexes, degrade organic compounds, and take up other inorganic ions such as ammonium, nitrate, and phosphate [28].

Nonetheless, comparing the uptake potential of different biomasses used by researchers may be a misleading way, because laboratory parameters are not regulated and even the method of treating biomasses varies. However, the removal potential of some analogous fungi is mentioned in this literature.

In corroboration with our findings, Bayramoglu et al., reported upper Cd removal by heat inactivated Lentinus edodes pellets comparing with the live mode [26].

Additionally, Zafar et al., found Cd biosorption (2.72–2.91 mg g^{-1} of biomass) by live *Aspergillus spp* at a contact time of 4 h [22].

Yan et al., used *Mucor rouxii* for the removal of Cd ions, which had a biosorption capacity of 6.94 mgg^{-1} by live fungi [40].

Results of removal tests by viable and nonviable biomass of *Rhodotorula rubra* yeast showed 4.5 mg g^{-1} and 10.5 mg g^{-1} Cd uptake respectively in 25 mg L^{-1} initial Cd concentration. They reported that nonviable biomass demonstrated higher metal uptake than viable biomass [41].

Genetic manipulation of heavy metal accumulator microorganism to promote accumulation ability of cells and applying hybrid microorganisms in real wastewater treatment plant are among the innovative technologies that have been introduced recently.

Use of plants, with hyper accumulating ability or in association with soil microbes including the symbiotic fungi, arbuscular mycorrhiza, are among the most common biological methods of treating heavy metals in the soil [42].

4. Discussion and Conclusion

In view of the above, it can be concluded that three biomasses of *A. versicolor* have great affinity and capacity for Cd removal. The best adsorbent was found to be dead biomass of *A. versicolor*. Heat treatment particularly through water vapor improved the uptake capacity of fungi. Biotreatment is considered to be an economic, eco-friendly, and efficient method to solve the environmental pollution of heavy metals. By optimizing environmental parameters, this fungus is posed to be a candidate for field remediation of environments contaminated with heavy metals.

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