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REMOVAL OF CADMIUM ION BY CADMIUM RESISTANT MUTANT OF *ASPERGILLUS NIGER* FROM CADMIUM CONTAMINATED AQUA - ENVIRONMENT

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ABSTRACT

N-methyl N'-nitro N-nitroso guanidine induced cadmium resistant mutant (Cd_2) of *Aspergillus niger* was selected for the study of removal of cadmium ions from cadmium contaminated aqueous systems like sewage, river and pond water. Growth studies of the Cd_2 mutant in plane and glucose supplemented sewage, river and pond water indicated that Cd_2 mutant could grow well both in plane and glucose supplemented river and sewage water but not as growth in glucose supplemented aqueous systems. The intracellular as well as extracellular Cd^{+2} concentration of the mutant grown in cadmium contaminated river water reflected the capability of the mutant for removal of cadmium ions both by the mycelial absorption and adsorption methods from an aqueous system. The enhancement of the mutant strengthened the support for high tolerance of the mutant to cadmium ions which favors the removal of cadmium ions.

Keywords: Cadmium resistant mutant, *Aspergillus sp*, mycelial biosorption & adsorption, reduced glutathione.

INTRODUCTION

The effect of heavy metals on global pollution through mining operation, industrialization and urbanization has gradually enhanced the potential of heavy metals in ecosphere. The industries of electronics, plating, battery and ammunition manufacture excrete heavy metal like cadmium, lead and zinc as waste materials in streams and contaminate the environment. Cadmium, one of the heavy metals widely used in industry affects human heart and liver through occupational (Ossola and Tomaro, 1995) and environmental exposure and also involved in cell proliferation, differentiation, apoptosis and other cellular activities and it may be considered as carcinogen due to its direct or indirect interaction with adenine and guanine in DNA (Hossain and Huq, 2002). People around Jinstsu river were attacked by painful Itai-Itai disease in eating rice cultivated in cadmium, zinc and lead contaminated soil with polluted water of the river. Agricultural soils were primarily contaminated with Cd^{2+} due to the excessive use of phosphate fertilizers, dispersal of sewage sludge and atmospheric deposition. Cd^{2+} was readily taken up by numerous crops including cereals, potatoes, rice and fruits (Ingwersen and Streck, 2005). Consumption of rice grown in paddy soils contaminated with Cd^{2+}, Cr^{6+} or Zn^{2+} may pose a serious risk to human health, because 22–24% of the total metal content in rice biomass concentrated in the rice grains (Wang *et al*., 2003). Thus, contamination by Cd^{2+} is increasing in both human food and overall in the agricultural environment. Plants readily take up Cd^{2+} from the soil. Microorganisms are of increasing importance in biotechnological processes applied for removal of heavy metal ions like biosorption (Kapoor and Viraraghavan, 1995), intracellular absorption (Pal and Das, 2005). Bioremediation of cadmium can be partially fulfilled by producing various mutants of different microorganisms and using the same to study biochemistry and molecular genetics of cadmium toxicity as reported in *Pseudomonus fluorescens* (Rossbach *et al*., 2000), *Paxillus involtus* (Courbot *et al*., 2004) and *Aspergillus niger* (Pal and Das, 2005), unicellular algae (Yoshida *et al*., 2006). The assay of intracellular uptake of cadmium ion, study of metallothionein and cadmium sensitive enzymes of cadmium resistant mutants of *Aspergillus niger* (Cd_1 and Cd_2) were described in our earlier paper (Pal and Das, 2005). In the present study the cadmium resistant mutant of *Aspergillus niger* has been used to verify the growth of the same in plane river , sewage and pond water with or without glucose. An attempt was also taken to investigate its cadmium tolerance and uptake (both intracellular and extracellular) capacity by using sewage and river water contaminated with cadmium ion and also to assay the content of reduced glutathione in cell-free extract of the mutant.

MATERIALS AND METHODS

Source of organism and composition of growth media

Aspergillus niger culture was obtained from the Department of Botany, University of Kalyani and N-methyl N'-nitro-N-nitroso guanidine induced cadmium resistant mutant (Cd_2) as reported earlier (Pal and Das, 2005) was selected for the present study. River, pond and

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sewage water were collected from Kalyani region. Czapex-Dox (CD) as broth (Raper and Thom, 1949) was used.

Growth of mutant in river water, pond water and sewage water:

Spore suspension of Cd_2 mutant (10^{10} conidia in one liter) of *A. niger* was separately inoculated to plane pond, sewage and river water (Ganges; Kalyani, W.B. India) and also supplemented only with variable concentration of glucose as a source of carbon and allowed to incubate at 30^o C for 96h in a shaker incubator. Harvested mycelia from each sample were washed repeatedly with sterile distilled water and dried completely in a vacuum drier.. The dried mycelia of each sample were weighed.

Spore suspension of Cd_2 mutant (10^7conidia/l) was separately inoculated to plane River and sewage water containing variable concentration of $CdCl_2$ and incubated at 30^0C for 96h in shaker incubator. The harvested mycelia collected from respective water source were washed repeatedly with sterile distilled water. The excess water was removed from mycelia and was placed in drier to obtain dried mycelia and weighed.

Estimation of biosorped/ mycelial adsorbed and absorbed cadmium:

Spore suspension of Cd_2 (10^{10} conidia in one litre) mutant was inoculated separately to river water containing varying concentration of $CdCl_2$, and incubated at 30^oC for 96 h in a shaker incubator. Harvested mycelia were washed repeatedly by sterile distilled water. The excess water was removed from mycelia and preserved at -20^oC. Squeezed water also was preserved for estimation of cadmium ion concentration. The repeatedly washed mycelia were ground with alumina (1:1) for the preparation of cell free-extract. Spectrophotometric method (dithiozone method) was followed as described earlier (Pal and Das, 2005) to estimate concentration of cadmium ion present in squeezed water and also in cell free-extract.

Measurement of reduced glutathione

Spore suspensions of wild type and the test mutant (Cd_2) were inoculated to CD broth medium and CD broth with variable concentration of $CdCl_2$ at a final concentration of 10^{10} conidia per liter and allowed to incubate in a shaker at 30^0C for 48 h. The harvested mycelia were washed with sterile distilled water under aseptic conditions. The excess liquid was removed and mycelia were preserved at -20^oC for the preparation of cell-free extract. The frozen mycelia were ground with neutral alumina (1:1) and extracted with 0.05M potassium phosphate buffer (pH 7.0) and 0.001M EDTA for the assay of reduced glutathione.

GSH content was assayed according to the method of Akerboom and Sies (1981). The reaction mixture (1.0ml) contained 50 µl Cell free extract, 50µl 5,5'-Dithiobis (2-benzoic acid) (DTNB) (1.5mg/100ml), 50µl gluthione oxidoreductase solution (5units/ml). 50 µl of NADPH (3.5mg/ml)and 0.8ml potassium buffer (0.05M, P^H 7.0). The mixture was allowed to incubate for 5 to 10 minutes at room temperature. The absorbance was measured after one minute and after 5 minutes at 412 nm..

RESULTS

Reduced GSH content in cell free extract of wild type and Cd_2 mutant

The reduced GSH content in cell free extract of the wild type grown in liquid CD medium. was found to be very low but the content sharply increased when the same strain was grown in liquid CD medium containing 0.5mM $CdCl_2$. Since then the rate of increment of GSH content was found to be infinitesimally small with further increase of $CdCl_2$ up to the tolerable concentration (1.5mM) of the same to the wild type (Fig. 1). The GSH content in Cd_2 mutant was found to be increased by 47.5% as compared to that of the wild type when the cell free extract was prepared from the mycelia grown in CD medium only. Whereas the GSH content of the Cd_2 increased linearly up to the concentration of 1.5mM $CdCl_2$, after that the increment rate of GSH content in the mutant was found to be almost the same as the wild type up to the tolerable concentration of $CdCl_2$ (3.0mM) to the mutant type.

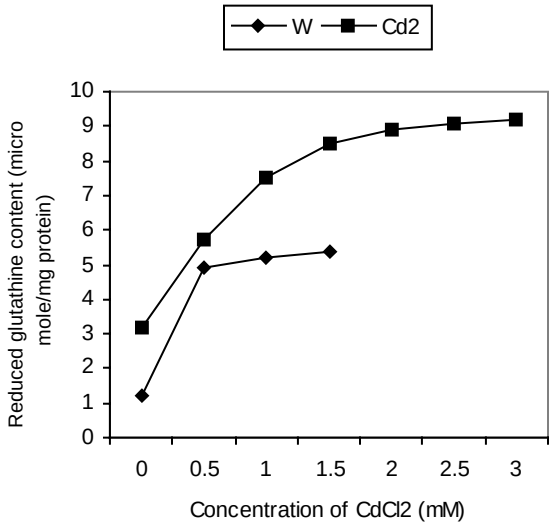

Fig. 1. Reduced Glutathione Content in cell free extract of Wildtype and Cd_2 in presence of $CdCl_2$.

Growth of Wild type and Cd_2 mutants in River's water, pond water and sewage water

Both the wild type and the Cd_2 mutant could grow in river/sewage water and solid agar medium prepared with

river/sewage water only (without addition of any nutrients exogenously) (Fig. 2a, 2b and 3a); but more growth for the both, the wild type and the Cd_2 mutant was found to be at 40 gram/ L glucose in river /sewage water without addition of any other nutrients (Fig. 2 and 3). There was no significant growth difference between the wild type and the mutant in plane pond and even in pond water supplemented with glucose only and also in solid agar medium prepared with pond water only. (Fig. 4 ,4a and 4b).

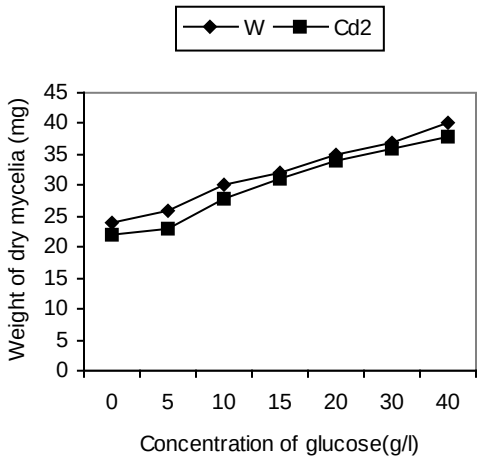

Fig. 2. Growth of Wild type and Cd_2 mutant in River water/ River water in presence of glucose only.

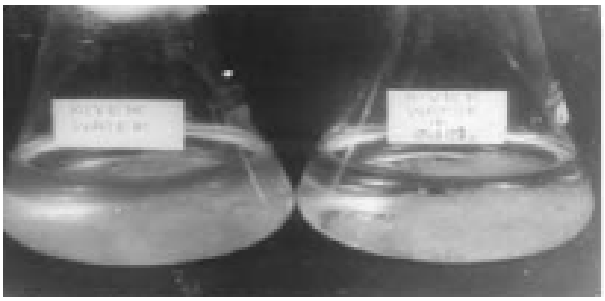

Fig. 2a. Growth of the Cd_2 mutant in plane river water and river water with supplemented with $CdCl_2$.

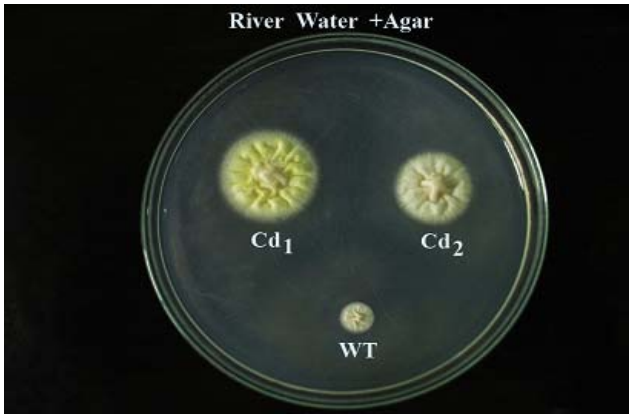

Fig. 2b. Growth of the Cd_1 and Cd_2 mutants in plane solid agar medium prepared with river water only.

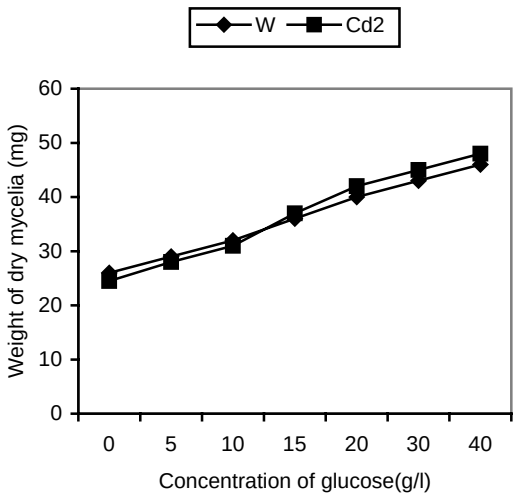

Fig. 3a. Growth of Wild type and Cd_2 mutant in sewage water / supplemented with glucose only.

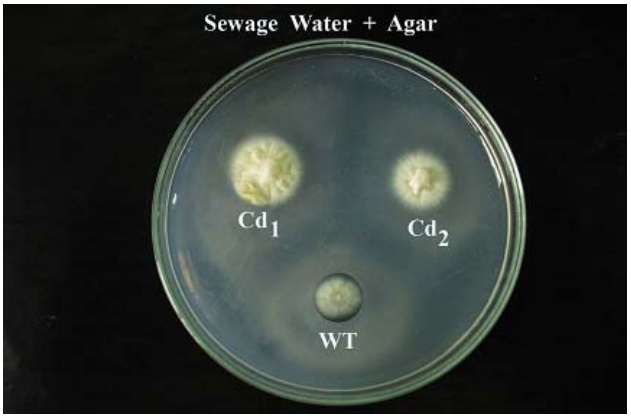

Fig. 3b. Growth of the Cd_1 and Cd_2 mutants in plane solid agar medium prepared with sewage water only.

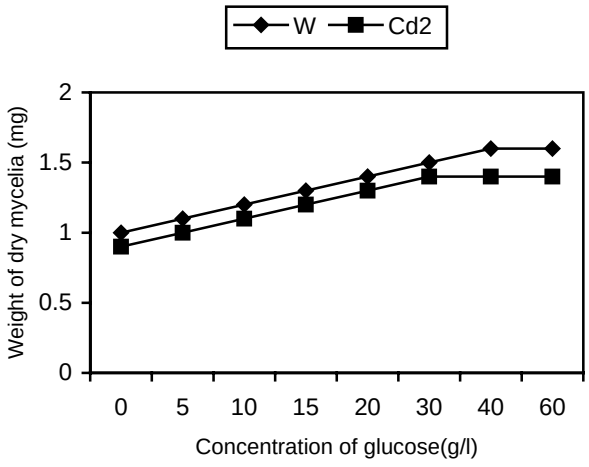

Fig. 4. Growth of Wild type and Cd_2 mutant in pond water/ supplemented with glucose only.

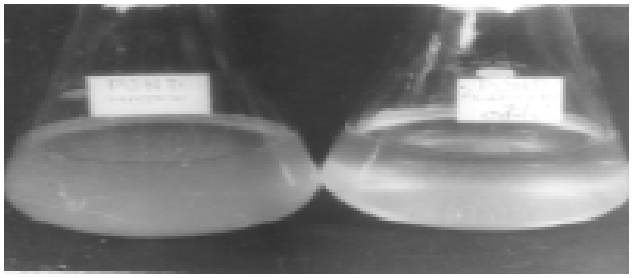

Fig. 4a: Growth of the Cd_2 mutant in plane pond water and pond water supplemented with $CdCl_2$.

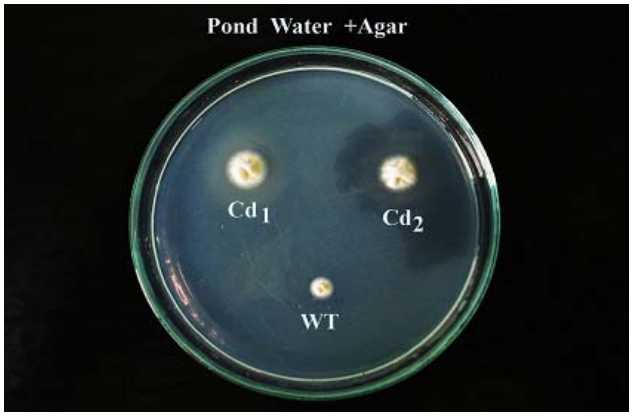

Fig. 4b. Growth of the Cd_1 and Cd_2 mutants in plane solid agar medium prepared with pond water only.

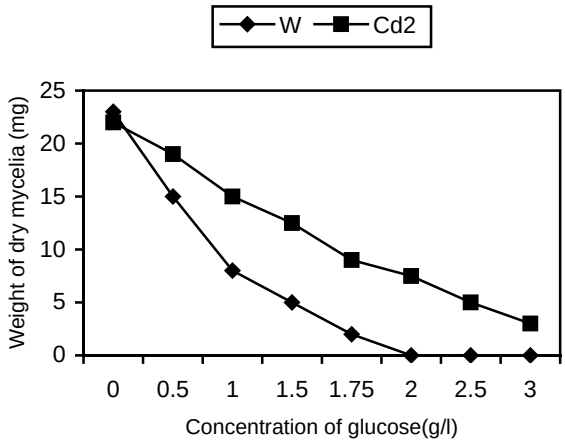

Fig. 5. Growth of Wild type and Cd_2 mutant in River water in presence of $CdCl_2$.

Growth of Wild type and Cd_2 mutant in River/ sewage water in presence of different concentration of $CdCl_2$

The Cd_2 mutant was found to grow both in River / sewage water in presence of $CdCl_2$ and also in solid agar medium prepared with river/ sewage water only (Fig. 2a, 2b, 3a, 5 and 6). Wild type produces very reduced amount of mycelia as compared to that of the mutant in presence of $CdCl_2$. But the mutant could grow even at 3.5mM $CdCl_2$; whereas the mutant showed about 10% less amount of dry mycelia with increase in $CdCl_2$ concentration from 0.5 to 1.0 mM. The wild type showed about 43 to 46% less amount of dry mycelia with the increase of $CdCl_2$ concentration from 0.5 to 1.0 mM and could not produce any mycelia at 2mM $CdCl_2$.

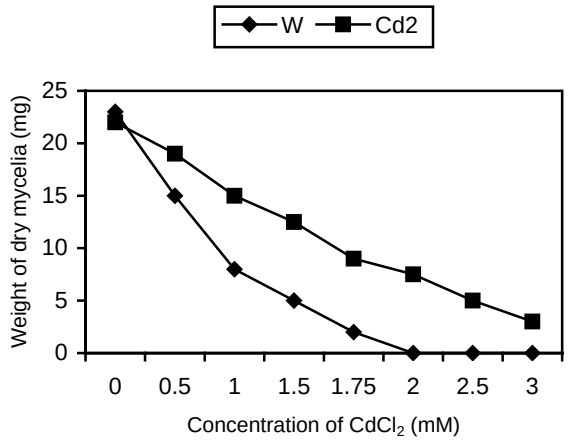

Fig. 6. Growth of Wild type and Cd_2 mutant in sewage water supplemented with $CdCl_2$.

Biosorped/ mycelial adsorbed (B.M) and absorbed (cell free extract) cadmium(A.M)

Growing mycelia / cell free extract prepared from the mycelia of the wild type absorbed Cd^{+2} maximally from river water containing 0.5mM $CdCl_2$ but the absorption capacity of the same strain declined gradually from the liquid media containing higher concentration of $CdCl_2$, whereas both of growing mycelia / cell free extract prepared from the mycelia of the Cd_2 mutant showed gradually increasing absorption capacity of Cd^{+2} till the tolerable concentration (Fig. 7).

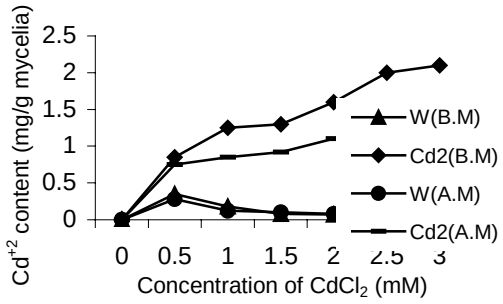

Fig. 7. Cd+2 content in biosorbed (squeezed water) and absorbed (cell free extract) mycelia of Wild type and Cd_2 mutant.

The results presented in Fig. 7 indicates that the Cd_2 mutant could biosorb extracellularly about 1.5 times more cadmium ion than that of the wild type, whereas absorbed cadmium ion prepared for cell free-extract of the Cd_2 was found to be about 1.7 times more compared with that of the wild type.

DISCUSSION

The wild type *A. niger* and its Cd_2 mutants could not grow in pond water as ponds usually accumulates water with limited micronutrients and carbon source from a specific area whereas the same strains could grow in sewage water, because the same water assimilates all nutrients from different geographical regions which are appropriate to the growth of all types of microorganisms (Bartsch and Mllum, 1997; Okun, 2002) and remarkable growth of the mutants has also been found in river water without addition of any sugar exogenously. Growth of the mutant in river water supplemented with $CdCl_2$ indicates that the mutants can tolerate $CdCl_2$ up to 3.5mM.

Investigation of growth of the mutants in sewage water indicated that, cadmium resistant mutant (Cd_2) can grow well in sewage rich in cadmium ion. It signifies that resistance mechanism of cadmium ion has been developed in the same mutant of *Aspergillus niger* as reported in *S.cerevisiae* (Joho *et al*., 1987).

The Cd_2 mutant had the capacity to take up cadmium ion from plane river water containing $CdCl_2$ only in both process of biosorption (extracellular) and absorption (intracellular) without inhibition of growth of the mutant as it showed increased metallothionein activity with partially defective cadmium-sensitive enzymes as reported earlier in the same mutant strain of *Aspergillus niger* grown in liquid CD medium containing $CdCl_2$ (Pal and Das, 2005). Similarly *Talaromyces helicus* (Romero *et al*., 2006) showed activity of biosorption of heavy metals. . The enhanced content of reduced GSH in the cell free extract of Cd_2 mutant indicated that it could bind more cadmium and allow the growth of the mutant type even at higher concentration of cadmium ion compared to the wild type as presented by Xiang and Oliver (1998) and strengthened the support for intake of cadmium ion by the Cd_2 mutant of *A. niger*.

The results presented in Figure 1 indicated that $CdCl_2$ induces the wild strain to synthesize more GSH for its survival under stress condition of cadmium ion as reported by Xiang and Oliver (1998). Since the Cd_2 mutant strain possessed high content of GSH in normal and Cd^{+2} stress condition compared to that of the wild type, the same mutant strain could tolerate higher concentration of $CdCl_2$ as reported by Dessislava *et al.* (2007).

As presented in the earlier paper the cadmium resistant mutants (Cd_1 and Cd_2) of *Aspergillus* niger (Dessislava *et al.*, 2007; Pal and Das, 2005) showing increased metallothionein activity along with partially defective cadmium sensitive enzymes and having more GSH content as presented here, have the capacity to take up Cd^{+2} intracellulary in different concentrations without inhibition of its growth, the mutant may be considered as a unique tool to be used for construction of a water purifying system by which cadmium can be removed from cadmium rich aqua-environment or grown mycelia or growing mutant cells can be used roughly in a perforated bag to be placed at the outlet of a sewage drain for remediation of cadmium ions from the waste water.

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