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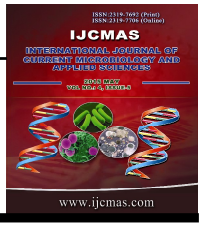

Original Research Article

Role of Morphology, Development and Characterization of Cd accumulating mutants of *Aspergillus nidulans*

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Keywords

Aspergillus nidulans, metabolic, inhibitors, kinetics, mutants, resistance

A B S T R A C T

Stable mutants of *Aspergillus nidulans*, resistant to 1 mM Cd were developed by step-by-step repeated culturing of the fungus on the medium containing higher concentrations of Cadmium chloride. Characterization of mutants could differentiate them into two categories CdR I and CdR II. Each category of mutants exhibited alterations in growth, conidial germination and melanin secretion both in Cd-free and Cd-containing media. CdR II mutants were little slow in growth with sparse mycelia and conidiation but showed high melanin secretion and higher Cd-uptake in comparison to CdR I mutant. Studies involving metabolic and translational inhibitors could prove that Cd accumulation was biphasic. The initial energy independent surface accumulation was found to be followed by energy dependent intarcellular uptake. Increase in the concentration of the metal in the medium or the time of exposure did not proportionately increase the metal uptake by the mutants. Cd -uptake followed Michaelis-Menten saturation kinetics, which was enhanced under optimum pH of 6.5–7.5 and reduced complexity of the medium due to free availability of ions. Resistance to Cd was found to be constitutive in CdRI mutant, and could be induced in CdRII mutant.

Introduction

The heavy metal Cadmium (Cd) is one of the essential micronutrients, required by various organisms for a range of structural organizations and metabolic activities. Cadmium is a cofactor for enzymes like hydrogenases, urease and methyl-S-coenzyme M-reductase, and some key enzymes in the metabolism of strictly anaerobic bacteria(Hausinger RP 1987). The toxic effects of the metal are a consequence of its ability to inactivate enzymes containing sulphydryl groups and may inhibit oxidative phosphorylation. Additionally, it can compete with other metals such as zinc and selenium for inclusion in metallo-enzymes and competes with calcium for the sites of connection with regulatory proteins such as calmodulin. These properties, together with its widespread use in batteries, make the

cadmium one of the most common environmental pollutants.Toxic levels of Cd inhibit growth and sporulation of various filamentous fungi and yeasts and cause reduction of RNA and protein synthesis and photosynthesis (Pucket KJ et al, 1979).

The study of interactions between heavy metals and microorganisms has been focused on mechanisms of transformation and conversion of metal ions by the reduction in different polluted environments, in the selection and use of resistant organisms as indicators of toxicity for other life forms, as well as analysis of mechanisms, determinants and transference of resistance.

Cadmium is recognized for its toxicity throughout the food chain, and is one of the toxic components of industrial and mining waste.

The metal exhibits mutagenic, carcinogenic, phytotoxic and ecotoxic effects, being extremely toxic, due to its concentration, for all living systems, particularly for mammals, including humans. (Kapoor A et al, 1995).

The purpose of this study was to evaluate the behavior of *Aspergillus nidulans*, grown in the presence of different concentrations of cadmium, regarding the growth, morphology behavior, and the ability of uptake of the metal ion(Peeper et al,2000).

Mainly, training the microorganisms to resistance by step-wise exposure to higher and higher levels of toxic element or by mutagenesis using a mutagen can lead to the development of microbial strains having resistance to high levels of metals. We developed strains of *Aspergillus nidulans*, having very high tolerance to Cd and characterised them for various aspects including Cd accumulation.

Material and Methods

Study Site and sampling

The present study was carried out in soil samples collected from the agriculture field of Khullad, located in Anugul district, Odisha. Tropical dry deciduous forest is considered to be the natural vegetation of these areas, but rapid development of industrialization led to the decline of forest cover due to the felling and biotic interferences. The districts were blessed with annual rainfall of about 128.7-151.5 cm from the South West monsoons from July to September (Rath et al., 2010). The temperature ranges from 16.3-47.4^{0}C (Patel & Behera, 2011). Sampling was done in accordance with soil microbiological study (Parkinson et al., 1971). The sample was brought to the laboratory in sterilized polythene packets.

Fungal isolates and acclimatization

The fungi, *Aspergillus nidulans*, used in this study were isolated from waste effluent sample. The isolation and identification of the fungi was carried out in the Environment and Sustainability Department of CSIR-IMMT, Bhubaneswar, Odisha. The fungi were identified by their colony characteristics as well as their vegetative and reproductive structures as observed under the electron microscope. Some macroscopic characteristics used for the identification include, colour of the colony, patterns of growth of colony and the by-products released by the organisms. Some of the microscopic characteristic as viewed under the microscope include, the shape of the conidia head, pattern of arrangement of spores on the conidia, shape of the spores and shape of the conidiophores. The isolates were further confirmed in the Department of Microbiology, OUAT, Bhubaneswar; Odisha.The organisms were isolated and

maintained on Sabouraud Dextrose Agar, SDA (Himedia, India) at pH 5. Inoculated slants were incubated for 7 days at 30°C and then stored at 4°C until used.

Culture media screening Cd by radial growth

For Screening to select the appropriate medium for experimental procedures was performed by using the radial growth. Pre-inoculation corresponding to cultures discs, with one centimeter in diameter, were inoculated in Petri dishes, containing different culture media: Malt Extract (ME), Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SAB) and Yeast Malt Agar (YMA), containing cadmium chloride, prepared in distilled and deionized water, pH 6.0, calibrated with the use of sodium hydroxide 1N and acetic acid to 10%, at concentrations of 1 mM, 2 mM and 3 mM. The cultures were incubated at 28 °C for 7 days.

Control-samples were grown according to the procedures cited without the metal. The growth was evaluated through the radial expansion, measured by the diameter of colonies, in millimeters, every twenty-four hours of incubation. The results are expressed as arithmetic average of triplicates.

Growth curves

Erlenmeyer flasks of 300 mL of capacity, containing 150 mL of liquid Sabouraud were inoculated and incubated at 28 °C, in a orbital shaker (250 Hertz), for fifteen days. Samples were collected with 3, 6, 9, 12 and 15 days of cultivation and submitted to lyophilization for determination of biomass production by dry weight. Control samples were prepared, under the same conditions, without cadmium. All results were expressed in average of five replicates.

Development of Cd -resistant mutants

Cd resistant mutants (Cd R) of *A. nidulans* were obtained by stepwise selection against increasing concentrations of Cd. The conidia from the fungal colonies were repeatedly subcultured in plates containing increasing concentrations of Cd in CDM agar until the growth of colonies was found stable and comparable with those on Cd -free medium. The process led to the selection of CdR strains that exhibited good growth and conidiation at 1mM concentration of Cd, beyond which the growth of the mutant strains was inhibited. Therefore, 1mM Cd was considered as the maximum tolerable concentration (MTC) of the metal for the fungus under study. To understand whether the mechanism of Cd-resistance was inducible, the conidia of mutant strains were allowed to germinate for first 8 h in CDM containing 0, 0.1 and 0.25 mM Cd and there after transferred to the medium having Cd concentration raised to 1mM. Fungal growth and accumulation of Cd in the fungal biomass was monitored for next 5 d at 24 h intervals.

Cd-accumulation study

The fungal biomoss raised in Cd containing liquid CDM was washed with normal saline, dried, weighed and incinerated at 6000C for 6h in a muffle furnace. The ash was dissolved in 2 mL of 1N HCl. In case of cytosol samples (as described below), 1 mL of the sample was mixed with 2 mL of 1N HCl. Suitable dilutions were made with deionised distilled water and subjected to Cd estimations at 232 nm in an atomic absorption spectrophotometer (AAS-Shimadzu: AA-6300). To estimate the accumulation of metal in the cell wall, the mycelial biomass from 72 h old culture was harvested, washed extensively in normal saline and blotted to dry. The mycelial mass

was homogenized in 1% sodium dodecyl sulphate (SDS), shaken at 200 rpm for over-night and centrifuged at 10,000 x g for 20 min at 40 C. The pellet containing cell walls was washed extensively with distilled water and then sequentially with 80% ethanol and ether. The cell wall preparation was dried at 800C, weighed and incinerated at 6000C. The supernatant contained the cytosolic fraction. Both the cell wall ash and the cytosolic fraction were subjected to Cd estimation by AAS.

Kinetics of Cd uptake

The Cd resistant mutant strain was grown for 60h in metal-free CDM and the mycelial biomass was harvested, washed extensively with normal saline and blotted to dry. One hundred mg of wet biomass was transferred to 10 mL of the liquid CDM and subjected to different treatments, which included varying concentrations of Cd, metabolic inhibitors, absence of carbon source, H^+ concentration, and the nature of the medium. Cd uptake was estimated in the biomass at 1 h intervals for 5 h. For determining the role of C-source, the pre-grown mycelia of the mutants were starved for 5 h by incubating in liquid medium without glucose, before subjecting to metal uptake assay. Uptake of Cd at external concentrations of 0.5-2.5 mM Cd was recorded after 3 h. The Km and V max for the uptake of Cd were calculated for the Cd R mutants. The Lineweaver-Burk plot was prepared using 1/S and 1/V values.

Protein estimation

Proteins were isolated and partially purified to determine their role in conferring Cd-resistance. Seventy two hour old mycelia from the cultures, grown under different experimental conditions, were harvested and washed in physiological saline and Tris-HCl buffer (10 mM, pH 8). One g of wet mycelium from each set was homogenized in 3 mL of Tris-HCl and the homogenate was centrifuged at 20,000 x g for 30 min in Beckman 70 refrigerated centrifuge (Kermasha et al,1993).The supernatant was heated at 600C in the heating block for 10 min and then centrifuged at 50,000 x g for 10 min. The supernatant, referred to as partially purifi ed fraction, was evaporated *in vacuo*. The residue was dissolved in 0.5 mL of Tris-HCl buffer for protein estimation and subjected to electrophoresis(Laemmli UK et al, 1970).

Results and Discussion

Effects of cadmium in the growth, morphology and ultrastructure of Aspergillus nidulans

With the aim of selecting a standard medium for the physiological and biochemical experiments an initial study related to different growth media was performed. Figure 1A-D shows results for the radial growth obtained for the cultivation of *Aspergillus nidulans* in Yeast Mold Agar, Malt Extract, Sabouraud and Potato Dextrose Agar media, in absence and presence of cadmium, at concentrations of 1, 2 and 3 mM, respectively. From an analysis of graphics one can infer that the cultivation in the absence of metal in different media, results in different growth profiles, estimated by colony radial expansion. However, comparing the different media, higher radial growth was noted for cultivation in the Sabouraud medium. This study evaluated the percentage of relative growth of the organism for each test condition in relation to control. The results presented reveal the influence of cadmium on the radial growth of colonies of *Aspergillus nidulans.* The cultivation in different media resulted in variations of colony radial expansion, and revealed the

inhibitory effect of the metal, which is directly related to its concentration.

The presence of the metal induces a significant reduction of growth, as determined by the cellular biomass, in relation to control culture. A decrease in the biomass production proportional to the concentration of metal used was verified. It was observed that after 3 days a growth of biomass in control and treated samples occurred. Notwithstanding, treatment induced a reduction in biomass production, which amounted to 46.31%, 32.62% and 29.99% of the control culture production for concentrations of 1, 2 and 3 mM, respectively. At the end of the trial period for the culture treated with 1mM of cadmium, biomass obtained corresponded to 64.89% of control culture. For the treatments with 2 mM and 3 mM cadmium, the biomass corresponded to 63% and 36.83% of the mycelia mass obtained for control culture, respectively.

Development of Cd resistant mutants: Most fungi accumulate metals by employing physico-chemical mechanisms and transport systems of varying specificity(Joho M et al,1995). However, both essential and non-essential metals in concentrations, higher than the optimal level, prove toxic to organisms

Under such conditions, these organisms may activate and adapt a mechanism of detoxifi cation to ensure survival(Gadd GM et al, 1992). We, in this study were successful in developing Cd-resistant (CdR) mutants from *Aspergillus nidulans* strain *riboA 1, biA 1* by step-by-step repeated culture and selection on the medium containing increasing concentrations of Cd. This process has successfully been employed for such purpose by many other workers. The selected mutants remained resistant to 1mM Cd, even after several subculturing on metal free medium and thus confirmed them to be genetically stable Cd R mutants.

Characterization of CdR mutants

The selection procedure provided two types of CdR mutants of *A. nidulans*. Mutants that formed relatively large colonies, dense bright green conidiation and secreted very little or no melanin were designated as CdR I (Fig. 3A). The mutants that formed medium size colonies, sparse mycelial growth and conidiation and secreted melanin profusely were designated as CdR II (Fig. 3B). The growth studies on solid as well as in liquid CDM showed that both CdRI and CdRII mutants, in spite of their tolerance Cd , showed extended lag phase, though later picked up the growth and became comparable to that of sensitive strain, grown on Cd free medium (Fig. 4A & B). In the medium containing even 0.5mM Cd, the hyphal growth of CdS strain after 120 h, was found to be inhibited by about 90 percent and CdRI and CdRII mutants by about 12 and 45 percent, respectively. At 1mM Cd concentration, the inhibition increased to 98 percent for CdS, 32 and 65 percent for CdRI, CdRII mutants respectively. The CdS strain showed almost no growth except for very title hyphal extension and no conidiation. In CdRI mutant, conidiation showed up by 72 h, while in CdRII was seen by 48 h and reached to maximum by 72 h. Such alterations in morphology, growth and morphogenesis of many other fungi due to exposure to heavy metals including Cd in the medium are well known and have been considered as a strategy by the organism to evade the toxicity for survival(Mohan PM et al,1983).Alterations in cell wall composition and accelerated production of melanin can be induced by toxic metals in the medium. Melanin in the cell wall is used as a defence mechanism as such structures are

impermeable to heavy metals and other toxicants. The addition of melanin to the Cu-containing culture of albino strain of *A. pullulans* reduced the metal toxicity to a certain extent(Gadd GM, 1993).

The CdR mutants, despite their tolerance to 1mM Cd, exhibited delayed and differential rates of conidial germination and developmental process irrespective of the presence of the metal in the medium. In first 8 h, conidia of CdRI mutant appeared in different stages of distensionwith only very few conidia showed the beginning of germ tube formation. Subsequently, the rate of further development of conidia into formations of germ tube, mycelia mats and balls continued to vary. The pattern of growth and development of CdRII mutant was similar to that of CdRI mutant except that comparatively high percentage of CdRII conidia showed faster rate of germination and growth. Mycelial mats of CdR mutants were much thinner irrespective of presence or absence of Cd in the medium but more so in Cd-containing medium. Mycelial balls of CdR mutants were fragmented at all the phases of their growth. The developmental aberrations brought about by Cd were irreversible as the conidia from a single mycelial ball, when transferred to Cd-free MM continued to produce colonies of fragmented mycelial balls of different sizes. The rate of disintegration was more rapid in CdS strain in Cd-free as well as in Cd-supplemented medium than in the mutants cultured in presence in Cd. The germination of conidia of CdS strain was completely inhibited by MTC level of Cd. The effect of Cd on delayed conidial germination of CdR mutants was further evident by assessment of their biomass during the first 24 h. CdRI andCd RII mutants grown in Cd-free medium registered only about 50% dry weight of the biomass formed by NiS in the same medium. However, in Cd-containing medium, both CdR mutants registered 4 to 6 folds higher biomass. In other studies (Cooley RN et al, 1986) the germination of conidia of the wild type strains of *A. nidulans* was found to be inhibited to 50 percent by 175μM Cu and 350μM of Cd, whereas the same or higher concentrations of these metals could slightly stimulate the germination of Cu- and Cd-resistant strains.

***Cadmium* uptake *by the CdR mutants*:** Both CdRI and CdRII mutant strains showed progressive increase in Cd uptake up to 72 h of their growth, there-after exhibiting a declining trend (Fig. 5). CdRII mutant accumulated two folds more Ni than the CdRI mutant. By 96 h, when the exponential growth had ended and mycelial disintegration had commenced, CdRII mutant retained more than 5 times Cd than the CdRI mutant. Thus, CdRII was better Cd-accumulator than CdRI. Very low content of Cd in the mutant biomass in later phases of growth can be attributed to the changes in the biosorptive properties of the fungus due to the chemical changes in the mycelium has been demonstrated in many filamentous fungi as well. Cell wall adsorption accounts for rapid, non-specific and energy-independent process resulting from ionic interactions,followed by a progressive energy-dependent phase of intracellular uptake. Similarly, when the fungal biomass from 60 h old cultures, grown in Cd-free medium, was exposed to metabolic inhibitors or the absence of carbon source in the CDM, after the initial accumulation in first 1 h, the CdR mutants did not register any significant increase in Cd uptake(Sized SM et al,1995). These results again confirmed that the initial energy-independent passive metal uptake by diffusion and surface binding, was followed by an active process requiring the availability of metabolic energy.

Acidic conditions though increase the metal availability; also increase H+ ion concentration that may compete for the binding to anionic sites on cell surface or for transport in the cell. Increase in alkalinity on the other hand, can precipitate the metal as hydroxide, oxide or carbonates of varying solubility and toxicity Such complexation of metal ions may lead to their unavailability for binding or uptake . The hydroxylated species may also associate more efficiently with the microbial cell than the corresponding metal cation. CdR mutants accumulated lesser amount of Cd when incubated in complete medium than in CDM (Fig.8). Several components of microbial media like agar, peptone, yeast extract, casamino acids and chelators like oxalate, citrate, glutamate, cysteine, EDTA, etc. are known to complex the metal ions affecting their bioavailability. Such complex formation may reduce the metal toxicity in certain microbial systems.

Studies on kinetics of Cd uptake

The kinetic studies with 60 h old mycelium of the mutants, grown in Cd-free medium,suggested that the amount of Cd accumulated by both CdRI and CdRII strains varied with the Cd concentration in the medium to a certain extent. There was a significant increase in Cd uptake in medium having concentration up to 1mM Cd during the first 3 h. Beyond this time, there was no appreciable enhancement in the metal uptake. The initial rapid Cd uptake was followed by more gradual uptake, which continued to saturation by 24 h. Further enhancement in the exposure time or the external concentration of the metal influenced the rate of uptake to a limited extent only. In these short term Cd uptake assays, higher efficiency of CdRII over CdRI mutant was again established.

A Lineweaver-Burk plot of the medium concentration of Cd (1/S) versus the rate of uptake (1/V) followed theMichaelis-Menten kinetics in both the mutants. The Km and Vmax values for Cd RI were 0.355mM and 0.098 mmoles-1 mg DW-1h, respectively and for CdRII mutant, 1.067 mM and 0.345 mmoles-1 mg DW-1h, respectively. A low half saturation constant (Km) for the uptake of Cd in Cd RI mutant indicated a higher affinity of the strain for the metal (Fig. 9). However, CdRII mutant showed significantly higher Vmax value suggesting that the mutant could efficiently accumulate metal. High affinity transport system for essential ions has been reported in several fungi. One of the Cd-resistant (CdR2) mutant of *N.crassa* was found to accumulate more Cd than the other Cd-resistant mutants. Transporters with differential affinity towards the same metal, reciprocal relationship between metal concentrations in the medium and the affinity of the transport system are a common phenomenon in metal-microbe relationship(Ross IS et al,1994)

Protein analysis

The inducibility of resistance mechanism in CdRII mutant was further investigated by using cyclo-heximide, an inhibitor of protein synthesis. IC 50 levels of cycloheximide for CdS and CdRII strains were 60μg mL-1 and for Cd RI mutant 40 μg mL-1. Presence of 1mM Cd along with IC 50 levels of cycloheximide inhibited the growth of CdS and CdRII strains to a much higher level as compared to CdRI strain.

However, Cd accumulation by the Cd R mutants was not inhibited in the presence of IC 50 levels of cyloheximide and the accumulated metal quantities were comparable to those accumulated in inhibitor-free medium (Fig. 10).

Table.1 Distribution of Cd in cellular fraction of Cd s and Cd R strains cultured in Czapeck – Dox broth containing 1 mM Cd for 72 hours at 370C 370c . □ = Cd s, ▲ = Cd R I, ■ = Cd R II

Cellular fraction	Cd^{S}	Cd^{R}	Cd^{R} II
Cell wall(μg mg DW^{-1}	3.4±0.31	15±1.97*	120.7±5.52*
Cytosol(μg ml^{-1})	6.36±1.03	150±9.11*	526.5±14.92*

Table.2 Cd uptake (μg mg-1DW) by Cd R mutants in Czapec-Dox broth containing 1 mM Cd and the metabolic inhibitors(NaN3 and 2,4-DNP)

Time h	Cd^{R} I			Cd^{R} II		
	Cd	$Cd+NaN_3$	Cd+DNP	Cd	$Cd+NaN_3$	Cd+DNP
0	0.06±0.01	0.07±0.01	0.05±0.01	0.27±0.01	0.26±0.01	0.58±0.04
24	0.98±0.04	0.95±0.02	1.19±0.06	5.50±0.21	4.98±0.08	4.24±0.23
48	6.02±0.07	3.51±0.21	4.16±0.18	9.71±0.30	4.55±0.19	4.95±0.24
72	56.81±2.44	4.57±0.22	5.72±0.20	125.8±2.34	6.56±0.33	6.08±0.23
96	20.00±0.86	4.933±0.03	5.96±0.29	94.65±4.36	5.81±0.29	6.61±0.26
120	5.82±0.32	0.28±0.014	0.35±0.021	28.22±1.47	3.01±0.09	4.30±0.16

Metal uptake by microorganisms is greatly influenced by pH(El-Morsy SM et al,2004). Cd uptake in both the CdR mutants was maximum at pH 6.5 (Fig. 6 and 7). The effect of pH can be accounted for then chemical behaviour of the ionic species involved

Fig.1 Radial growth of Aspergillus nidulans in different culture media, aiming to select a standard medium for the physiological and biochemical investigations. A- Control ; B- Treated with cadmium 1 mM; C- Treated with cadmium 2mM; D- Treated with cadmium 3mM. YMA (Yeast Malt Agar); ME (Malt Extract); PDA (Potato Dextrose Agar) and SAB (Sabouraud Dextrose Agar)

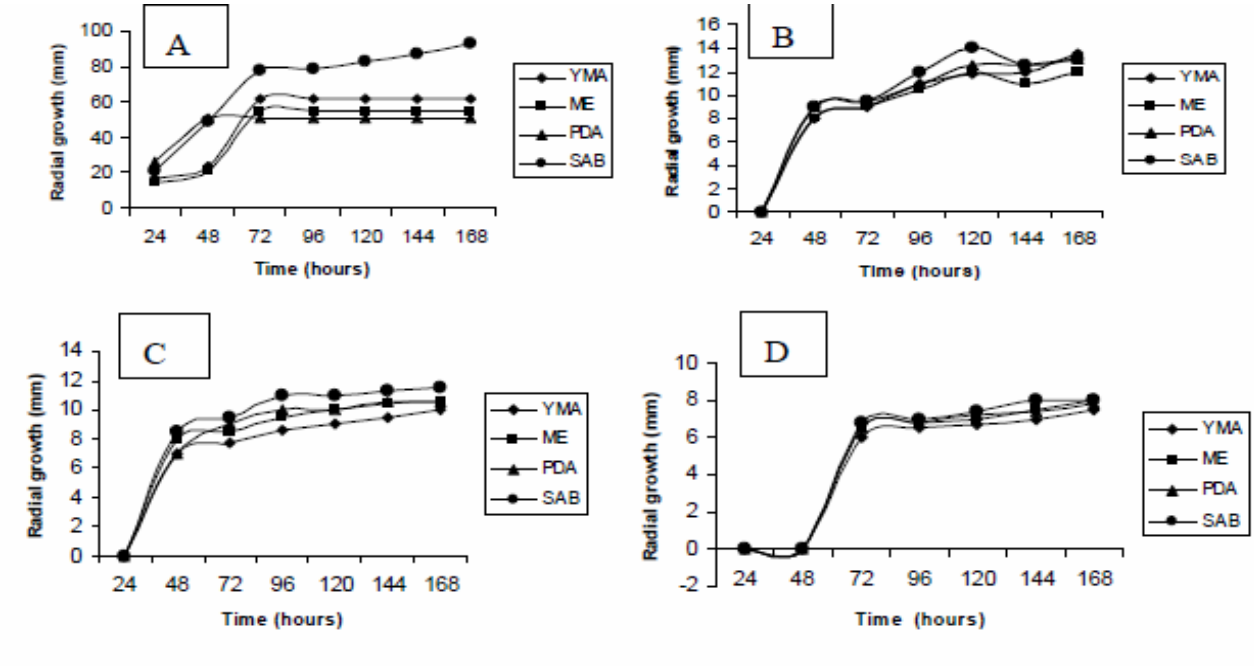


Figure.2 shows the results of the biomass production of *Aspergillus nidulans* cultures in the presence and absence of cadmium

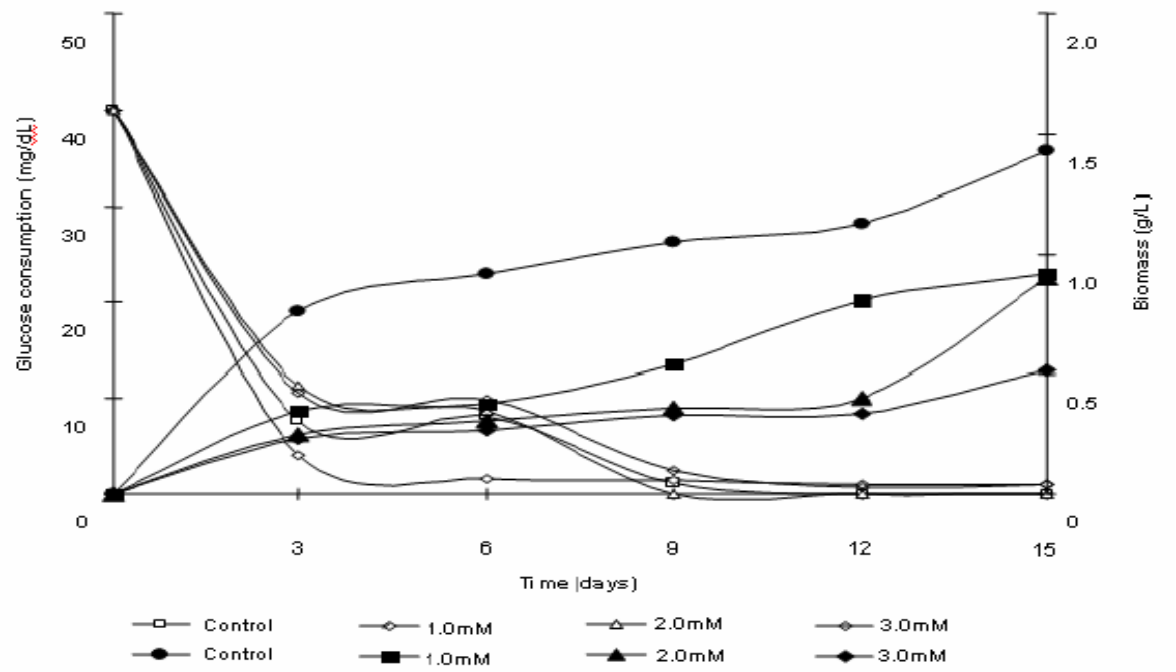


Fig.3 Colonies of CdR mutants on supplemented Czapec-Dox agar containing 1 m M Cd at 370c after 120 h.A: Cd R I,B. Cd R II.Growth of Wild type control strain(Cd s) is highly inhibited. (marked by →)

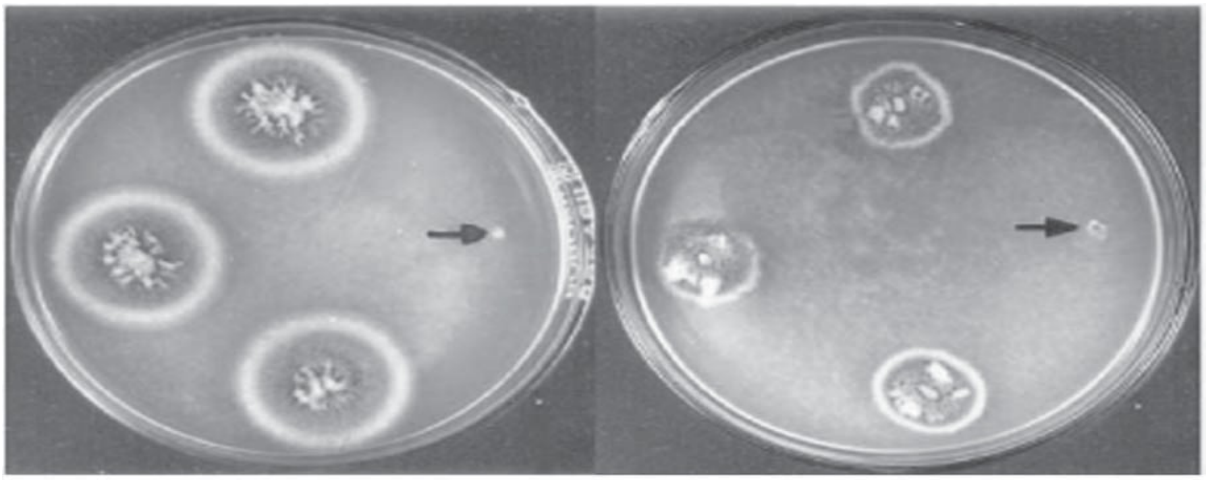


Fig.4 Growth of Cd s and Cd R strains on supplemented Czapec-Dox agar at 370c .A: Cd free medium , □ = Cd s, ▲ = Cd R I, ■ = Cd R II.B: Cd(1Mm) containing medium □ = Cd s, ▲ = Cd R I, ■ = Cd R II

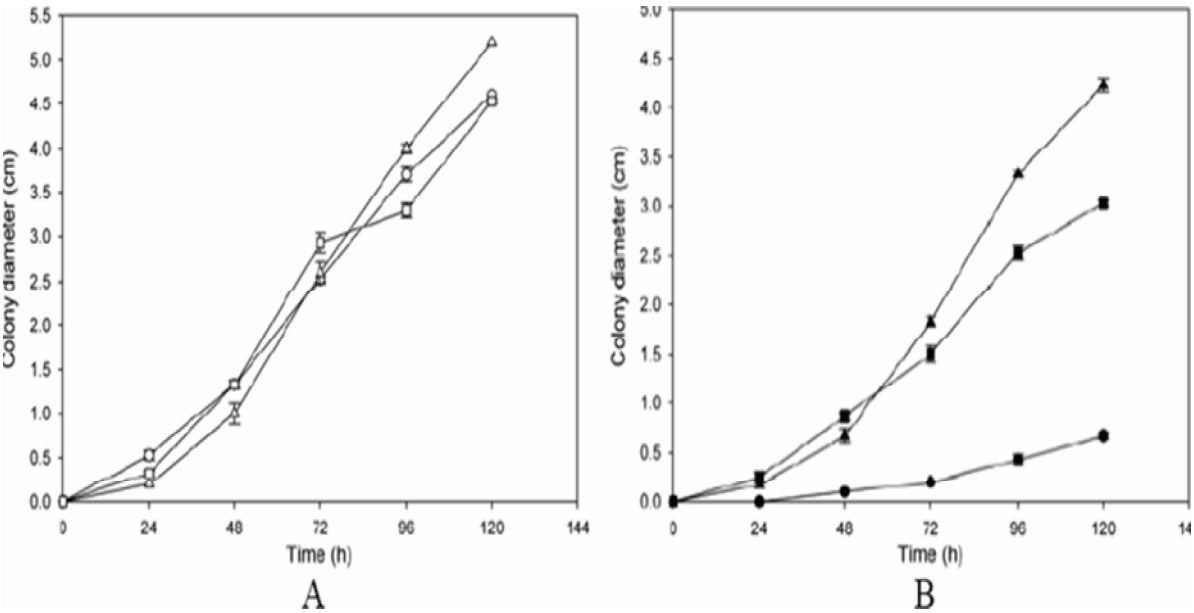


Fig.5 Cd uptake by Cd s and Cd R strains in liquid Czapec-Dox medium containing 1 mM Cd over a period of 120 hours at 370c . □ = Cd s, ▲ = Cd R I, ■ = Cd R II

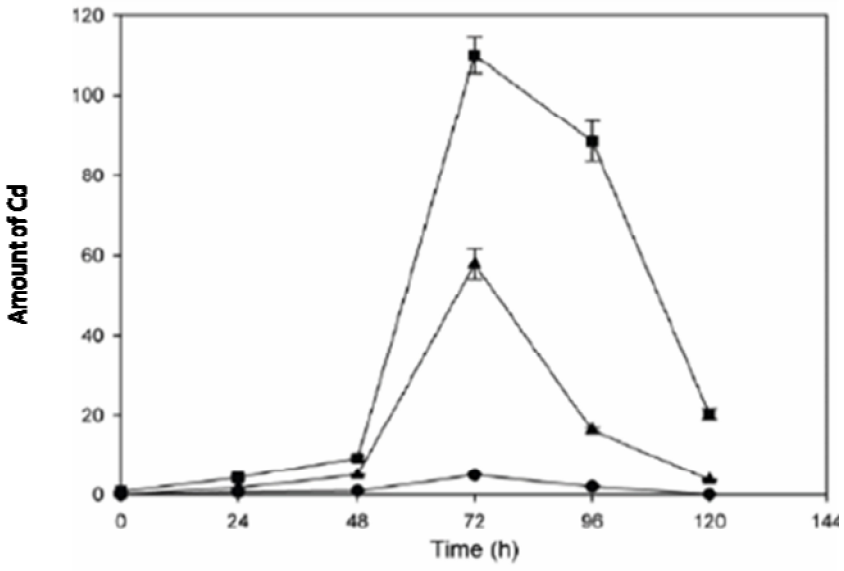


Fig.6 Effect of pH on Cd uptake by CdR mutants in liquid Czapeck-Dox medium. Mycelia grown for 60 h in Cd-free medium were transferred to medium containing 1mM Cd. CdRI M

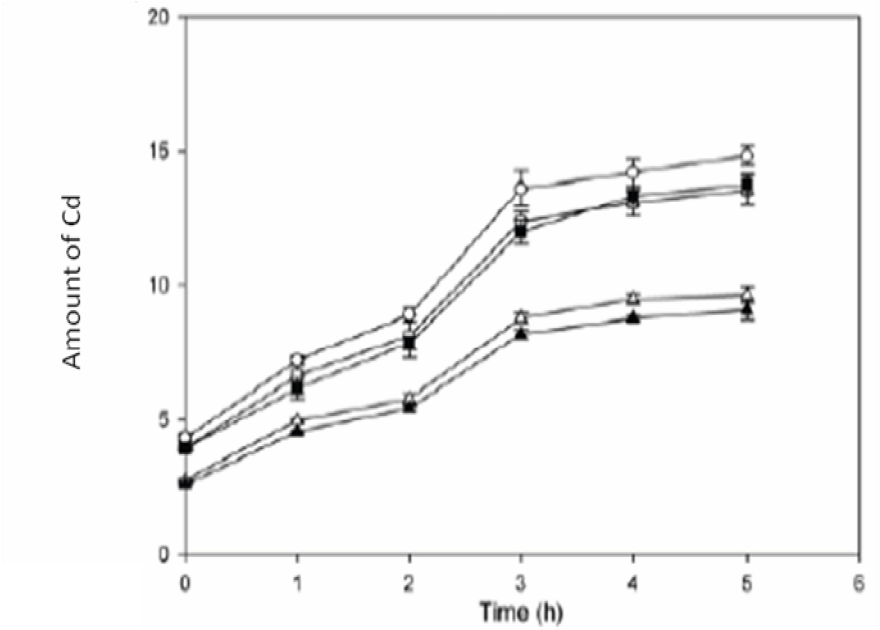


Fig.7 Effect of pH on Cd uptake by CdR mutants in liquid Czapeck-Dox medium. Mycelia grown for 60 h in Cd-free medium were transferred to medium containing 1mM Cd. CdRII M

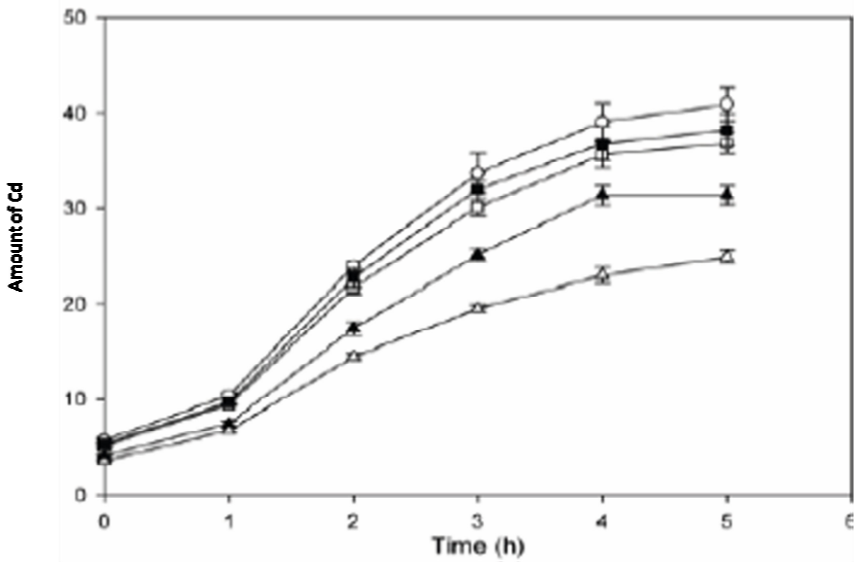


Fig.8 Effect of the complexity of the medium on Cd uptake. Mycelia of CdR mutants grown in Cd- free medium for 60 h were transferred to CM containing 1mM . =Cd R I in MM+Cd; = Cd R II in MM+Cd (Control); = Cd R I in CM+Cd; = Cd R II in CM+Cd

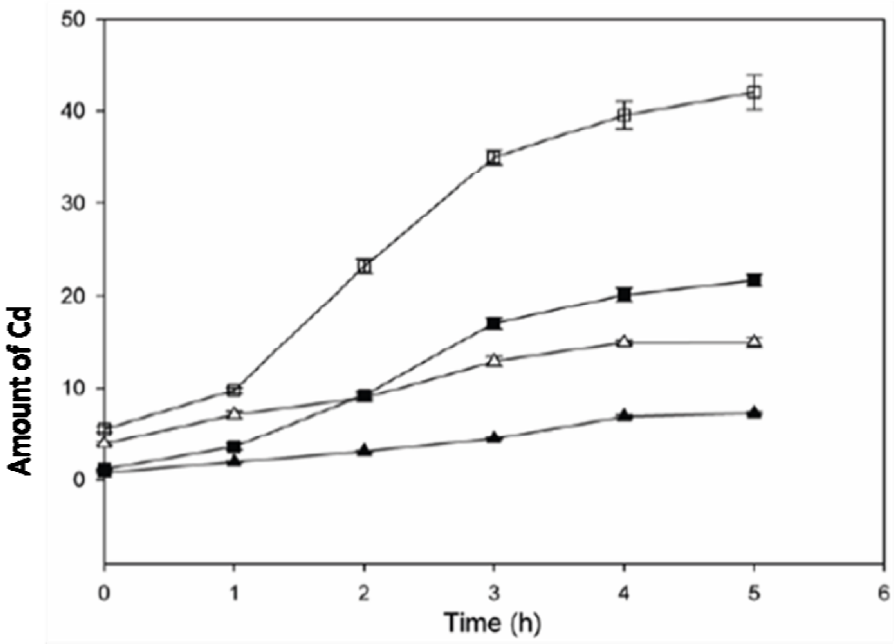


Fig.9 Lineweaver-Burk graph for uptake of Cd □ = Cd R I, ● = Cd R II

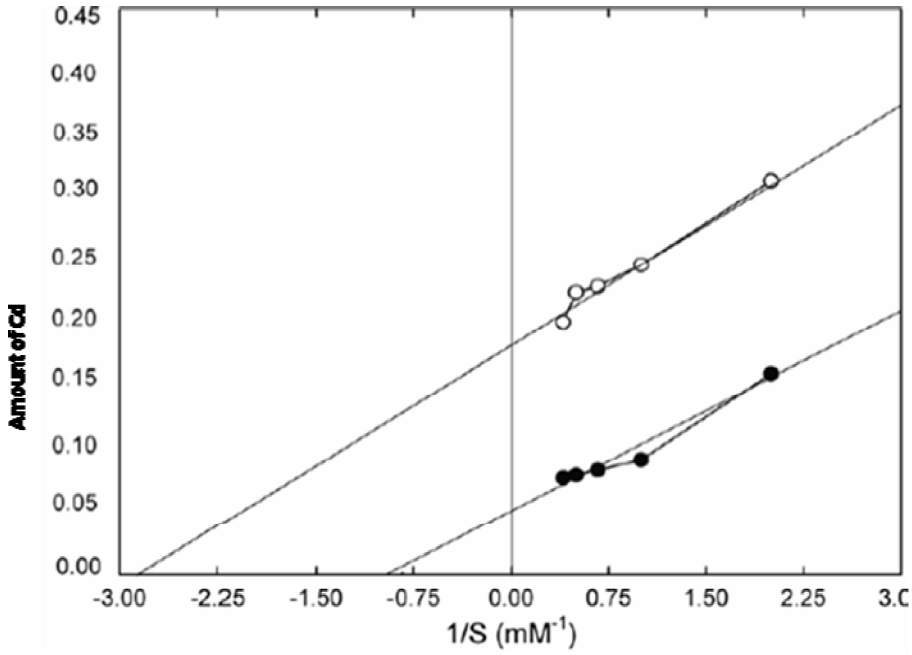


Fig.10 Cd accumulation by mutants grown in medium containing Cd(1mM) and cyclohexamide as its IC 50 value over period of 120h.Cd uptake in medium containing only Cd is the control.MM+Cd : . =Cd R I; = Cd R II+ cycloheximide; = Cd R I : = Cd R II

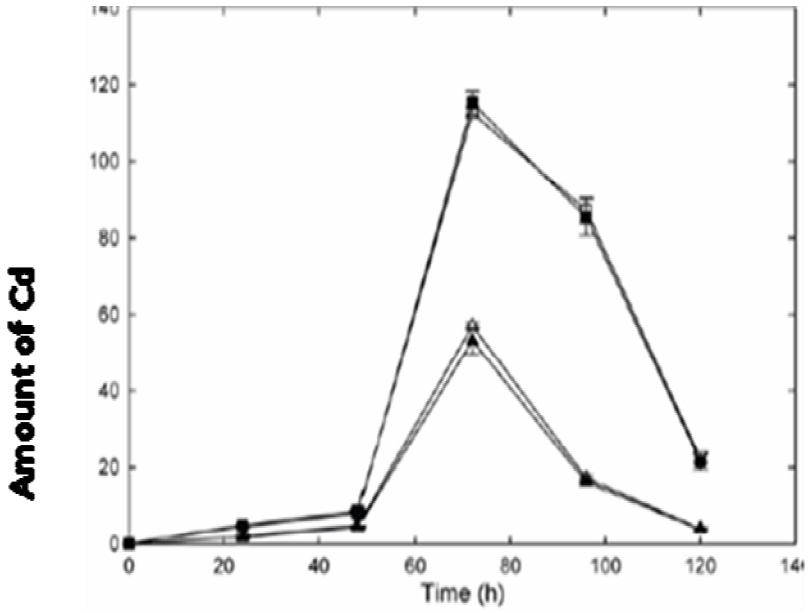
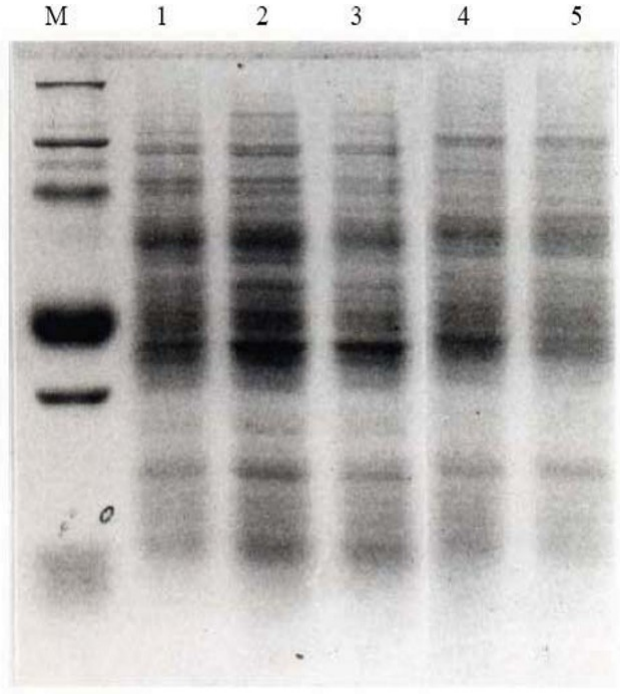


Fig.11 SDS-PAGE analysis of partially purified protein extract of CdR strains. Lanes: M - Markers, 1 - CdRII mutant pre-exposed to 0.1 mM Cd and simultaneously treated with cycloheximide, 2 - CdRII mutant pre-exposed to 0.1 mM Cd, 3 - CdRII mutant grown directly in 1mM Cd, 4 - CdRI mutant grown directly in 1mM Cd, 5 - Wild-type grown in the absence of Cd



To understand the possible involvement of protein(s) in the regulation of metal uptake, the strains were grown under appropriate conditions.

SDS-PAGE analysis of partially purified protein extract of CdR strains. Lanes: M - Markers, 1 - CdRII mutant pre-exposed to 0.1 mM Cd and simultaneously treated with cycloheximide, 2 - CdRII mutant pre-exposed to 0.1 mM Cd, 3 - CdRII mutant grown directly in 1mM Ni, 4 - CdRI mutant grown directly in 1mM Cd, 5 - Wild-type grown in the absence of Cd. Purified protein preparation from 72 h old biomass revealed that CdRII mutant after induction by 0.1 mM Cd produced a protein having MW of 125 kDa (Fig. 11). This protein could not be detected in CdS or CdRI strains.

In this study, a strain of *Aspergillus nidulans* grown in the presence of cadmium, showed growth ability in high concentrations of cadmium, being, however, the growth profile related to the concentration of the metal ion.Under un-induced condition or cycloheximide treatment during induction, the amount of this protein was reduced to several folds. Thus, 0.1 MM Cd appears to stimulate production of this protein in CdRII mutants. These results indicated some role of this high MW protein in inducing resistance against Cd in CdRII mutant. It could also be inferred that *de novo* protein synthesis was not required for Cd accumulation by the Cd R mutants of *A. nidulans* and the protein synthesised by CdRII mutant did not regulate the entry of metal, instead employed other mechanism to detoxity the effect of Cd. Additionally, the results for the efficiency of removing the metal of the means of cultivation, over cell growth, point to the potential of the isolated for studies of remediation of metals.Cu-resistance in *S. cerevisiae* can be induced by Cu to produce metallothionein, which may bind other metals also. Such metal stimulated, metal binding polypeptides have also been reported from *Candida glabrata* and several *Mucor* species(Mehra RK et al, 1991). Cd-resistant strains of *S. cerevisiae* accumulated large amounts of histidine in response to intracellular Cd indicating the role of amino acid in combating Cd-toxicity. Thus, this study while providing a procedure to develop metal resistant accumulating fungal strain has yielded efficient Cd accumulating strains of *A. nidulans*. The information generated regarding the characteristic of the strains and the nature of the Cd-resistance can be exploited to develop bio-inoculants of this fungus for bioremediation of Cd from contaminated environment.Additionally, the results for the efficiency of removing the metal of the means of cultivation, over cell growth, point to the potential of the isolated for studies of remediation of metals.

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