Biochemical characterization of N-methyl N′-nitro-N-nitrosoguanidine-induced cadmium resistant mutants of *Aspergillus niger*

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Two cadmium resistant mutants (Cd_1 and Cd_2) of *Aspergillus niger*, among the six isolated by mutagenization with N-methyl N′-nitro-N-nitrosoguanidine (MNNG) at pH 6·4 were selected for the study. Analysis of lipid composition of the mutants and the wildtype indicated that total lipid as well as individual lipids of the cadmium resistant mutants were changed as compared with that of the wildtype. The increased activities of metallothionein and reduced activities of D-xylose isomerase and L-phenylalanine ammonia lyase in cell free extract of the cadmium resistant mutants suggested that mutants could allow high concentration of cadmium salt as compared with that of the wildtype. The respiratory activity and intracellular as well as extracellular Cd^{2+} concentration of the mutants reflected the high tolerance of the Cd mutants to cadmium ion.

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1. Introduction

It is now well established that cadmium is very toxic to human and other higher organisms as well as to microorganisms. The heavy metal resistant mutants of *Saccharomycess cerevisiae* (Wu *et al* 1993), *Neurospora crassa* (Levine and Marzluf 1989) and *Candida albicans* (Riggle and Kumamoto 2000) have been used as biochemical and genetic tools for studying the biochemistry and genetics of toxicity and detoxification of the metal concerned. Cadmium resistant mutants of *N. crassa* were isolated following mutagenization with chemical mutagens and UV irradiation to study transportation of cadmium ion through cell wall membrane of the organism (Levine and Marzluf 1989). The cadmium-tolerant strains of *S. cerevisiae* (Inouhe *et al* 1989) were used for understanding the molecular genetics of cadmium toxicity and its detoxification. Cadmium-tolerant strains of *Aspergillus carbonarius*, *Penicillium* sp. (Razak 1989) and *Paxillus involutus* (Courbot *et al* 2004) could also be used for investigation of genetical mechanism of cadmium sensitivity and detoxification from cadmium-contaminated biological systems. Upon exposure to metal ions, fungi synthesizes metallothioneins (MT, class-II type) and phytochelatins (PC, class-III type of MT). Cellular resistance to heavy metal cytotoxicity in fungi is mainly mediated by binding of metal ions either to MT or PC. The present report is concerned with isolation of cadmium-resistant mutants of *A. niger*, following mutagenesis with the chemical mutagen N-methyl N′-nitro-N-nitroso-guanidine (MNNG), and biochemical characterization of the mutants.

2. Materials and methods

2.1 *Source of organism and composition of growth media*

A. niger culture was obtained from the Department of Botany, University of Kalyani. Six cadmium-resistant

Keywords. *Aspergillus niger*; cadmium; enzymes; mutants

Abbreviations used: CD, Czapek-Dox; MNNG, N-methyl N′-nitro-N-nitrosoguanidine; MT, metallothioneins; PC, phytochelating; PMSF, phenyl methyl sulphonyl fluoride; TTC, 2-3-5-triphenyl tetrazolium chloride.

mutants were isolated of which Cd_1 and Cd_2 were selected for further studies as it tolerated maximum concentration of cadmium ions. Czapek-Dox (CD) as broth (Raper and Thom 1949) and as solidified media (CDA) were used. The pH of the medium was adjusted to 6·4 before autoclaving. Cadmium-resistant mutants after isolation were maintained on CDA, and grown on CD or CD containing 1·2 g/l of cadmium chloride.

2.2 *Mutagenesis with MNNG and isolation of cadmium-resistant mutants*

Spores from 8-day-old culture of *A. niger* were suspended in Tris-maleate-buffer (0·1 mol/l: pH 6·4) at a concentration of 10^6 conidia/l, with Tween 80 (0·1 g/l) as surfactant. Aliquots of spore suspension were mixed separately with different concentration of MNNG and shaken in a flat shaker in the dark for 35 min at 30°C. The mutagenized spores were washed thrice with sterile distilled water. Diluted spore suspension was plated on CDA containing 1·2 g/l of $CdCl_2$. The plates were incubated for 4 days at 30°C. Colonies formed on the medium were considered as cadmium-resistant mutants. The mutants were further purified by single colony isolation technique. Mutant character was further verified by growing on CDA containing 1·2 g/l of $CdCl_2$ for a second time.

2.3 *Analysis of mycelial lipid composition*

Lyophilized mycelia of the Cd mutants (Cd_1 and Cd_2) and the wildtype were powdered separately and the lipid was extracted successively, using the solvent systems as shown below.

Solvent system	Ratio
$CHCl_3$: CH_3OH	2 : 1
$CHCl_3$: CH_3OH	1 : 2
$CHCl_3$: CH_3OH : H_2O	1 : 2 : 0·8

Total extract was washed with it's half volume of chloroform: water (1 : 1 v/v). The separated organic phase was dried over anhydrous Na_2SO_4 and finally evaporated *in vacuo* at room temperature and weighed. Thin-layer chromatography (TLC) was used to separate the polar and neutral lipids of the individual strain using acetone as a developing solvent. In this process polar lipids were at the origin of TLC plate and neutral lipids along with the pigment moved almost to the solvent front. Individual lipids were identified by development with iodine vapour. Neutral lipids and pigments were scrapped off and extracted with $CHCl_3$. Finally extracted neutral lipids were evaporated *in vacuo* and weighed. Polar lipids were also scrapped off from the base line with the solvent mixture, chloroform : methanol : water (69 : 25 : 4 v/v/v), evaporated to dryness and weighed.

Total phospholipids in the total lipids were measured according to the method of Ames and Dubin (1960). A known amount of total lipid was heated with 0·15 ml of 10% $Mg(NO_3)_2$ solution in ethanol in a pyrex test tube until the brown fumes disappeared. This step was continued for further 2 min. The tubes were allowed to cool down to room temperature, and 1·5 ml of 0·5 (N) HCl was added and boiled for 30 min at 80°C. After cooling, 3·5 ml of reagent mixture containing ammonium molybdate and freshly prepared ascorbic acid (10%, 6 : 1 v/v) was added and incubated at 45°C for 30 min. The absorbance was measured at 420 nm against appropriate blank. An aliquot of total lipid (10–15 mg) was dissolved in chloroform and mixed with 3 ml of $FeCl_3$–H_2SO_4 colour reagent. The reaction mixture was then incubated in ice-bath for 10 min and absorbance at 540 nm was recorded using the appropriate blank to estimate the amount of total sterol.

2.4 *Estimation of cadmium in intact mycelia and cell-free-extract*

Spore suspension of Cd_I and Cd_2 (10^{11} conidia in one litre) mutants were separately inoculated to liquid CD medium containing variable concentration of $CdCl_2$, and incubated at 30°C for 96 h in a shaker incubator. Harvested mycelium was washed repeatedly by sterile distilled water. The excess water was removed from mycelium and it was preserved at – 20°C. Both mycelia and squeezed water were preserved for estimation of concentration of cadmium ion at the respective phases. This repeatedly washed mycelium was ground with alumina (1 : 1) for the preparation of cell free-extract. Spectrophotometric method (dithizone method) was used to estimate concentration of Cd^{2+} ion. Cd^{2+} reacts with dithizone under optimum conditions to form a red colour, which can be extracted with $CHCl_3$. The volume of 5 ml of the extract was made up to 25 ml with double distilled water, and pH was adjusted to 2·8 with Thymol blue indicator. One ml of aqueous sodium potassium-tartarate solution (250 g/l), 5 ml of NaOH–KCN solution (40% NaOH + 1% KCN in 1 litre distilled water), 1 ml NH_2OH, HCl (20%) and 15 ml dithizone (0·01% in $CHCl_3$) were mixed-well and shaken for 1 min. The $CHCl_3$ layer was taken in 25 ml cold tartaric acid and the residue layer was further extracted with 10 ml of chloroform. A mixture of 0·025 ml NH_2OH, 15 ml HCl, dithizone solution and 5 ml of NaOH–KCN solution was added into chloroform extract and it was shaken for 1 min; the $CHCl_3$ layer was further extracted. The absorbance of the chloroform extract was measured at 518 nm against the reagent blank.

2.5 *2,3,5-Triphenyl tetrazolium chloride experiment*

Wildtype, *A. niger* and its cadmium-resistant mutants (Cd_1 and Cd_2) were point inoculated in the solid CD medium and allowed to grow at 30°C for 96 h. The molten agar (1%) containing 1·5% TTC was overlaid on CD plate and incubated at 30°C in the dark until turned pink.

2.6 *Measurement of rate of respiration*

The uptake of oxygen was measured polarographically with Clark-type oxygen electrode at 30°C with oxygen monitor (YSI Model No. 53) with the reaction mixture containing glucose (0·08 mol/l), and $MgCl_2$ (0·005 mol/l) with intact mycelia (0·005 g) of the wildtype and it's Cd-resistant mutants (Cd_1 and Cd_2).

2.7 *Assay of metallothionein protein and cadmium sensitive enzymes*

A spore suspension of wildtype and the test mutants were inoculated to CD broth medium and CD broth with 3·5 mM of Cd^{2+} respectively at a final concentration of 10^{11} spores per litre and allowed to incubate in a shaker at 30°C for 48 h. The harvested mycelium was washed with sterile distilled water under aseptic conditions. The excess liquid was removed and preserved at – 20°C for the preparation of cell-free extract. The frozen mycelium was ground with neutral alumina (1 : 1) and extracted with the appropriate buffers particular for the assay of the enzymes and protein examined in this study. After thorough homogenization, cell debris was removed by centrifugation at 13000 *g* for 20 min. The assay was done within 2 to 3 h. All operations were carried out at 0–4°C.

Metallothionein was assayed according to the method of Viarengo *et al* (1997). In the preparation of sample, mycelium of both wildtype and mutants were homogenized separately in a mixture of 0·5 mol/l sucrose, 0·02 mol/l Tris-HCl buffer (pH 8·6) containing 6×10^{-6} mol/l leupeptine, 5×10^{-3} mol/l phenyl methyl sulphonyl fluoride (PMSF) as antiproteolytic agent and 0·01% *b*-mercaptoethanol as reducing agent. The homogenate was then centrifuged at 30000 *g* for 20 min to obtain the supernatant containing metallothionein. The collected supernatant was then treated with ethanol-chloroform as described by Kimura *et al* (1979). Cold (– 20°C) absolute ethanol (1·05 ml) and 80 µl of chloroform were added to aliquot of 1 ml of supernatant; the samples were then centrifuged at 6000 *g* for 10 min at 0–4°C. The collected supernatant was combined with 1 mg RNA and 40 µl 37% HCl and subsequently with three volume of cold ethanol. The sample was maintained at – 20°C for 1 h and centrifuged in a swinging rotor at 6000 *g* for 10 min. The pellets containing metallothionein was then washed with 87% ethanol and 1% chloroforms in homogenizing buffer (mentioned earlier), centrifuged at 6000 *g* for 10 min and dried under nitrogen stream. The pellets were re-suspended in 150 µl NaCl (0·25 mol/l) and subsequently 150 µl HCl (1 N) containing EDTA (0·004 mol/l) and 4·2 ml NaCl (2 mol/l) containing 5,5-dithiobis-2-nitrobenzoic acid (DTNB) ($4{\cdot}3 \times 10^{-4}$ mol/l) buffered with 0·2 mol/l Na-phosphate (pH 8) were added. The mixture was finally centrifuged at 3000 *g* for 5 min. The absorbance of supernatant was measured spectrophotometrically at 412 nm. The metallothionein concentration was estimated considering reduced glutathione (GSH) as reference standard.

D-xylose isomerase was assayed according to the method of Yamanaka (1966). The reaction mixture (1·0 ml) contained 0·5 ml maleate buffer (0·05 mol/l), (pH 6·6); 0·05 ml of $MnSO_4$ (0·01 mol/l) and 0·1 ml of cell-free-extract. After equilibration for 5 min at 35°C, 0·05 ml of D-xylose (0·1 mol/l) was added. The mixture was then incubated at 35°C for 10 min. The reaction was stopped by adding 0·05 ml of 50% trichloro-acetic acid. Heat-inactivated enzyme blank was used for every determination. The cystine-carbazole reaction was carried out at 35°C for 20 min and the absorbance was measured spectrophotometrically at 480 nm.

L-phenylalanine ammonia lyase was assayed according to the method of Subba Rao and Towers (1971). One ml of 0·05 mol/l Tris-HCl buffer, 0·5 ml of 0·01 M mol/l L-phenyl alanine, 0·1 ml of cell-free-extract and double distilled water were mixed to make the volume up to 2 ml and incubated at 30°C for 1 h. The reaction was stopped by adding 0·5 ml of 1 (N) HCl and the cinnamic acid was extracted with peroxide-free ether. One ml aliquot was removed from organic layer and dried under a jet of air. The residue was dissolved in 3 ml of 0·05 (N) NaOH solution and the absorbance was recorded spectrophotometrically at 268 nm. Protein in the cell-free extract was measured according to the method of Lowry *et al* (1951).

3. Results

3.1 *Mutagenesis with MNNG and isolation of cadmium-resistant mutants*

The applied concentration of MNNG for the isolation of cadmium-resistant mutants of *A. niger* is shown in figure 1. It may be noted that the effective range of the concentration was 200 mg/l to 600 mg/l in Tris-maleate buffer at pH 6·4 with the survival percentage of 29 to 15 at 30°C for 35 m in a shaking incubator and the highest frequency of cadmium resistant mutants was obtained at 400 mg/l of MNNG.

3.2 *Growth characteristic of wildtype and the mutants*

The growth characteristics of the Cd_1 and Cd_2 mutants and the wildtype are shown in figures 2–4. Good growth of Cd_1 and Cd_2 mutants was found in the solid CD medium containing 6 mM cadmium chloride after 48 h of incubation at 30°C, whereas growth of the wildtype was found to be negligible even after 6 days incubation under the same conditions. Studies on the growth of cadmium-resistant mutants (Cd_1 and Cd_2) and the wildtype culture indicated that 4 mM and 10 mM of $CdCl_2$ were sufficient for growth inhibition of the cadmium-resistant mutants in CD broth and CDA media respectively, whereas 2·5 mM of $CdCl_2$ in broth medium and 5 mM in CDA medium caused growth inhibition of the wildtype.

3.3 *Lipid analysis of wildtype and the mutants*

Figure 5 shows the mycelial lipid composition of the cadmium resistant mutants (Cd_1 and Cd_2) and the wildtype. It is found that the total mycelial lipid of Cd_1 and Cd_2 mutants were about 50% and 48% respectively less than that of the wildtype. The total phospholipid of Cd_1 and Cd_2 mutants was about 25% and 10% less; sterols were 40% and 60% less compared with the wildtype; whereas the neutral lipid of the test mutants were respectively 25% and 35% higher than of the wildtype.

3.4 *Measurement of rate of respiration*

Table 1 shows the respiratory activities of the cadmium-resistant mutants and the wildtype. It may be noted that the respiratory activity of mutant was more or less same that of the wildtype in the presence of glucose, but the activity of Cd_2 mutant was about 12% less than of the wildtype. In the absence of glucose, Cd_1 and Cd_2 mutants showed 25% less and about 61% higher respiratory activity.

3.5 *Measurement of Cd^{2+} ions*

Table 2 shows the measurement of concentration of Cd^{2+} in intact mycelia and cell free extracts of the Cd mutants. It may be noted that the intact mycelia of both Cd_1 and Cd_2 mutants could adsorb almost same amount of Cd^{2+} extracellularly, whereas the cell free extract of the Cd_1 mutant prepared from washed mycelia contained 66·6% more Cd^{2+} than that of the Cd_2 mutant. The results mentioned in table 2 indicated that both Cd_1 and Cd_2 mutants could take Cd^{2+} intracellularly in different concentrations without the inhibition of growth of the Cd mutants.

3.6 *Assay of metallothionein protein and cadmium-sensitive enzymes*

The results of the assay of metallothionein protein and cadmium sensitive enzymes in cell free extract of Cd_1, Cd_2 and the wildtype are shown in table 3. It may be noted that both the Cd_1 and Cd_2 mutants possessed about 40% and 25% higher metallothionein activity in comparison to that of the wildtype. The assay of cadmium-sensitive enzymes, D-xylose isomerase and L-phenylalanine ammonia-lyase in cell free extracts showed that both Cd_1 and Cd_2 mutants possessed 50% and 30% less D-xylose

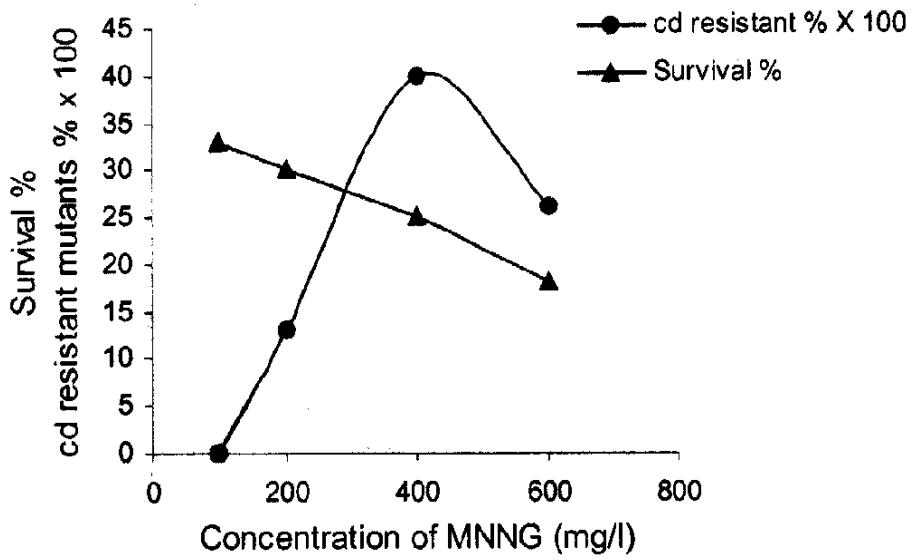

Figure 1. Effect of MNNG on the wildtype for isolation of cd resistant mutants.

isomerase activity, respectively, but Cd_1 showed about 30% less activity of L-phenylalanine ammonia lyase and Cd_2 mutant retained the normal level of the same as compared with the wildtype culture.

4. Discussion

The most effective pH is found to be 6·4 for mutagenesis of *Aspergillus* sp. In isolation of other mutants, same mutagen was used to suspend the same in a buffer of acidic pH; but mutational condition of other physical parameters are found to be different for different microorganisms (Wunderlich *et al* 1995; Dutta and Das 2004); but failure repeatedly in studies on the mutation with same chemical mutagen at alkaline pH to get the same type of mutant in *A. niger* predicted that the alkaline pH was not suitable for the same, which was similar as reported in *Escherchia coli* (Adelberg *et al* 1965), *A. nidulans* (Armitt *et al* 1975) and *N. crassa* (Catheside 1954) but differed the physical conditions including concentration of MNNG for genetic mutation of this strain indicated that the effective concentration of MNNG as well as physical parameters for mutagenic action depends

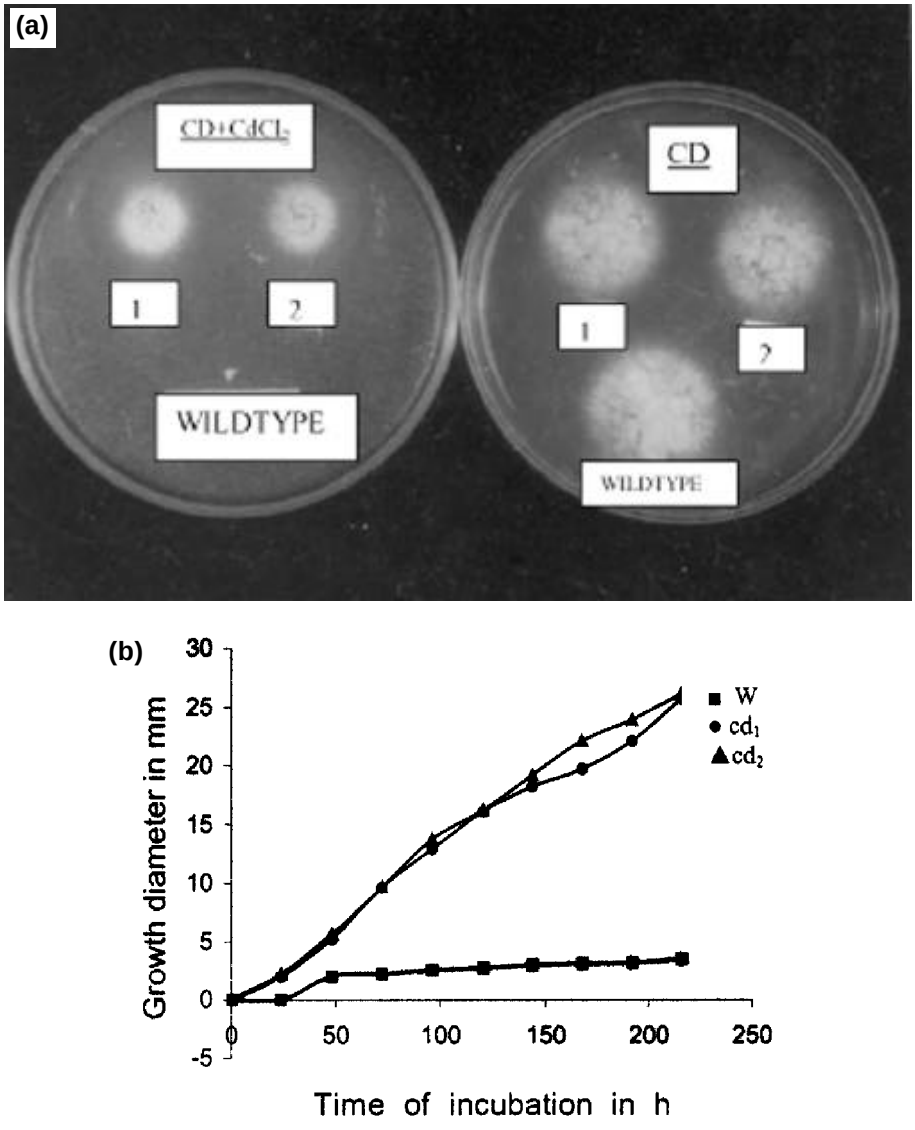

Figure 2. (**a**) Growth of *A. niger* (wildtype), Cd_1 (1) and Cd_2 (2) mutants on solid CD (CDA) medium containing 1·2 g/l $CdCl_2$. (**b)** Growth curves of *A. niger* (w, wildtype), Cd_1 and Cd_2 mutants on solid CD (CDA) medium containing 1·2 g/l $CdCl_2$ with variable time.

on the micro-organisms and their physiological state (Wunderlich *et al* 1995). *A. niger* has been used since it is an lower eukaryote and can be considered as a bridge between lower and higher organisms. Cadmium-resistant mutants of the same may provide with a suitable tool for studies on molecular biology, biochemistry and genetics of cadmium toxicity and it's detoxification. Moreover, the mutants may accumulate a variety of heavy metal pollutants and therefore may be used in the removal of heavy metal ions from the heavy metal contaminated environments (Mackay *et al* 1993).

The results presented in the figures 3 and 4 signified that Cd_1 and Cd_2 mutants tolerated higher concentration of $CdCl_2$ (almost double) in comparison to that of the wildtype; as there may be either alteration of membrane permeability, or change in activities of any of the biochemical events related to Cd-sensitivity of the Cd-mutants. These findings and the results of lipid assay (figure 5) suggested that there was a change of permeability in mycelial membrane in order to prevent entry of Cd^{2+} through membrane of the cadmium-resistant mutants as reported in the respiratory-deficient mutants of *A. niger* (Mandal *et al* 1978). The measurement of rate of respiration in the Cd mutants and the changed colour (to purple) of 2,3,5-triphenyl tetrazolium choride (TTC) signified that both the Cd mutants were almost respiratory-competent as the wildtype, and that the cadmium-resistant mutants of *A. niger* might allow TTC to pass through the mycelial membrane as the wildtype did and TTC entered intracellulary both in Cd mutants and the wildtype to interact with normal flavoprotein for changing the colour as pink. The isolated Cd mutants of *A. niger* might not change the permeability criteria as the Cd mutants of *N. crassa* could not allow to uptake of cadmium ion due to change of cell wall or plasma membrane permeability as reported by Levine and Marzluf (1989).

Both Cd_1 and Cd_2 mutants were able to takeup Cd^{2+} intracellularly in different concentrations without inhibition of growth of the Cd mutants – the metallothionein, a low molecular weight cysteine-rich metal-binding pro-

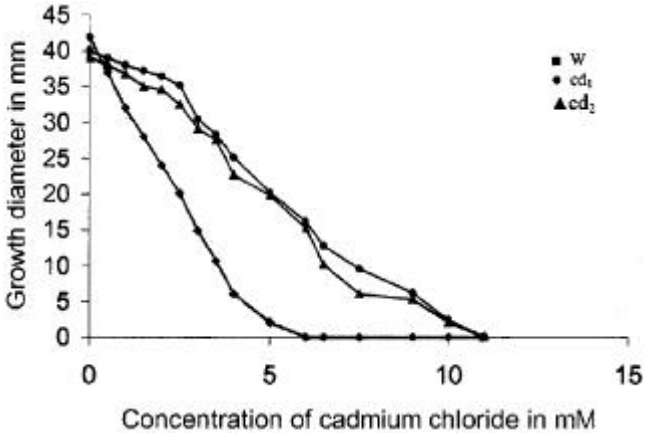

Figure 3. Growth curves of *A. niger* (w, wildtype), Cd_1 and Cd_2 mutants on solid CD (CDA) medium in the presence of different concentration of $CdCl_2$.

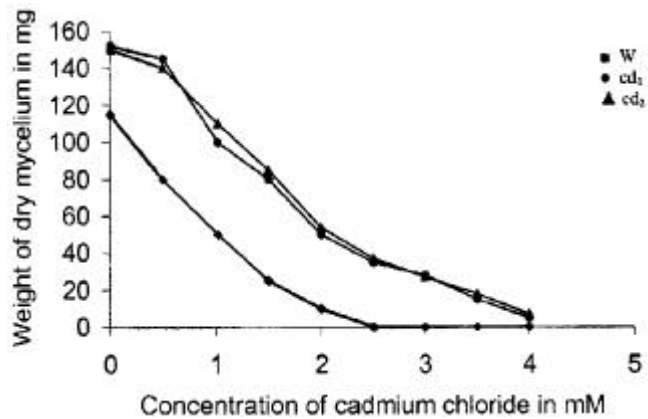

Figure 4. Growth curves of *A. niger* (w, wildtype), Cd_1 and Cd_2 mutants on liquid CD (CD broth) medium in the presence of different concentration of $CdCl_2$.

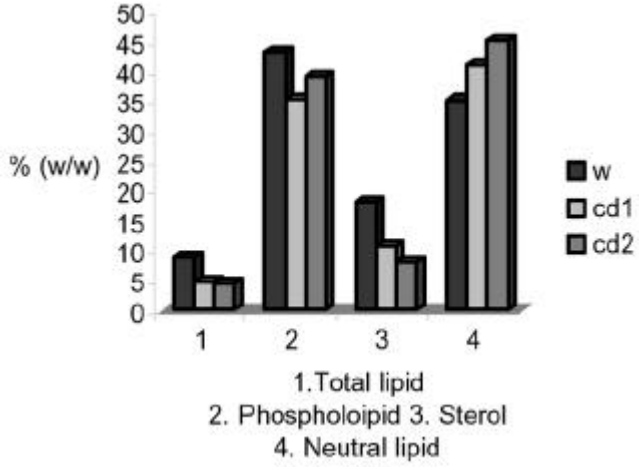

Figure 5. Mycelial lipid composition of *A. niger* (w, wildtype), Cd_1 and Cd_2 mutants.

Table 1. Respiratory activities of *A. niger*, wildtype and its mutant type, Cd_1 and Cd_2.

	Respiratory activity[a]	
Strain	With glucose	Without glucose
Wildtype	31·2	12·8
Cd_1	30·5	9·6
Cd_2	27·3	20·7

[a] μl oxygen consumed h^{-1} mg^{-1} dry mycelia.

Table 2. Estimation of Cd^{2+} content in intact mycelia and cell free extract of Cd_1 and Cd_2 mutants of *A. niger*.

Concentration of $CdCl_2$ in CD broth (mM)	Cd^{2+} content (mg/g of mycelia)					
	Intact mycelia			Cell free extract		
	Wildtype	Cd_1	Cd_2	Wildtype	Cd_1	Cd_2
0·5	0·4	0·8	0·9	0·3	0·7	0·5
1·0	0·2	1·1	1·0	0·1	0·8	0·5
1·5	0·1	1·3	1·0	0·1	0·9	0·6
2·0	(–)	1·5	1·6	(–)	1·0	0·6
2·5	(–)	1·8	1·6	(–)	1·2	0·8
3·0	(–)	1·9	1·7	(–)	1·2	0·8

Table 3. Assay of cadmium sensitive/binding enzymes/protein in cell free extract of wildtype *A. niger* and cadmium resistant mutants (Cd_1 and Cd_2).

Strain	Specific activities enzymes/protein in cell free extract		
	Metallothionein[a]	D-Xylose isomerasec[b]	L-Phenylalanine ammonia lyase[c]
Wildtype	$20{\cdot}625 \times 10^{-5}$	0·0020	0·0555
Cd_1	$29{\cdot}700 \times 10^{-5}$	0·0010	0·0377
Cd_2	$25{\cdot}850 \times 10^{-5}$	0·0014	0·0555

[a]μmol metallothionein mg^{-1} protein.
[b]OD h^{-1} mg^{-1} protein.
[c]μmol cinnamic acid formed h^{-1} mg^{-1} protein.

tein, can bind heavy metal ions, such as Cu, Zn, Cd and Hg (Viarengo 1989), and acumulation of heavy metal ions by this protein up to a certain amount does not affect the growth of living cell. The enhanced metallothionein activity in the cell free extract of Cd mutants (Cd_1 and Cd_2) indicated that it could bind more cadmium and allow the growth of the mutants even at higher concentration of cadmium ion compared to the wildtype. In fact, the increase in heavy metal concentration in the cell stimulates the *de novo*-synthesis of apothioneins that can bind metal cation in nontoxic form resulting in the reduction of deleterious effect. Tohoyama *et al* (1985) and Roesijadi (1992) reported that a Cd mutant of *S. cerevisiae* produced multiple copies of *CUP1* gene encoding metallothionein, whereas the wildtype produced only one. Moreover, cadmium-resistant mutants (Cd_1 and Cd_2) could tolerate the high concentration of Cu^{2+} to some extent, but not as the amount of Cd^{2+}; it could not tolerate any one of the heavy metal ions either Co^{2+} or Ni^{2+}. These results signified that the partially defective cadmium-sensitive enzymes as well as high level of metallothionein of the Cd_1 and Cd_2 mutants could make the mutants cadmium-resistant in *A. niger* as some of the main mechanisms involved in metal detoxification with the chelation of metal ions in the cytosol with thiol containing compounds, such as phytochelatin or metallothionein (Courbot *et al* 2004). These mutants could tolerate higher concentration of Cd^{2+} without hampering the growth of the same. The novelty of these Cd mutants of *A. niger* are to show increased metallothionein activity along with partially defective cadmium-sensitive enzymes, D xylose isomerase and L-phenylalanine ammonia lyase. Hence, this mutant type may be a unique model of microbial strain by which cadmium ion can be removed from cadmium rich polluted aqua-environment.

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