January 2008 *Volume 3 Issue 1*

Trade Science Inc.

Environmental Science

An Indian Journal

Current Research Papers

ESAIJ, 3(1), 2008 [24-31]

A powerful cadmium resistant strain of a fungus

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Received: 25th July, 2007 ; Accepted: 30th July, 2007

ABSTRACT

The six Cd^{+2} resistant strains of fungus screened by the cadmium supplementation experiment from the local sewage water of local waste water treatment center, were tentatively identified as *Aspergillus niger* on the basis of study of the morphology and conidial color. One of the six strains was found to show a quantitative sorption of Cd^{+2} by more than 60% of the initial Cd^{+2} ion concentrations tested. Growth of the cadmium resistant strain was found to be maximum at 10mM and was inhibited totally in Czapex Dox (CD) broth containing 30mM Cd^{+2}. The same strain could uptake Cd^{+2} intracellularly and could concentrate the same in different cellular compartments. Uptake of Cd^{+2} was studied at different time of incubation and also at different pH. The biosorption of high concentration of Cd^{+2} reflected the exceptionally high tolerance of the Cd^{R} strain to Cd^{+2}. Removal of cadmium by the fungal strain was further substantiated by scanning electron microscopy (SEM), and energy-dispersive X-ray spectroscopy (EDX), which indicated an accumulation of cadmium in the fungal mycelium.

KEYWORDS

Aspergillus niger;
Bioremediation;
Biosorption of cadmium ion;
Cadmium resistant strain;
Scanning electron microscopy.

INTRODUCTION

Heavy-metal contamination of environment brings a potential health hazard that can cause metal toxicities in animals and humans[31]. Heavy metals like, lead, cadmium, copper, and mercury etc., identified as very toxic heavy metals, have been found in the environment at increased concentrations, because a wide variety of industrial activities has accelerated the release of these metals at higher rates than natural geo-chemical cycling processes as operative in the earth[16]. Automobile and leather factories, wood processing, plating battery and ammunition manufacturing units located in the important cities all over the countries have been identified as the potential sources of heavy metal containing effluent contamination of aquifers and rivers. Rivers are the source of drinking water, irrigation, recreational and fishing activities, and also are considered as important ecological reserve. Microorganisms may be considered as biological tool for metal processing as they can concentrate, remove and recover metals from the aqua-environment contaminated with heavy metal ions[24].

Different microorganisms such as fungi, yeasts and bacteria were examined for their ability to bind heavy metals and also to assess their biosorption potential[32]. In recent years, filamentous fungi are getting increasing importance as they are capable of removing heavy metals by biosorption as well as by intracellular uptake mechanisms. Process of biosorption could be considered as an alternative treatment option for cleaning of heavy metal from waste waters which is concerned mainly with the use of fungi[12] . Several cell wall components like proteins, lipids, and functional groups like phosphate, sulphate, amino acids and carboxylate bind the heavy metals[6,15,23] and could be used to remove heavy metal ions. However, little information is available about the use of *Aspergillus sp*. for sequestering heavy metals from solutions[1]. The study as presented here is to identify preliminary the fungal isolate likely to be a strain as *Aspergillus niger* resistant to cadmium and also to use the same strain for characterization with respect to growth and uptake of cadmium ion in the presence of different cadmium ion concentrations under different physical parameters. Scanning electron microscopy study was used to confirm the accumulation of cadmium ion in mycelium of Cd^R.

EXPERIMENTAL

Organism and growth medium

1. Samples

Sewage sediment sample was collected from the flowing sewage water of treatment center, Kalyani, West Bengal, India, using a sediment grab sampler. The sample was preserved at 0°C until use. The sample was diluted serially with a sterile 145mM NaCl solution .and thoroughly shaken . The properly diluted sample was used to spread onto solid Czapex Dox (CD) plates containing 20mM $CdCl_2$

2. Isolation of microorganisms

The isolation and enumeration of microorganisms were carried out in solid CD medium as described by Raper and Thom[21], 1949 that contained (per liter): KH_2PO_4(1g), $NaNO_3$(2g), $MgSO_4$(0.5g), KCl(0.5g), $FeSO_4$(0.01g), $ZnSO_4$(0.01g), glucose(40g). The pH of the medium was adjusted to 7.0 before autoclaving. The medium was solidified with 2% agar as solid CD medium(CDA). Streptomycin was added to the medium for arresting the bacterial growth.. All of the plates were allowed to incubate at 30^0C in an incubator for 72 hrs for the fungal growth. The best grown fungal colony with black conidia was primarily identified as high cadmium ion tolerant strain and the same was preserved in CDA slant or CD containing 20mM $CdCl_2$ for further purification.

3. Preparation of pure culture and its maintenance

The conidia of the preserved strain was taken in sterile water containing one/two drops of Tween 80 and shaken vigorously. The properly diluted conidial suspension was taken for spreading onto CDA medium supplemented with $CdCl_2$ and allowed to grow in an incubator at 30^0C for 72hrs to get the countable colony. The best grown colony having black conidia was preserved in CDA slant and selected for the present study.

The strain was maintained at 30^0C in CDA medium. Slant cultures were routinely sub-cultured every 1 month prior to experimental use on the same medium; 8 day old spore suspension was used as inoculum.

Preliminary identification of the cadmium tolerant strain of the fungus

Lactophenol-cotton blue staining technique was followed to stain the freshly grown mycelia and the morphology of the fungal strain was examined under Phase contrast microscope.

Growth of Cd^R strain in CD and CD supplemented with different concentration of $CdCl_2$

1. Growth of Cd^R strain determined by measurement of colony diameter

Appropriate amount of $CdCl_2$ was separately added to CDA and the plates were point inoculated with the Cd^R strain and incubated for different times in hour at 30^0C. The diameter of the colony was measured against the control (CDA without $CdCl_2$).

2. Growth of Cd^R strain measured by weighing of biomass

Spores suspension of Cd^R strain(10^{10} conidia in one litre) was separately inoculated to liquid CD broth and CD broth medium containing different concentrations of $CdCl_2$ and allowed to incubate at 30^0C for different

times in a shaker incubator for production of biomass. Harvested biomass from each sample was washed repeatedly with sterile de-ionised distilled water and dried on pre-dried and pre-weighed Whatman filter paper no.1 at 85^{0}C till a constant weight was obtained. The increase in biomass weight reflects fungal growth.

Estimation of total cadmium uptake(intra-and extra-cellular cadmium)

1. Growth of mycelia

Spores suspension of Cd^R strain(10^{10} conidia in one litre) was separately inoculated to liquid CD broth and CD broth medium containing different concentrations of $CdCl_2$(5,10,15,20, and 25mM) and allowed to incubate at 30^{0}C for 96 hrs in a shaker incubator. Harvested mycelia (1g) from each sample was separately acidified with concentrated HNO_3 and preserved at -5^{0}C for further processing.

2. Preparation of mycelial sample

The acidified mycelia of each sample was mineralized with the mixture of perchloric acid and concentrated nitric acid (1:3) in a thermostatic bath at 90±10^{0}C till the volume was made to 1ml. After completion of mineralization, 9ml of de-ionised water was added. The samples were then analyzed for cadmium estimation.

3. Estimation of cadmium by Atomic absorption spectrometer

The samples were analyzed by an atomic absorption spectrometer(AAS) (*Perkin Elmer AA400*). The cadmium content in each sample was estimated with the measurement of absorption of cadmium in a cadmium hollow-cathode lamp of AAS considering a known concentration of $CdCl_2$ as reference standard the of wavelength 228.8 nm, slit-width 0.5 nm, lamp current 6 mA, laminar flow burner, air flow 3.5 L/min, and acetylene flow 1.5 L/min.

The effects of pH, time of incubation and initial concentrations of cadmium ions on bioaccumulation were also measured.

Estimation of cadmium ion in sub-cellular fraction

1. Preparation of sub-cellular fraction

Subcellular fractionation was carried out according to the method of S7 as described in Optiprep™ application sheets. The harvested mycelia of Cd^{+2} grown in CD broth medium containing 10mM $CdCl_2$, was washed repeatedly with sterile de-ionized water. The washed mycelia were ground with neutral alumina (1:1) for the preparation of cell-free-extract. The ground mycelia were extracted with the solution of 0.25M sucrose, 1mM EDTA and 20mM Hepes-KOH (pH-7.4) and cell debris was removed by centrifugation at 500g for 20min. The supernatant was centrifuged at 1000g for 10min for collection of nuclear fraction as pellet. This was dissolved in solution of 0.25 M sucrose, 25mM KCl, 5mM $MgCl_2$,and 20mM HEPES-KOH (P^H7.4). The supernatant was then centrifuged at 3000g for 10 min at 4^{0}C for collection of heavy mitochondrial fraction as sediment which was dissolved in a solution of 0.2M mannitol, 50mM sucrose and 1mM EDTA, 20mM HEPES-KOH(P^H 7.4). The supernatant was centrifuged again at 16000g for 10 minutes at 4^{0}C to isolate the light mitochondrial fraction as pellet which was dissolved in the same buffer as used in the earlier step. All the operations were carried out at 0^{0}C.

2. Sample preparation and estimation of Cd^{+2}in each sub cellular fraction

1 ml sample each taken from each- subcellular fractions was acidified separately with 3ml of concentrated HNO_3 and frozen until analysis. The sample was mineralized with the same acid mixture as used before. The cadmium content of each fraction was estimated by the AAS method followed in the earlier experiment.

Electron microscopic study on $CdCl_2$ treated Cd^R strain

In preparation of sample for electron microscopic studies, the freshly grown mycelium of Cd^R strain was washed repeatedly with phosphate buffer(pH 7.0) and was fixed in 5%gluteraldehyde using the same buffer of pH 7.0 for 4 hours. The sample was washed again with the same buffer and dried in graded acetone solution ranging from 30% to 100% twice with each, half an hour time interval. The dried sample was mounted on carbon tape and coated with platinum for 45 seconds. The sample was then analyzed by a field emission scanning electron microscope JEOL JEM 6700F operated at 5.0kV and 20.0.eV.

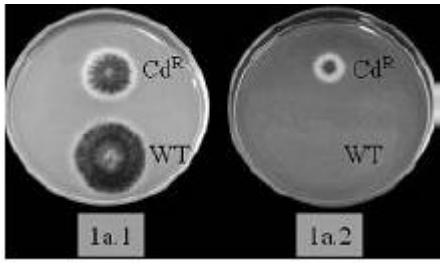

Figure 1(a) : Growth of A.niger (wild type, WT) collected from botany department, university of Kalyani and cadmium resistant (Cd^R) strain on solid CD and CDA medium containing 40mM of $CdCl_2$

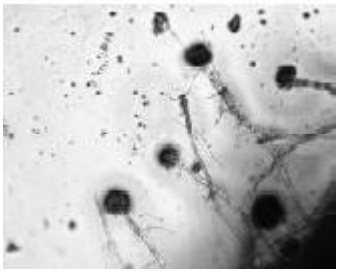

Figure 1(b) : Morphology of the Cd^R strain of the fungus

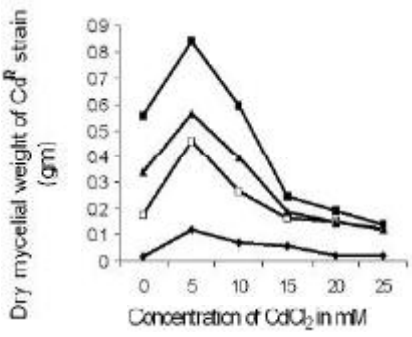

Figure 2: Growth of Cd^R strain in CD broth containing various concentrations of $CdCl_2$
Incubation time: 24 h (♦); 48 h (□); 72 h (▲); 96 h (■)

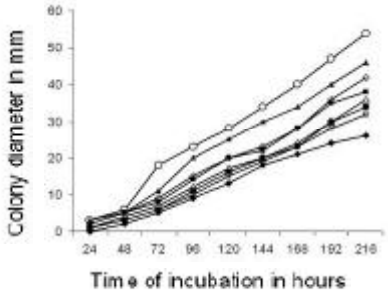

Figure 3: Growth of Cd^R strain on solid CD (CDA) medium containing different $CdCl_2$ concentrations
$CdCl_2$: 2.5mM (Î); 5mM (▲); 10mM ( ); 15mM (■); 20mM (Δ); 25mM (♦); 30 mM (□□); 40mM (♦)

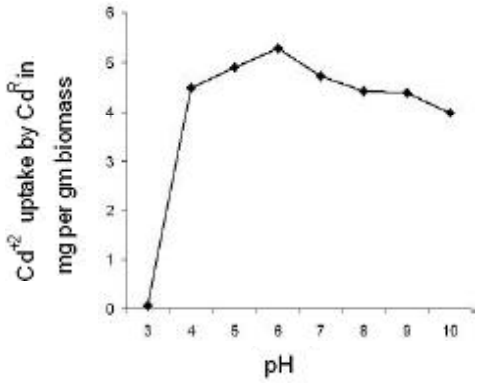

Figure 4: Effect of pH on cadmium biosorption by Cd^R strain

RESULTS

Growth and morphology of Cd^R strain in CD and CD supplemented with different concentration of $CdCl_2$

Both the wild type, *Aspergillus niger* and the Cd^R strain could grow on CDA medium; but no appearance of growth of the wild type was found in CDA medium containing 40mM $CdCl_2$ whereas Cd^R strain showed the remarkable growth in the same medium (Figure 1a) . Color of conidia was found to be black and characteristics of conidiophores and hyphae of Cd^R strain of the fungus investigated under phase contrast microscope is presented in figure1b.

Growth of the CdR srtain was found to increase initially in CD broth supplemented with $CdCl_2$ up to the concentration of 5.0mM, since then growth of the same strain gradually declined and remarkable growth was found in the same medium containing 15mM $CdCl_2$. (Figure 2). Visible growth was appeared till the concentration of $CdCl_2$, 25mM.

Figure 3 shows the growth rate of Cd^R strain in CDA medium containing of different concentrations of $CdCl_2$. It was found that the growth rate of the strain decreased with increasing concentration of $CdCl_2$. The same strain of the fungus tolerated at least 40mM concentration of $CdCl_2$.

Total cadmium consumption by Cd^R strain (intra- and extra-cellular cadmium)

Figure 4 presents the effect of pH. on Cd^{+2} sorption by Cd^R strain. Sorption of Cd^{+2} by Cd^R strain was found to increase sharply with the increase of pH from 3 to 4, then rate of increase was marginalized and

reached maximally at P^H 6 (under acidic pH), finally it started to decline with further increase of P^H from above 6 to 10 i.e at the alkaline range of P^H.

Figure 5 shows measurement of total sorption of Cd^{+2} by Cd^R strain grown in different concentrations of $CdCl_2$. It was found that consumption of Cd^{+2} by Cd^R strain increased with the increase of concentration of $CdCl_2$and reached maximally at a concentration of 10.0mM $CdCl_2$. since then it was found to decrease gradually with further increase of the concentration of $CdCl_2$.

Maximum absorption of Cd^{+2} was found to be at 48 hrs of incubation and then it was decreased with further increase of incubation period (Figure 6).

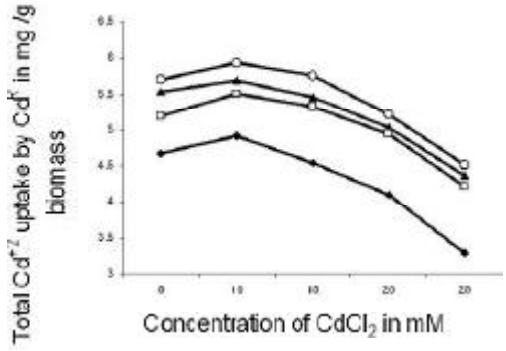

Figure 5: Effect of Cd concentration on metal uptake by Cd^R strain; Incubation time: 24h (♦); 48h (O); 72h (▲); 96h (□)

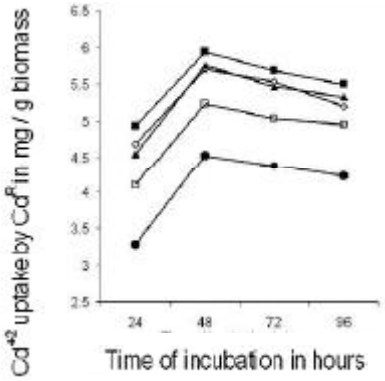

Figure 6: Effect of incubation period on Cd uptake by Cd^R strain; $CdCl_2$: 5mM (◊); 10mM(■); 15mM(▲); 20mM (□); 25mM (♦)

Cd^{+2} content in mitochondrial and nuclear fraction

Figure 7 shows the distribution of cadmium in mitochondrial and nuclear fractions of fungal cell. The content of Cd^{+2} was found to be minimum in light mitochondrial fraction, 1.5 fold higher content in heavy mitochondrial fraction and 2.1 fold higher content in nuclear fraction than that of light mitochondrial fraction . However, the Cd^{+2} content in the total mitochondrial fractions was higher than that of nuclear fraction.

Removal Cd^{+2} ion from spent medium

Cd^R strain removes maximum percentage(40% to 65%) of Cd^{+2} from a CD broth containing 5mM $CdCl_2$; whereas removal of Cd^{+2} ion percentage decreases from about 30% to 15% with the increase of $CdCl_2$ concentration from 10mM to 25mM. In every concentration of $CdCl_2$ as studied here (Figure 8), removal of Cd^{+2} percentage gradually increases with the increase of time of incubation period.

Electron microscopic study on $CdCl_2$ treated Cd^R strain

The SEM micrographs of cadmium-loaded Cd^R strain are shown in figure 9

Cells were grown aerobically in CD liquid medium containing 5mM cadmium and harvested after 96hrs incubation at 30^0C .The top picture is an SEM micrograph of the strain Cd^R under different magnifications showing the electron-dense surface layer and biosorption of cadmium ion. The middle and bottom pictures are EDXS pictures showing the distribution of

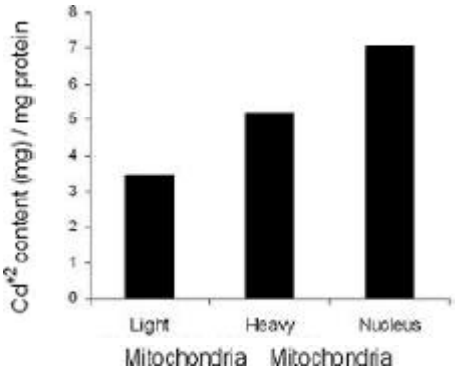

Figure 7: Distribution of Cd^{2+} ion in subcellular parts of Cd^R strain

carbon, oxygen and cadmium respectively. Electron micrographs and energy-dispersive microanalysis indicated that cadmium was bound to the mycelium of Cd^R.

DISCUSSION

The fungus with the growth of maximum diameter

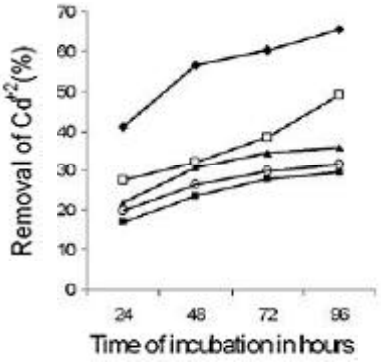

Figure 8: % removal of cadmium by Cd^R strain in different incubation period; $CdCl_2$: 5mM (♦); 10mM (□); 15mM (▲); 20mM (○); 25mM (■)

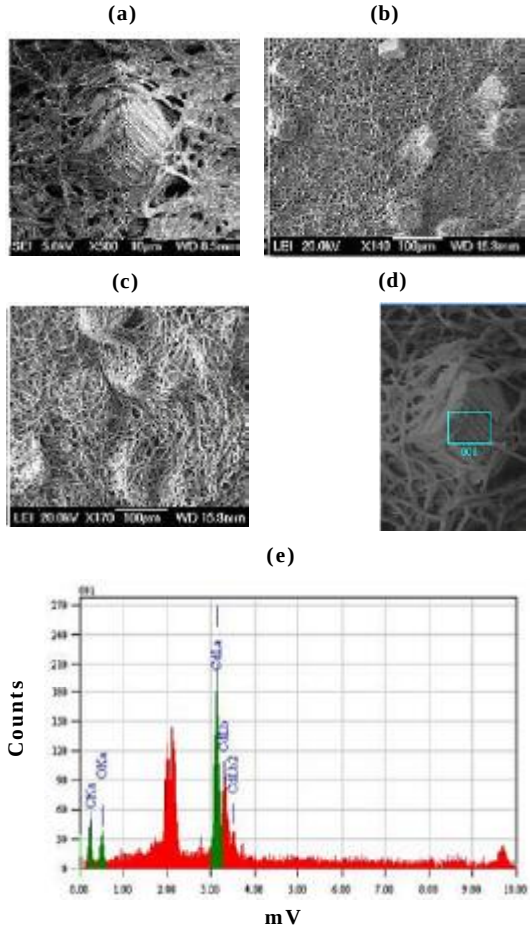

Figure 9: Electron micrographs in scanning electron microscope JEOL JEM 6700F operated at 5kV and EDXS analysis of strain Cd^R; (a) at 5.0eV X500; (b) at 20.0eV X140; (c) at 20.0eV X170; (d) identification of presence of Cd (□marked as 001) in mycelia by EDXS, confirmed from the graphical analysis (e) and results in TABLE 1

in CDA medium containing 30mM $CdCl_2$ may tentatively be identified as high cadmium ion resistant strain.

The conidial color, structure of conidiophores and hyphae of the Cd^R strain as obtained from microscopic observation indicate that the screened fungal strain is likely to be a strain of *Aspergillus niger*. The Cd^R strain showed the capacity to tolerate higher concentration of $CdCl_2$ as compared to that of the cadmium resistant strains of others as reported earlier[5]. There may be change either in its membrane permeability or may have increased the content of short or long peptides like GSH, metallothioneins as reported in different strains of fungi[18,31]. Moreover it was found that low concentration of $CdCl_2$ promoted the growth of the Cd^R strain initially, while higher levels gradually demoted the rate of growth of the strain, which was found to be mostly proportional to the concentration of $CdCl_2$; but the strain could be allowed to grow remarkably up to the Cd^{+2} concentration of 40mM. These findings indicated that under stress conditions, the strain might initially be induced multiply with higher rate to prevent lethal effect of toxic metallic ion similar to as reported in Wood-Rotting Basidiomycete *Daedalea quercina*[7]. However, remarkable growth of the strain at higher concentration of Cd^{+2} signified that the strain may possess specific inherited or acquired resistance to metallic ion similar to as reported in *E.coli, S.aureus* and others, with the phenomenon of extra-chromosomal resistance to metallic ions[17]. *M.luteus* and *Azotobactor* sp. could immobilize 99% of lead from broth containing large concentrations of lead salts[30]. Hence, it may be predicted that cell wall immobilization of inorganic ions may be one of the important physical mechanisms by which the strain Cd^R may consume metallic ions through development of acquired cadmium ion resistant character as reported in some marine fungi[29]. Finally, the increased growth of the strain at 5mM Cd^{2+} concentration in the broth may support the phenomenon of Arndt-Schulz effect[14] rather than a possible requirement of cadmium ion for the growth of the strain .

Effect of pH on cadmium uptake indicated that pH of the growth medium of Cd^R could influence the uptake of cadmium from the broth containing cadmium ion,since the process of uptake of Cd^{2+} may follow the principle of ion exchange mechanism as reported in *Aspergillus sp*[3,27]. It was found that cadmium uptake of *Citrobacter* strain could be enhanced by shifting of pH from 2.0 to 7.0 whereas the uptake decreased at

the pH above 7.0[20]. Similar effect of pH on uptake of Cr(III) was also reported in *Aspergillus sp*[2,3]. At low pH, very small amount of cadmium uptake was carried out by the Cd^R strain, may be due to the competition between hydrogen (H^+)/hydronium(H_3O^+) ions and Cd^{2+} ions[9]. The findings of increasing cadmium uptake with the increase of pH suggest that gradual decrease of H^+ concentration may result less ionic competition with Cd^{2+} and may thus favor more Cd^{2+} uptake[26]. At alkaline pH (8.0 or higher) Cd^{2+} ions may be hydrolyzed and possibly precipitate as sparingly soluble hydroxide and the reduction in the solubility may reduce the uptake rate[13,19].

In the study of sub-cellular localization of Cd^{+2}, it was found that the Cd^R strain could concentrate more cadmium ion in nuclear fraction as compared to the mitochondrial fraction, while lesser amount of cadmium was concentrated in light mitochondrial fraction than that of the heavy fraction. The result may be a reflection of membrane permeability difference of sub-organelles towards Cd^{+2} due to having variable composition of protein and lipid in different organelles. It is reported that the transportation of divalent cations through membrane of filamentous fungi[10] are energy dependent ;once cadmium is transported to the cytoplasm, cadmium ion may be distributed to intracellular metal binding components or distributed among the cytoplasmic sub-organelles.

Intracellular uptake of total cadmium ion content by the Cd^R strain was found to be increased with increase in cadmium chloride content of the media from 5mM to 10mM. This may seem to be contrary to our finding that spent medium, initially supplemented with 10mM metal concentration, has greater metal content than that with 5mM metal concentration. The anomaly can be explained by taking into account the increased production of biomass at low concentration of $CdCl_2$, which may be the reason for scavanging higher amount of cadmium ion[11].Another reason may be that 5mM metal content in the medium is not sufficient for saturating all the cadmium binding sites outside and inside the fungal mycelia of the produced biomass. It may also be stated that10mM metal concentration may be lethal for the fungus as revealed from the study of growth rates and further this metal concentration is more than sufficient to saturate almost all the cadmium binding sites of the produced biomass as indicated by the presence of higher amount of cadmium in the spent medium. A drastic reduction in metal uptake at higher initial metal content in medium may be due to the reduction in biomass production and change in cell membrane permeability.

It has also been reported that the synthesis of metallothioneins and phytochelatins could be induced in the microbes by Cd^{2+} ion which became effective in complexing cytoplasmicCd^{+2}[22,25].

The Cd^R strain of the isolated fungus could grow well in CD medium containing $CdCl_2$ at the concentration of 5mM and removes cadmium ion from cadmium supplemented CD medium. Similar report was available in the case of removal of chromium by *Aspergillus s*[2,28]. Growth of the isolated fungal strain over a wide range of concentrations of $CdCl_2$ as well as its efficiency of cadmium removal can be considered as an important criteria of the isolated Cd^R strain for bioremediation of Cd^{+2} ion from cadmium contaminated aqua system.

The SEM micrograph of the Cd^R strain under different resulations showing the electron-dense surface layer as well as biosorption of cadmium ion, reflects the results as obtained in the study of the growth of Cd^R strain in different concentration of $CdCl_2$ and also the removal of Cd^{+2} ion from the spent medium. Moreover, the EDSX analysis confirmed the presence of cadmium in the fungal mycelium as reported for chromium accumulation in the mycelia of *Aspergillus niger*.

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