AN INVESTIGATION ON THE EFFECT OF SOME HEAVY METALS ON SOIL MYCOFLORA OF URBAN ENVIRONMENT

THESIS SUBMITTED TO UNIVERSITY OF KALYANI FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PH.D) IN SCIENCE

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TO WHOM IT MAY CONCERN

I have the pleasure to certify that Shri Kaoushik K Mukherjee, has completed his Ph.D. work entitled "**AN INVESTIGATION ON THE EFFECT OF SOME HEAVY METALS ON SOIL MYCOFLORA OF URBAN ENVIRONMENT**", under my supervision. Shri Mukherjee has completed his work through repeated laboratory analysis in the Department of Environmental Science, University of Kalyani, Nadia, West Bengal. This work is original and has not been submitted earlier for any degree or award.

(Prof. S. C. Santra)

Supervisor

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(Kaoushik K Mukherjee)

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INTRODUCTION

Fungi are ubiquitous in natural environments and important in industrial processes. A range of morphologies are found from unicellular yeasts to polymorphic and filamentous fungi, many of which have complex macroscopic fruiting bodies. Their most important roles are as decomposers of organic materials, with concomitant nutrient cycling, as pathogens and symbionts of animals and plants and as spoilage organisms of natural and synthetic materials like wood, paint, leather, food and fabrics.

The fungi are utilized as producers of economically important substances, e.g. ethanol, citric acid, antibiotics, polysaccharides, enzymes and vitamins. The study of the interaction between toxic metals and fungi has long been of scientific interest. This is because metal toxicity has been, and remains the basis of many fungicidal preparations used in the control of plant pathogens and to preserve natural and synthetic materials (**Horsfall, 1956; Ross, 1975**). Early work often had a phytopathological perspective relating to the assessment of toxicity (**Somers, 1961**). Subsequent observations on the ability of fungi to resist and adapt to toxic metals led to further work on the physiological, bio- chemical and genetical explanations for these phenomena (**Ashida, 1965; Ross, 1975; Gadd 1986a**). Research has been stimulated particularly by the continuous refinement in biochemical and molecular techniques and by the general appreciation of filamentous fungi and yeasts as model eukaryotic cells systems. In an environmental context, accelerating pollution of the natural environment by toxic metals, metalloids, radionuclides and organo-metal(loid)s have also led to increased interest because of the ubiquitous and sometimes dominant presence of fungi in metal-polluted habitats, their uptake and translocation of toxic metals and radionuclides to fruit bodies of edible fungi and the significance of mycorrhizal fungi in polluted habitats (**Gadd, 1986a; Brown & Hall, 1990**). While amelioration of metal phytotoxicity by mycorrhizal fungi is relevant to land reclamation (**Colpaert & van Assche, 1987**), another area of applied interest is the use of fungal (and other microbial) biomass for the detoxification of metal/radionuclide-containing industrial effluents (**Gadd, 1986b, 1990a, 1992a**, **2010**).

Physico-chemical, biochemical and genetical aspects of cellular interactions are included mainly with reference to 'microfungi' and yeasts, though mention is also made for mycorrhizas and macrofungi in polluted habitats. Biotechnological discussion relates only to the use of fungal biomass and products for environmental protection. Several important topics such as the roles of essential metal ions in fungal metabolism and differentiation **(Jones & Gadd, 1990**), yeast flocculation (**Kuriyama, et al., 1991**), iron acquisition and function (**Winkelmann, et al., 1987; Winkelmann, 1992**), interactions of metals with lichens (**Nieboer, et al., 1978; Nash, 1990**) and (organo) metal-containing fungicides and their effects (**Cooney & Wuertz, 1989**) are not covered in detail.

1.2 Essentiality of metals

Metals are directly and/or indirectly involved in all aspects of fungal growth, metabolism and differentiation. While many metals are essential, e.g. K, Na, Mg, Ca, Mn, Fe, Cu, Zn, Co and Ni, many others have no apparent essential function, e.g. Rb, Cs, Al, Cd, Ag, Au, Hg and Pb. However, all these elements can interact with fungal cells and can be accumulated by physico-chemical mechanisms and transport systems of varying specificity (**Gadd, 1988**). Most essential and inessential metals exhibit toxicity above a certain concentration, which vary depending on the organism, the physico- chemical properties of the metal and environmental factors. This may necessitate expression of a detoxification mechanism if the organism is to survive (**Fig. 1**). Although the connotations of toxicity and bioaccumulation are most often linked with inessential metals, the potential toxicity of many essential metals should not be overlooked. A good example is provided by calcium, an important intracellular second messenger in fungal growth and differentiation, which can also be highly toxic within cells owing to precipitation of phosphates. Fungi, like other eukaryotic cells, maintain cytosolic free Ca^{2+} at around 0 1 µM by means of a variety of transport systems located on the plasma and vacuolar membranes which effect influx/efflux and vacuolar compartmentation; cytosolic free Ca^{2+} may also be modulated by interaction with calcium-binding proteins (**Miller, et al., 1990; Gadd & Brunton, 1992**). Many attempts have been made to define metals in relation to biological effects using their physical, chemical and biological properties. Metals and metalloids can be considered to include all the elements except the noble gases and H, B, C, N, 0.

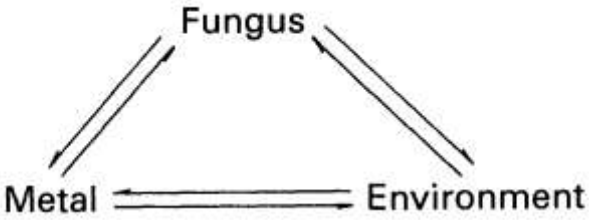

Fig.1.1 Diagrammatic representation of metal-fungal interactions.

The metals are broadly classified as type A, type B and transition metal. The details are given in Table - 1.

Table 1.1 Classification of metal ions

Type-A metal cations	Transition metal cations	Type-B metal cations
Electron configuration of inert gas	1–9 outer shell electrons	Electron number corresponds to Ni^0, Pd^0 and Pt^0 (10 or 12 outer shell electrons)
	Not spherically symmetric	Low electronegativity
Low polarizability		High polarizability
'Hard spheres'		'Soft spheres'
(H^+), Li^+, Na^+, K^+, Be^{2+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Al^{3+}, Sc^{3+}, La^{3+}, Si^{4+}, Ti^{4+}, Zr^{4+}, Th^{4+}	V^{2+}, Cr^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Ti^{3+}, V^{3+}, Cr^{3+}, Mn^{3+}, Fe^{3+}, Co^{3+}	Cu^+, Ag^+, Au^+, Ga^+, Zn^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, Sn^{2+}, Tl^{3+}, Au^{3+}, In^{3+}, Bi^{3+}

Borderline metal ions do not show a clear preference for the above metal binding donor atoms and ligands, affinities which depend on a variety of factors. While 'type-A' metal ions are sharply separated from borderline metals, the distinction between 'type-B' and borderline categories is not so clear. Among borderline metal ions, 'type-B' character increases in the order:

$Mn^{2+} < Zn^{2+} < Ni^{2+} < Fe^{2+}\ Co^{2+} < Cd^{2+} < Cu^{2+} < Pb^{2+}$

Metal ions compete with H^+ for sites on ligands and H^+ may, in fact, be regarded as a borderline ion (**Nieboer & Richardson, 1980**). An increase in acidity will therefore result in protonation of the ligand anion and an increase in the concentration of the free metal ion (**Hughes & Poole, 1991**). The above classifications of metals may be useful in defining or predicting certain interactions with organisms, as well as in assessing metal speciation in environmental samples or growth media (**Hughes & Poole, 1991**). Other definitions may be based on toxicity and environmental impact although this can be highly variable and dependent on, e.g. the organism, element, speciation, concentration, physico-chemical factors and anthropogenic activities

(**Babich & Stotzky, 1980; Gadd, 1990b**). In fungi, metal effects can vary between organisms, strains, the stage of growth and even different vegetative and reproductive forms of the same organism (**Gadd & Mowll, 1985; Sabie & Gadd, 1990**). A given organism may exhibit several mechanisms by which potentially toxic metal species are detoxified, both dependent and independent of metabolism and all affected by environmental factors. Furthermore, other elements not considered to be true metals according to chemical definitions may have some metallic properties and exhibit varying degrees of toxicity as well as accumulation by fungi. These include metalloids, and the actinides and lanthanides, e.g. uranium and thorium. The alkali metals are not usually included in discussions on metal toxicity but nevertheless exhibit a dramatic range of biological properties as exemplified by K^+, Na+ and Cs^+ as respectively representing an essential non-toxic, an essential but potentially-toxic, and an inessential potentially-toxic metal ion. Caesium, and many actinides, are of current concern because of environmental contamination, accumulation by the biota, including fungi, and transfer to other organisms including humans (**Gadd, 1992a**). Current definitions of the term 'heavy metal' are particularly variable. These are often defined as a group of approximately 65 metallic elements, of density greater than 5, with the general ability to exert toxic effects on microbial and other life forms (**Gadd & Griffiths, 1978; Nieboer & Richardson, 1980**). A further complication is that 'heavy elements' may be defined as Pb, As, Cd, Hg, Sb, Se, TI, In, Bi, and Te (**Fergusson, 1990**). This is again a diverse group and, in fact, As, Sb, Bi, Se and Te have elemental structures more typical of non metals (**Fergusson, 1990; Morgan & Stumm, 1991**). Organometallic compounds, simply defined as a compound containing at least one metal-carbon bond (**Thayer, 1988**), frequently exhibit enhanced toxicity, hence they are used as fungicides. When such compounds contain metalloid elements, the term organo-metalloid may be used. Such substances should also be included in discussions of metal interactions with fungi because of their significance as environmental pollutants and as fungicides and also because some may be synthesized by fungi as a result of exposure to and transformation of the 'parental' metal species.

The present study was planned to isolate and characterize metal tolerant fungal strains from metal contaminated natural sites and also to assess their possible utility in soil/waste water/industrial effluent bioremediation.

The brief outline of the thesis is given below:

Chapter – 1: Introduction

Chapter – 2: Review of Literature

Chapter – 3: Aims and Objective

Chapter – 4: Materials and Methods

Chapter – 5: Results

Chapter – 6: Discussion

Chapter – 7: Conclusion

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Chapter – 9: Publications

REVIEW OF LITERATURE

2.1 Metals toxicity of fungi

Toxic metals can exert harmful effects principally as a result of their strong coordinating abilities (**Ochiai, 1987**). Toxic effects include the blocking of functional groups of biologically important molecules (e.g. enzymes and transport systems for essential nutrients and ions), the displacement and/or substitution of essential metal ions from biomolecules and functional cellular units, conformational modification, denaturation and inactivation of enzymes and disruption of cellular and organelles membrane integrity (**Ochiai, 1987**). Because of the wide spectrum of potentially toxic interactions between metals and fungi, almost every aspect of their metabolism, growth and differentiation may be affected, depending on the organism, metal species and concentration and physico-chemical factors (**Gadd, 1986a, c; Gadd & White, 1989a**). Thus, toxic symptoms may vary widely between different fungi and for different metal species. A prerequisite for direct toxic interactions is contact between the active metal species and cellular components **(Gadd & White, 1989a**). The cell membrane is an obvious initial site of action for a toxic metal species and membrane damage can result in loss of mobile cellular solutes, e.g. K^+, and increased permeability of the cell to external materials (**Norris & Kelly, 1977; Kuypers & Roomans, 1979; Mowll & Gadd, 1983; White & Gadd, 1987 a, b; Laurence, et al., 1989**). Indirect mechanisms of metal toxicity may involve free radicals, which are deleterious to cells as they can take part in chain reactions which involve the breakdown of biological macromolecules. Consequently, aerobic organisms possess protective enzymes such as superoxide dismutases, which are metalloenzymes containing either Mn, Fe or Cu/Zn (**Greco et al., 1990**), which eliminate the radicals produced by normal metabolism. Major targets in cells are membranes, where lipid peroxidation, whereby the alkyl chains of lipids are converted to peroxyalkyl radicals and fatty acid hydroperoxides, is initiated (**Mehlhorn, 1986**). Lipid-soluble complexes of transition elements such as Fe (II) may undergo the Fenton reaction [(1), (2)] with the hydroperoxides and accelerate this process (**McCord & Day, 1978**).

$Fe^2 + H_2O_2$-* Fe^{3+} +OH^-+OH (1)

O_2-+ Fe^3+ O> 0_2+ Fe^{2+} (2)

Complexes and free ions of these cations may also undergo this reaction in aqueous solution. In animal systems, metal ions such as Hg^{2+}, Cd^{2+}, Pb^{2+} and Ag^{2+} induce free radical toxicity as a result of their reactions with thiols or enzymes which normally protect against these reactive species (**Mehlhorn, 1986**). A little work on this aspect has been carried out with fungi except for Cu^{2+} and *Saccharomyces cerevisiae* (**Greco et al., 1990**).

Organometallic compounds are of increasing environmental significance because of their use in the chemical and petroleum industries and as biocides. Organometals are generally more toxic towards fungi than corresponding free metal ions and the toxicity of their compounds varies with the number and with the identity of the organic groups **(Blunden, et al., 1984; Cooney & Wuertz, 1989**). Major effects of organotins and organoleads are disruption of mitochondrial membranes and action as C1-/OH- ionophores. In this way, they depolarize electrochemical gradients and consequently interfere with energy conservation **(Blunden et al., 1984; Cooney & Wuertz, 1989**). In fact, organotin-resistant mutants of *S. cerevisiae* can exhibit increased respiration rates (**Dupont et al., 1990**) and modification of the organotin-binding site on the inner mitochondrial membrane (**Lancashire & Griffiths, 1975**). Organometals may also damage membranes by the production of free radicals since the carbon-metal bond readily reacts with available radicals to produce peroxyalkyl radicals which can result in lipid peroxidation (**Mehlhorn, 1986**). The organometallic compounds may also exert a disruptive effect on cell membranes and cause a loss of K^+ (**Cooney et al., 1989**).

2.2 Resistance and tolerance

Fungal survival in the presence of toxic metals mainly depends on intrinsic biochemical and structural properties, physiological and/or genetic adaptation including morphological changes and environmental modification of metal speciation, availability and toxicity The relative importance of each often been difficult to determine **(Gadd & Griffiths, 1978; Gadd, 1990 b, 1992 b**). Arbitrary terms such as ' resistance ' and ' tolerance ' which are used rather loosely and often interchangeably in the literature are generally based on the ability to grow on a certain metal concentration in laboratory media (**Trevors, et al., 1986; Gadd, 1992b, c**). It is

probably more appropriate to define 'resistance' as the ability of an organism to survive metal toxicity by means of a mechanism produced in direct response to the metal species concerned, e.g. synthesis of metallothioneins or y-glutamyl peptides (**Mehra & Winge, 1991**). 'Metal tolerance' may be defined as the ability of an organism to survive metal toxicity by means of intrinsic properties and/or environmental modification of toxicity (**Gadd, 1992b, c**). Intrinsic properties that can determine survival include possession of impermeable pigmented cell walls, extracellular polysaccharide and metabolite excretion, especially where this leads to detoxification of the metal species by binding or precipitation (**Gadd, 1990b**). However, in many cases distinctions are difficult because of the involvement of several direct and indirect physico-chemical and biological mechanisms in survival in field and laboratory. Biological mechanisms implicated in fungal survival (as distinct from environmental modification of toxicity) include extracellular precipitation, complexation and crystallization, transformation of metal species by, e.g. oxidation, reduction, methylation and dealkylation, biosorption to cell walls, pigments and extracellular polysaccharide, decreased transport or impermeability, efflux, intracellular compartmentation and precipitation and/or sequestration **(Fig. 2.1)** (**Ross, 1975; Gadd & Griffiths, 1978; Gadd, 1988, 1990a, b, 1992a,b,c; Brown & Hall, 1990; Mehra & Winge, 1991**). A particular organism may directly and/or indirectly rely on several survival strategies. For example, metallothionein synthesis is a mechanism of Cu^{2+} resistance in *Saccharomyces cerevisiae* yet Cu^{2+} binding or precipitation around the cell wall and intracellular transport are also components of the total cellular response (**Gadd & White, 1989a**). Fungus species *Cunninghamella elegans* has great ability to accumulate phosphorus in the form of poly phosphate (**Lima et al., 2003**) to metabolize xenobiotic recalcitrant substances such as PAH (**Cerniglia, 1997**); azodyes used in textile industry (**Ambrosio and Takaki,** 2004); Oxidation of dibenzothiophene (**Schlenk, et al., 1994**); biotransformation of drugs (**Zhang et al,. 1996**); as well as able to tolerate and remove cadmium (**Lima et al,. 2013**). Detailed accounts of metal resistance and tolerance of fungi can be found elsewhere (**Ross, 1975; Gadd, 1986a, 1990b, 1992b,2010; Mehra & Winge, 1991**).

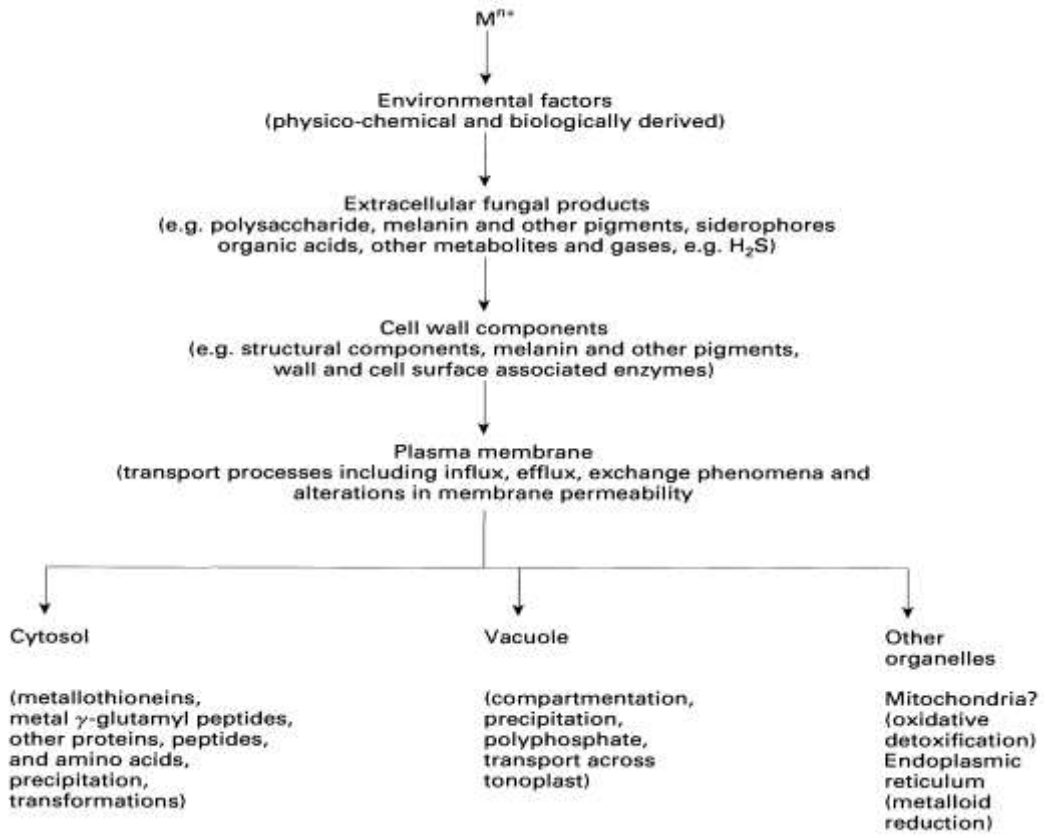


Fig. 2.1 Diagrammatic representation of metal interactions with fungi. The metal species are represented in cationic form. All the interactions shown may be involved in fungal survival and/or metal detoxification.

2.3 Environmental influence on heavy metal toxicity towards fungi

The physico-chemical properties of a given environment or growth medium determine metal speciation and therefore biological availability and toxicity as well as other essential and inessential metal-organism interactions. Where such factors as pH, Eh, the presence of other anions and cations, particulate and soluble organic matter, and clay minerals decrease biological availability and toxicity may be reduced (**Gadd & Griffiths, 1978**). It is therefore clear that the concentration of free metal ion, defined as pM = -log [M] (**Hughes & Poole, 1991**), may be an important consideration in many cases, although toxicity may still be exerted by many inorganic and organic metal complexes in the absence of free metal cations, M^+.

In simple terms, if excess metal ion (M) is in the presence of a strongly binding ligand (L), $[M_{free}] = [M_{total}] - [L_{total}]$. If the ligand is in excess, and unprotonated, $L_t = ML + L$, $[ML] \sim [M_t]$ and the pM may be calculated:

$K_1 = [ML]/[M][L] = [M_t]/[M][L_t - M_t]$,

$pM = -\log [M] = \log K_1 + \log ([L_1 - M_1]/[M_t])$.

If the ligand is protonated, the calculation becomes $Lt = ML + L + HL + H_2L \ldots H_nL$ (**Hughes & Poole, 1991**). In relation to the complex situation which occurs in the environment and in growth media, the multiplicity of metal-ligand binding interactions that can occur may mean that calculation of pM is not possible.

The pH can affect metal-fungal responses by effects on metal speciation and mobility and indirectly by influencing other aspects of cell physiology and metabolism. Increasing pH can result in the formation and precipitation of metal hydroxides or oxides. In aqueous solution, divalent metal cations form multiple hydroxylated species, some forming precipitates (**Collins & Stotzky, 1989; Hughes & Poole, 1991**).

At higher pH values, some precipitates re dissolve giving anionic hydroxometallate complexes, e.g. $Zn(OH)_4^{2}$ (**Hughes & Poole, 1991**). The pH values at which hydroxylated species form varies between different metals. The different hydroxylated forms possess different toxicities (**Collins & Stotzky, 1989**). The formation of hydrolysis products may also favour biosorption.

Though decreasing pH may increase the concentration of free metal ions in solution as mentioned previously, H^+ may compete with metal ions for cellular binding sites and reduce potential interactions with cells. External pH may also affect the speciation of metal-binding ligands and therefore metal complexation. Many toxic interactions of heavy metals with fungal cells can be interpreted in terms of effect of pH on metal speciation and availability (**Newby & Gadd, 1986; Gadd, 1986a**). Fungal toxicity of Cd may increase with increasing pH. A reduction in metal toxicity at low pH may result from H^+ competition and also pH effects on intracellular uptake. This has been described for those fungi, e.g. *Penicillium ochrochloron*, that are capable of growing in saturated $CuS0_4$ and high concentrations of other heavy metal salts (**Gadd et al., 1984**). With increasing pH intracellular Cu accumulation

dramatically increased in *P. ochrochloron* and at pH 6 and above this fungus was sensitive to quite low concentrations of Cu (**Gadd & White, 1989**).

Oxidation-reduction potential (Eh) may affect heavy metal availability and toxicity. In a reducing environment (negative Eh), insoluble metal sulphides may form which exhibit little or no toxicity. Sulphide precipitation is a detoxification mechanism found in several fungi (see later). The E_h may also effect speciation, e.g. chromium exists as Cr^{6+} or Cr^{3+} depending on the E_h. Cr^{6+} exhibits the greater toxicity towards hyphal growth and sporulation in several fungi (**Collins & Stotzky, 1989**).

Inorganic anions may affect toxicity by forming inorganic complexes, as mentioned previously and by precipitation of (**Gadd & Griffiths, 1978**). Increasing Cl^-, for example, decreased Cd toxicity towards several filamentous fungi, possibly as a result of the formation of negative Cd-Cl coordination complexes (**Babich & Stotzky, 1982; Collins & Stotzky, 1989**). Other cations may affect fungitoxicity of heavy metals by competing for binding sites on cell surfaces including membranes and for transport mechanisms. Heavy metal toxicity towards fungi was also reduced in hard water an effect attributed to CO_3^{2-} **(Babich & Stotzky, 1981**). Many heavy metal salts are of low solubility, e.g. sulphides and phosphates, and therefore readily precipitate out of solution. Silver chloride is highly insoluble and the concentration of free Ag+ in solution may be negligible in many cases, particularly in growth media (**Hughes & Poole, 1991**).

Clay minerals can adsorb heavy metal cations and reduce their potential toxicity (**Gadd & Griffiths, 1978; Babich & Stotzky, 1980**). In general, minerals with a high Cation Exchange Capacity (CEC) are more effective in reducing toxicity than minerals with a lower CEC (**Babich & Stotzky, 1977a, b; Bewley & Stotzky, 1983**). Montmorillonite was more effective than kaolinite, with a lower CEC, in protecting fungi from Cd, Ni and Pb toxicity **(Babich & Stotzky, 1978, 1979, 1982**). Such effects are also being influenced by soil pH (**Collins & Stotzky, 1989**).

Dissolved and particulate organic matter in the environment, and in growth media, can influence metal toxicity by complexation and binding, generally reducing toxicity. Examples of such organic materials include natural and synthetic chelating agents, including plant, fungal and other microbial exudates and metabolites, humic acids and soil colloids, and soluble organic chemicals and mixtures. It is commonly observed

that metal toxicity towards fungi is reduced in complex media compared to simple defined media and such effects are also being influenced by pH for the reasons outlined previously (**Gadd & Griffiths, 1978; Collins & Stotzky, 1989**).

2.4 Fungi in polluted habitats

A range of fungi from all major taxonomic groups may be found in metal-polluted habitats and the ability to survive and grow in the presence of potentially toxic concentrations is frequently encountered (**Ross, 1975; Gadd, 1986a; Baldi, et al., 1990; Turnau, 1991**). In general terms, toxic metals are believed to affect fungal populations by reducing abundance and species diversity and selecting for a resistant/tolerant population (**Babich & Stotzky, 1985; Duxbury, 1985**). However, the effect of toxic metals on microbial abundance in natural habitats varies with the metal species and organisms present and with environmental factors (**Gadd & Griffiths, 1978; Duxbury, 1985**).

General reductions in fungal numbers have often been noted in soils polluted with Cu, Cd, Pb, As and Zn (**Babich & Stotzky, 1985**). Along a steep gradient of Cu and Zn in soil towards a brass mill, fungal biomass decreased by 75 % (**Nordgren, et al., 1983**) while fungal numbers were reduced in glucose-supplemented soil by Cd or Zn, the former metal being of greater toxicity (**Bewley & Stotzky, 1983**). However, numerical estimates alone which have built-in inadequacies may provide little meaningful information unless possible changes in fungal groups and species are considered. There is evidence that such changes can occur in response to metal exposure.

In Cu and Zn polluted soil, Geomyces and *Paecilomyces sp*. and some sterile forms increased with increasing pollution, whereas *Penicillium* and *Oidiodendron sp*. declined at polluted sites (**Nordgren et al., 1983**). *Trichocladium asperum, Trichoderma hamatum*, *Zygorrhynchus moelleri* and *Chrysosporium pannorum* were isolated more frequently from an organomercurial treated golf green than from untreated locations, whereas Chaetomium, *Fusarium, Penicillium* and *Paecilomyces* sp. were greatly reduced (**Williams & Pugh, 1975**). Several other studies have indicated sensitivity of *Penicillium sp*. towards heavy metals (**Duxbury, 1985**). However, some of the best examples of microbial metal tolerance are also found in the genus *Penicillium*, which underlines the fact that metal responses may be strain

specific. As noted above, *P. ochrochloron* can grow in saturated $CuSO_4$ and it is frequently isolated from industrial effluents (**Stokes & Lindsay, 1979**) whereas *P. lilacinum* comprised 23% of all fungi isolated from soil polluted by mine drainage (**Tatsuyama et al., 1975**). In soil polluted with cadmium dust, *Strobilurus tenacellus*, *Mycena ammoniaca*, *Auriscalpium vulgare* and *Armillaria lutea* were the most common Basidiomycetes (**Turnau, 1991**).

Heavy metal pollution of plant surfaces is wide spread and while there may be significant decrease in total microbial numbers (including bacteria) on phylloplanes, numbers of filamentous fungi and non-pigmented yeasts are generally little affected (**Bewley, 1979, 1980; Bewley & Campbell, 1980; Mowll & Gadd, 1985**) and if metal-supplemented isolation media are used only fungi may be isolated (**Bewley, 1980**). On polluted oak leaves *Aureobasidium pullulans* and *Cladosporium sp.* were the most numerous organisms and a greater proportion were metal-tolerant when compared with control isolates **(Bewley & Campbell, 1978; Bewley, 1980**). In fact, numbers of *A. pullulans* showed a good positive correlation with lead concentrations, whether derived from industrial or vehicular sources and it often became the dominant organism, in some cases comprising up to 97% of the leaf surface population on a numerical basis (**Bewley & Campbell, 1980; Mowll & Gadd, 1985**). However, adaptation was unnecessary for growth of *A. pullulans* on polluted leaf surfaces and the ability to tolerate high concentrations of lead occurred in isolates from the unpolluted site (**Gadd, 1984; Mowll & Gadd, 1985**).

In contrast, the numbers of the ballistospore producing yeast, *Sporobolomyces roseus* and heterotrophic bacteria were very low or absent from polluted samples while numbers of *S. roseus* showed a significant negative correlation with increasing lead concentrations (**Mowll & Gadd, 1985**). **Smith (1977)** also studied the effect of heavy metals on phylloplane fungi and found that *Aureobasidium pullulans, Epicoccum sp.* and *Phialophora verrucosa* were tolerant, *Gnomia platani, Cladosporium* and *Pleurophomella sp.* were of intermediate tolerance while *Pestalotiopsis* and *Chaetomium* sp. were sensitive. Mercury tolerant fungi have been isolated from the surfaces of seeds treated with mercury compounds. These include *Pyrenophora avenae, Penicillium crustosum, Cladosporium cladosporoides, Syncephalastrum racemosum* and *Ulocladium atrum* (**Ross & Old, 1973; Greenaway, et al., 1974**).

It seems therefore that elevated concentrations of heavy metals can affect the qualitative and quantitative composition of fungal populations though it must be stressed that it may be difficult to separate metal effects from those of other environmental components. Phylloplane mycoflora may be subject to the influence of other potential toxicants, e.g. SO_2 (**Babich & Stotzky, 1980**), while polluted soils may be nutrient-poor, of variable pH and also contain additional toxicants (**Gadd & Griffiths, 1978**). These factors may affect fungal populations. Furthermore, there are well known difficulties in obtaining meaningful assessments of fungal numbers and diversity which cannot be ignored. Despite this, it is less apparent that certain fungi can exhibit considerable tolerance towards toxic metals and can become dominant microorganisms in some polluted habitats. However, while species diversity may be reduced in certain cases, resistance/tolerance can be exhibited by fungi from both polluted and non-polluted habitats. Fungal numbers were reduced and there were alterations in species composition in polluted soil near a zinc smelter (**Jordan & Lechevalier, 1975**). However, there was little difference in the zinc tolerance of fungi isolated from either site and most could exhibit 50% growth at 700ppm Zn^{2+}. At control sites, Zn-tolerant genera included *Bdellospora, Verticillium* and *Paecilomyces* with *Penicillium, Torula* and *Aureobasidium* at polluted sites. In another study, fungal populations in soil contaminated with Ni, Cn, Fe and Co were not significantly different from those at control sites (**Freedman & Hutchinson, 1980**). Cu and Nitolerant fungi (defined as being capable of growth at approximately 16 mM Cu^{2+} and/or Ni^{2+}) were isolated from both control and contaminated sites, the predominant genera being *Penicillium* (60%) followed by *Trichoderma, Rhodotorula, Oidiodendron, Mortierella* and *Mucor*. Examination of microfungi isolated from unpolluted and copper-polluted forest soil confirmed that species from the polluted site were usually copper-tolerant although there was little evidence for adaptation in isolates from sites with short or long histories of pollution (**Arnebrant, Baath & Nordgren, 1987**). Such studies indicate that in many cases survival must be dependent on intrinsic properties of the organisms rather than adpative changes (which are generally studied under laboratory conditions) and physico-chemical properties of that environment. These will include changes associated with the metal pollution, which may modulate toxicity and affect species composition (**Gadd, 1986a, 1990b**).

2.5 Interactions between toxic metals and fungi

In general, microbe mediated metal removal or recovery from contaminated site/reservoir may involve the following pathways:

a) Metal cations may bind on cell surface (biosorption), within the cell wall (bioaccumulation) and in turn, metal uptake is augmented through microprecipitation;

b) Metal ions may be actively translocated inside the cell through metal binding proteins;

c) Metal precipitation may occur when heavy metals react with excellular polymers or with anions (e.g. sulphide or phosphate) produced by microbes;

d) Metal volatilization through enzyme mediated biotransformation (Fig. 2.2).

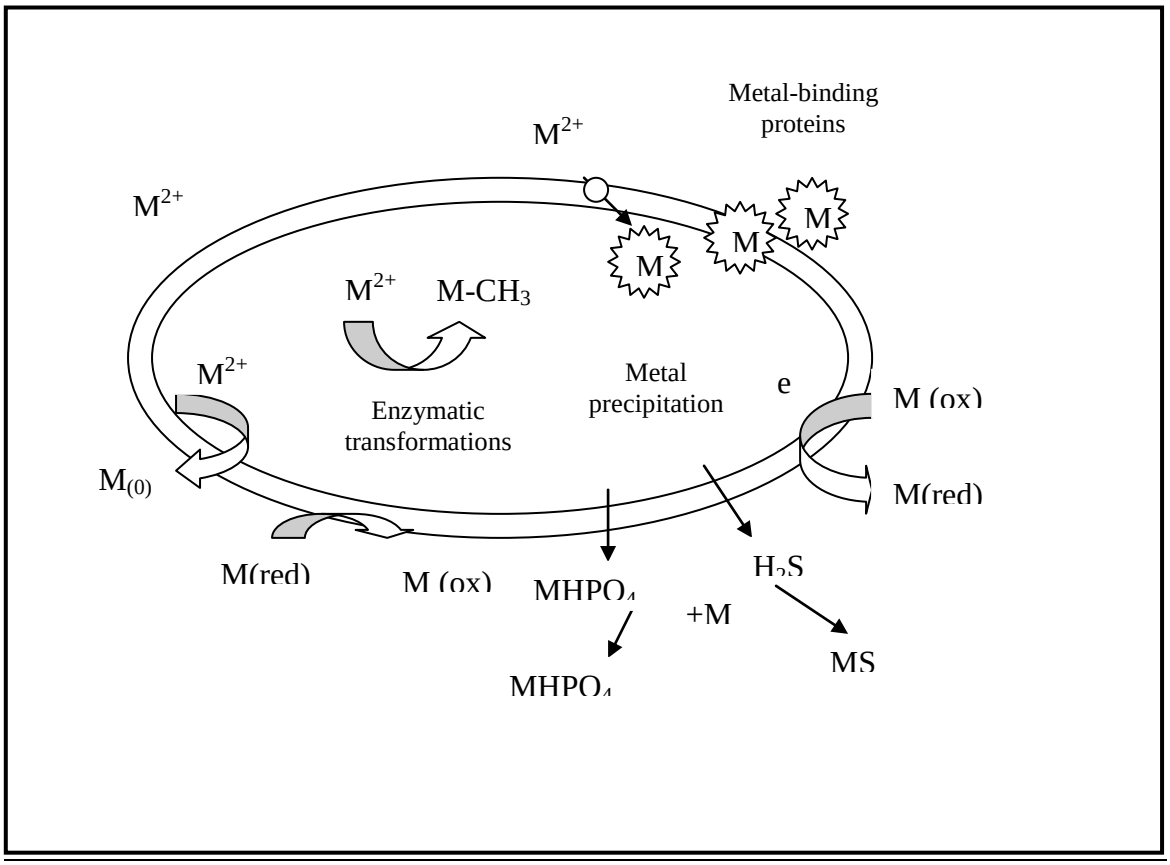


Fig. 2.2. Mechanisms of heavy metal uptake on microbial surface

2.5.1 Extracellular precipitation and complexation

Many extracellular fungal products can complex or precipitate heavy metals. Citric acid can be an efficient metal-ion chelator and oxalic acid can interact with metal ions to form insoluble oxalate crystals around cell walls and in the external

Table 2.1 Macromolecular constituents of fungal cell walls (adapted from **Peberdy, 1990**)

Skeletal elements	
Chitin:	β-1–4-linked homopolymer of *N*-acetyl-D-glucosamine
β-glucans:	β-1,3-glucan homopolymer comprised of D-glucose units with β-1,3 and β-1,6-glucosidic bonds (R-glucan)
Cellulose:	β-1,4-linked homopolymer of glucose
Matrix components	
α-glucan:	α-1,3-homopolymer of glucose (S-glucan)
	α-1,3- and α-1,4-linked glucan (nigeran)
Glycoproteins	
Mannoproteins	
Miscellaneous components	
Chitosan:	β-1,4-polymer of D-glucosamine
D-galactosamine polymers	
Polyuronides	
Melanins	
Lipids	

medium (**Murphy & Levy, 1983; Sutter, et al, 1983**). The production of H_2S by yeasts can result in precipitation of metals as insoluble sulphides predominately in and around cell walls (**Ashida, et al, 1963; Minney & Quirk, 1985**). For strains of *S. cerevisiae* growing on a copper-containing medium, resultant colonies may appear dark in colour owing to formation of copper sulphide (**Ashida et al., 1963**). Iron is of fundamental importance to living cells and many filamentous fungi and yeasts release high affinity Fe-binding molecules called siderophores (**Winkelmann et al., 1987; Winkelmann, 1992**). The externallyformed Fe^{3+} chelates may subsequently be taken up into the cell (**Raymond, et al, 1984; Adjimani & Emery, 1987**). In several fungi, the excretion of such iron-binding molecules is markedly stimulated by Fe deficiency and such compounds may also bind Ga (**Adjimani & Emery, 1987**). *Debaryomyces hansenii* produced riboflavin or a related compound when grown in Fe-deficient media or in the presence of copper, cobalt and zinc. Polarographic analysis showed that the pigment was capable of Fe^{3+} binding (**Gadd & Edwards, 1986**).

2.5.2 Metal binding to cell walls

The wall is the first cellular site of interaction with metal species and metal removal from solution may be rapid although rates will depend on factors such as type of

metal ion and biomass, concentration of metal and environmental factors. Metabolism- independent association of metal species to fungal walls may include ion exchange, adsorption, complexation, precipitation and crystallization (**Mullen et al., 1992**). 'Biosorption' is a term that is frequently used to describe such non-directed physico-chemical interactions between metals (including radionuclide) species and microbial biomass particularly in a biotechnological context (**Shumate & Strandberg, 1985**).

The fungal cell wall thus has important protective properties and so may act as a barrier, controlling the uptake of solutes into the cell including potentially toxic metal species (**Gadd & Griffiths, 1980 a; Gadd, 1986a, b; Ono, et al, 1988**) and also indirectly affecting the intracellular ionic composition by restriction of cellular water (**Peberdy, 1990**). The wall is mainly composed of polysaccharides, some of which may have associated protein, with other components including lipids and melanins (Table 2.2). All filamentous fungi, with the exception of the Oomycetes, contain chitin with (1-3)-fl-glucan as skeletal components. The *Oomycetes,* together with Hyphochytridiomycetes possess cellulosic walls (**Peberby, 1990**). In yeasts, skeletal components consist of (1-3)-fl-D-glucans with (1-6)-,8-linkages at branch points, the degree of branching determining crystallinity (**Bartnicki-Garcia, 1973**). Matrix components in yeasts comprise a mannan-protein complex, while (1-3)-cc-glucan normally occurring at the outer surface of the wall, is the major matrix polymer in filamentous fungi (**Peberdy, 1990**). Heterogeneous glycoproteins are also found in filamentous fungal walls (**Peberdy, 1990**).

It is clear, therefore, that metal biosorption in fungal cell walls may be complex, involving different components and mechanisms, and variable depending on wall structure and composition, the later itself being affected by the presence of toxic metals (**Gadd & Griffiths, 1980a; Newby & Gadd, 1987; Venkateswerlu & Stotzky, 1989; Venkateswerlu, et al, 1989**). A variety of potential sites may be involved in metal sequestration including carboxyl, amine, hydroxyl, phosphate and sulphydryl groups although their relative significance is usually difficult to resolve (**Strandberg, et al, 1981**). However, primary interactions probably involve binding to carboxyl and phosphate groups which may be enhanced by electrostatic attraction to other negatively charged functional groups (**Tobin, et al, 1990**). Metabolism-independent biosorption is frequently rapid and unaffected over moderate ranges of

temperature, e.g. 4-30 °C (**Norris & Kelly, 1977; De Rome & Gadd, 1987a, b; Junghans & Straube, 1991**). In *Rhizopus arrhizus*, biosorption was related to ionic radius for, e.g. La^{3+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Ba^{2+}, Hg^{2+}, Pb^{2+}, UO_2^{2+} and Ag+, but not Cr^{3+} or the alkali metal cations, Na^{+}, K^{+}, Cs^{+} and Rb^{+}, which were not taken up by the cell walls (**Tobin, et al, 1984**). Low external pH often decreases biosorption of, e.g., Cu^{2+}, Cd^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+} (**Fuhrmann & Rothstein, 1968; Paton & Budd, 1972; Roomans et al., 1979; Gadd & Mowll, 1985; De Rome & Gadd, 1987 a, b; Venkateswerlu & Stotzky, 1989; Gadd, 1990a**) while other anions and cations may have the same effect by, e.g. precipitation by phosphates, and removal of metal from solution or by competition for binding sites, e.g., Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+} and Mn^{2+} (**Tobin, et al, 1987, 1988; Gadd, 1990a**). The later effects may be dependent on the relative concentrations and chemical behaviour of the different metal species present. Biomass concentration may also effect fungal biosorption. At a given equilibrium concentration, the uptake of metals when expressed on a unit dry weight or cell basis, is generally greater at lower cell densities than at high ones (**Itoh, et al, 1975; De Rome & Gadd, 1987a; Junghans & Straube, 1991**). Since growth conditions affect the structure and composition of fungal cell walls, their manipulation offers potential for biosorbency for specific purposes (**Volesky, 1990**). Chitin and chitosan have received attention as significant metal-biosorbing substances in cell walls of fungi. Chitin is a polymer of N-acetyl-D-glucosamine; chitosan is deacetylated chitin (**Volesky, 1990**). A variety of metal ions are readily bound by chitin although alkali metals, NH^{4+}, Ca^{2+} and Mg^{2+} are not sequestered (**Muzzarelli, et al, 1986**). In *R. arrhizus* coordination of uranium to the amine nitrogen of chitin and simultaneous adsorption in the cell wall chitin structure are followed by slower precipitation of uranyl hydroxide (**Tsezos & Volesky, 1982a**). Accumulation of hydrolysis products continues until final equilibrium (**Tsezos, 1983**). The mechanism of thorium deposition involved localization at the outer region of the wall in *R. arrhizus*. Coordination to amine nitrogen still occurred but the hydrolysis of Th^{4+} to $Th(OH)_4$ at pH 4 resulted in deposition at the chitin surface. The formation of hydrolysis products favours biosorptionat pH 2. Where Th^{4+} is the main thorium species, lower biosorption occurred with a general dispersal of Th^{4+} through the wall (**Tsezos & Volesky, 1982 b**). The chitin-chitosan content of fungal walls varies between species, e.g. *Neurospora crassa* (26 % of the wall dry weight) and *R. nigricans* (53 Go), and this may change during growth (**Volesky, 1990**). Purified chitin and chitosan

derivatives may also have a biosorptive ability although this may be influenced by the extraction method employed. Isolated chitin from *R. arrhizus* showed decreased uranium biosorption in comparison with intact biomass (**Tsezos, 1986**). Chitin phosphate and chitosan phosphate were able to remove metal and actinide species from solution, both substances showing a markedly greater affinity for UO_2^{21} than other metal species including Cu^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+} Zn^{2+} Mg^{2+}, Ni^{2+} and Ca^{2+} (**Sakaguchi & Nakajima, 1982**). Non-phosphorylated chitin and chitosan were not efficient biosorbents **(Sakaguchi & Nakajima, 1982)**. Insoluble chitosan-glucan complexes and glucans possessing amino- or sugar acid groups from *Aspergillus niger* exhibit biosorptive properties and may efficiently remove transition metal ions from solution (**Muzzarelli et al., 1986**). Synthesized chitosan derivatives, e.g. N-[2-(1,2- dihydroxyethyl)tetra-hydrofuryl] chitosan, can remove uranium from brines at an order of magnitude greater than the intact biomass of *R. arrhizus* (**Muzzarelli et al., 1986; Macaskie & Dean, 1990a**). Hydroxide treatment of fungi to expose chitin/chitosan, may also improve biosorptive abilities (**Wales & Sagar, 1990**).

Melanins are important fungal pigments which enhance the survival of many species in response to environmental stress (**Bell & Wheeler, 1986**). Fungal melanins are located in and/or exterior to cell walls where they may appear as electron-dense deposits and granules on electron micrographs. These granules may be released into the external medium and be termed 'extracellular melanin', although this is generally of identical composition to wall-associated melanin. 'Extracellular melanins' are more correctly defined as melanins synthesized exterior to cells by secretion of phenol oxidases which oxidize phenolics or secretion of phenols that are subsequently oxidized (**Bell & Wheeler, 1986**). Fungal phenolic polymers and melanins contain phenolic units, peptides, carbohydrates, aliphatic hydrocarbons and fatty acids and therefore possess many potential metal-binding sites (**Saiz-Jimenez & Shafizadeh, 1984; Senesi, et al, 1987; Sakaguchi & Nakajima, 1987**). Oxygen-containing groups in these substances, including carboxyl, phenolic and alcoholic hydroxyl, carbonyl and methoxyl groups may be particularly important in metal binding (**Gadd, 1988**). The order of binding ability of fungal phenolic polymers and melanins followed the sequence Cu > Ca > Mg > Zn (**Zunino & Martin, 1977**).

A variety of melanin types occur in fungi. Although it is apparent that many fungi can form extracellular DOPA (3,4-dihydroxyphenylalanine)melanin via tyrosinase-

mediated oxidation of tyrosine, there is still no firm evidence for the existence of DOPA- melanin in fungal cell walls (**Bell & Wheeler, 1986**). Melanins in cell walls of Basidiomycetes are derived from y-glutaminyl-3,4-dihydroxybenzene (GDHB) or catechol. In Ascomycetes and Deuteromycetes, wall melanins are generally synthesized by the pentaketide pathway via 1 ,8-dihydroxynaphthalene (DHN) as the immediate precursor. Other extracellular dark pigments may be referred to as 'heterogeneous' melanins (**Bell & Wheeler, 1986**). A variety of heavy metals can induce or accelerate melanin production in fungi and melanized cell forms, e.g. chlamydospores, can have high capacities to adsorb metals with virtually all the metal being located in the cell wall (**Mowll and Gadd, 1984**). Melanin from *Aureobasidium pullulans* can bind significant amounts of Cu^{2+} and Fe^{3+} (**Gadd, 1984; Senesi et al., 1987; Gadd & De Rome, 1988**) as well as organometallic compounds, e.g. tributyltin chloride (**Gadd, 1990**).

2.5.3 Transport of toxic metal cations

The plasma and vacuolar membranes are the main transport membranes of fungi and the basic energetics as well as detailed kinetic analysis have been established for a variety of solutes, particularly carbon and/or nitrogen sources (**Sanders, 1988, 1990**). Most work on metal ion transport in fungi has concerned K^+ and Ca^{2+} largely because of their great importance in fungal growth, metabolism and differentiation. The transport of toxic metal species is still poorly understood. Transport systems in fungal (and plant) cell membranes are usually classed as either carrier or channel systems. In the former the conformational changes in the transport protein are believed to result in alternate exposure of the transport binding site(s) on each side of the membrane. Carriers include all metabolically-coupled and H^{+}-gradient driven transport systems. Fluxes through such systems saturate with respect to ligand concentration and if a current is carried with respect to the membrane potential ($\Delta\Psi$) (**Sanders, 1990**). Ion channels are a class of proteins that function as gated pores in the plasma membrane allowing the flow of ions down electrical and/or chemical gradients (**Gustin et al., 1986**). Channels have higher turnover rates than carriers, 10^{7-8} s^{-1} compared to 10^{2-4} S^{-1} respectively (**Sanders, 1990**). To date, the main primary transport systems which have been characterized in fungi derive energy from ATP hydrolysis and pump H^+ electrogenically from the cytosol, creating a transmembrane electrochemical H^+ gradient ($\Delta\sim\mu_H{}^+$), negative and alkaline inside. Such an electrochemical gradient

($\Delta\sim\mu_{H^+}$) has an electrical component, the membrane potential ($\Delta\Psi$) ,a chemical component and the pH gradient (ΔpH) which are interconvertible and which can drive the transport of ionizable substances across membranes. Such secondary gradient-coupled transport systems exist for a variety of inorganic and organic solutes which are energized by coupling with passive reflux of H^+; proton and solute fluxes may be in the same (symport) or opposing (antiport) directions (Sanders, 1990). In fungi and yeasts, three main classes of H^+- pumping ATPases have been identified in mitochondrial, vacuolar and plasma membranes (**Serrano, 1984, 1985**) though it is now suggested that transport ATPases include a Ca^{2+}-ATPase located on the endoplasmic reticulum (**Goffeau et al., 1990**). The main function of the mitochondrial ATPase is ATP synthesis via the mitochondrial respiratory chain. It is the plasma membrane and vacuolar ATPases that are associated with ion transport and intracellular compartmentation and regulation of intracellular pH (**Jones & Gadd, 1990**). The transport of monovalent cations is linked to the action of the plasma membrane H^+-ATPase that expels protons creating the transmembrane electrochemical proton gradient ($\Delta\sim\mu_{H^+}$) previously described. K^+ uniport in fungi appears to be electrically coupled with H^+ efflux. Under usual cellular and environmental conditions, H^+ is extruded in an apparent 1: 1 ratio to K^+ uptake with H+ able to be extruded against 50: 1 concentration gradients and K+ to be taken up against 1000:1 such gradients (**Borst-Pauwels, 1981**). However, it is now known that under K^+-deficiency, K^+ influx may be coupled with H^+ influx in a H^+-K^+ symport system. It has been suggested that observations of stoichiometric K^+/H^+ exchange arise because the H^+ pump maintains electroneutrality during K^+ uptake by efflux of one equivalent of H^+ per equivalent of charge flow mediated by symport (**Sanders, 1988**); the net inflow of positive charge is approximately twice the net K^+ uptake (**Rodriguez-Navarro et al 1986**). One kind of K^+ uniport is via channels in the fungal plasma membrane (**Gustin et al., 1986**). Since such channels are not active at the hyperopolarizing (negative) potentials found in intact cells, their physiological role is unclear (**Sanders, 1988**). It now appears that the plasma membrane of *Saccharomyces cerevisiae* may have at least 2 classes of K^+-selective channels, the first of which is voltage gated (**Gustin et al., 1986**) and the second possibly operated via second messengers and sensitive to blocking by divalent cations (**Van de Mortel et al, 1990**).

Potentially toxic metal ions, e.g. Cu^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, Mn^{2+}, mercurials and other organometallic compounds, can inhibit the yeast plasma membrane ATPase by means of various binding interactions, both specific and non-specific (**Ochiai, 1987; White & Gadd, 1987 a, b**). Such deleterious effects lead to a reduced ability in maintenance of the electrochemical gradients described. For H^+ efflux, heavy metal inhibition generally increased with increasing metal concentration with a toxicity sequence of $Cd^{2+} > Cu^{2+} > Ni^{2+} > Zn^{2+} > Co^{2+}, Mn^{2+}$. These metals also inhibited K^+ uptake with a toxicity sequence of $Cd^{2+} > Cu^{2+} > Ni^{2+} > Co^{2+}, Mn^{2+} > Zn^{2+}$. K^+ uptake was considerably more affected by these metals than was H^+ efflux which suggested that the inhibition of K^+ uptake was not solely a result of inhibition of the H^+-ATPase and other factors such as increased membrane permeability to K^+ may have contributed (**White & Gadd, 1987 a, b**).

Many divalent cations, such as Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+}, which are essential for growth and metabolism need to be accumulated from the external environment. However, above certain concentrations, many are toxic and may cause impairment of cell metabolism including ionic nutrition and ultimately cell death. Inessential metals may also be accumulated, e.g. Cd^{2+} and Hg^{2+}. Some divalent cations, such as Ca^{2+} and Mg^{2+}, may enter *S. cerevisiae* as low-affinity substrates of the monovalent cation transport system but this is probably of little significance in relation to the systems that will now be described.

In filamentous fungi and yeasts, energy-dependent transport of many divalent cations has been demonstrated, the apparent affinity series being Mg^{2+}, Co^{2+}, $Zn^{2+} > Mn^{2+} > Ni^{2+} > Ca^{2+} > Sr^{2+}$ (**Fuhrmann & Rothstein, 1968**). However, differences in accumulation may not be due to differences in affinities for the transport mechanism as some reductions in the net rate of Ca^{2+} and Sr^{2+} uptake, for example, may be due to increased leakage or efflux of these cations. In addition, depending on the metal and its concentration, there may be effects on the structural integrity of the membrane (**Gadd & Mowll, 1983; White & Gadd, 1987a**). As with monovalent cations, uptake of divalent cations is inhibited or halted by metabolic inhibitors, low temperatures and the absence of energy-yielding substrates (**Gadd & White, 1989 a; Sabie & Gadd, 1990; Starling & Ross, 1990**). It follows that uptake thus depends on the metabolic state of the cell and may vary with different growth media and conditions. For maximal uptake rates, cells require adequate K^+ and phosphate (**Fuhrmann &**

Rothstein, 1968; Okorokov et al., 1975; Roomans et al., 1979) and the phosphate requirement may be indirect and related to the energetic state of the cell as shown for Mn^{2+} (**Borst-Pauwels, 1981**).

Divalent cation transport is dependent on plasma membrane H^+-ATPase activity and a similar affinity sequence of divalent cations for stimulation of the ATPase as for transport has been demonstrated (**Fuhrmann & Rothstein, 1968**). Diethylstilboestrol (DES) is an effective in vitro and in vivo inhibitor of the plasma membrane H^+-ATPase (Bowman et al., 1978; Serrano, 1985) and also inhibits the uptake of divalent cations (**Borst-Pauwels, 1981; White & Gadd, 1987a**). However, a direct role of the H^+- ATPase has been discounted for several reasons. Initial transport rates of Mn^{2+} and Sr^{2+} in *S. cerevisiae* were similar, yet Mn^{2+} strongly stimulated the plasma membrane H^+-ATPase whereas Sr^{2+} had no effect (**Roomans et al., 1979; Nieuwenhuis, et al, 1981**). Even though influx rates of these two ions were similar, much more Mn^{2+} was subsequently accumulated than Sr^{2+} which suggested efflux systems and vacuolar Mn^{2+} compartmentalization (**Nieuwenhuis et al., 1981**). Calcium ions enter cells via ligand or voltage-gated channels. These are membrane proteins which, when in the open position, allow passive flux of Ca^{2+} down the electrochemical gradient, $\Delta\sim\mu_2 ca^{2+}$ (**Miller et al., 1990**). Influx of Ca^{2+} may be difficult to distinguish because of its energy-dependent accumulation in fungal vacuoles (**Eilam et al., 1985 a**).

It appears that the major role of the plasma membrane H^+-ATPase in the uptake of divalent cations is to energize the cell membrane by creating the electrochemical gradient previously mentioned. Transport depends on the membrane potential (**Boutry, et al, 1977; Borst- Pauwels, 1981; Eilam & Chernichovsky, 1987; White & Gadd, 1987a; Budd, 1988, 1989**) and uptake is inhibited by substances, such as protonophoric uncouplers and high concentrations of external K^+, which depolarize the cell membrane (**Roomans et al., 1979; Borbolla & Pena, 1980; Gadd & White, 1989 a**). Conversely, divalent cation uptake may be enhanced under conditions where the membrane potential is increased, e.g. by enhanced K^+ efflux (**Eilam, 1984; Eilam, et al, 1985 b; Gadd & Mowll, 1985; Eilam & Cherni- chovsky, 1987**). For *S. cerevisiae* treated with high concentrations of ATPase inhibitors, e.g. DCCD, the resulting hyperpolarization of the membrane caused increased Ca^{2+} uptake. The increase in A3f balanced the decrease in pH so that the overall value of ($\Delta\sim\mu_H{}^+$)

remained almost constant (**Eilam, et al 1984**). However, changes in ($\Delta\Psi$) only partly account for glucose-stimulated Ca^{2+} influx in this yeast with intracellular acidification also being involved (**Eilam, et al, 1990**). Furthermore, it should be stressed that inhibitor studies should be treated with caution since the enhancement of M^{2+} uptake in yeast induced by several plasma membrane ATPase inhibitors was due to increased cation permeability of the membrane rather than hyperpolarization (**Borst-Pauwels, 1988**). Similar conditions may apply to interactions of yeast with those metal cations or xenobiotics which are potentially toxic and induce K^+ efflux (**Gadd & Mowll, 1983; Gadd, 1986 a, c; Theuvenet et al., 1987; Borst-Pauwels, 1988; Belde et al., 1988**).

2.5.4 Intracellular fate of toxic metals

Metal-binding proteins and peptides of filamentous fungi and yeasts

Metal-binding proteins are important in the modulation of intracellular concentrations of both potentially-toxic and essential metal ions. The super family of proteins called metallothioneins, first discovered in mammalian systems, may achieve these roles by binding metal ions to cysteine thiolate groups (**Hamer, 1986**). It is now clear that a variety of metal binding polypeptides are found in animals, plants and fungi and there has been some progress in devising a coherent nomenclature (**Rauser, 1990**). Polypeptides are designated 'metallothioneins' if they have properties which include low molecular mass, high metal content, high cysteine (Cys) content, lack aromatic amino acids and histidine, possess abundant Cys-X- Cys sequences (where X is an amino acid other than Cys), spectroscopic properties characteristic of metal thiolates, and metal-thiolate clusters, all are the characteristics of equine renal metallothionein. Sub division into three classes has been recommended (**Rauser, 1990**):

Class I: Polypeptides with locations of cysteine closely related to those in equine renal metallothionein.

Class II: Polypeptides with locations of cysteine only distantly related to those in equine renal metallothionein.

Class III: Atypical, non-translationally synthesized metal thiolate polypeptides.

Class I includes most vertebrate forms as well as some fungal metallothioneins, e.g. those of *Neurospora crassa* and *Agaricus bisporus* (**Lerch & Beltramini, 1983;**

Munger & Lerch, 1985). Class II metallothioneins are found in *Saccharomyces cerevisiae* (**Steffens, 1990**). Algae, plants and certain fungi, e.g. *Schizosaccharomyces pombe* and *Candida glabrata* produce class III metallothionein (**Rauser, 1990**). The last occur as metal-binding complexes which behave like entities of relative molecular mass 10000-13800 in gel filtration (**Rauser, 1990**). However, these complexes are aggregates of a heterogeneous group of polypeptides for which no single name is suitable. In this article, the term metal y-glutamyl peptide will be used (**Winge et al., 1989**) although, in the context of plants, this may be inappropriate because of the diversity of y-glutamyl di- and tripeptides which may occur (**Rauser, 1990**).

Metallothioneins are small, cysteine-rich polypeptides that can bind essential metals, e.g. Cu and Zn, as well as inessential metals such as Cd.

Copper resistance in *S. cerevisiae* can be mediated by the induction of a 6573 Da cysteine-rich protein, copper-metallothionein (Cu-MT) (**Hamer, 1986; Butt & Ecker, 1987; Fogel, et al, 1988**). This protein normally functions to maintain low concentrations of intracellular copper ions and thus prevent futile transcription of the CUP1 structural gene (**Wright, et al, 1988**). Although yeast metallothionein can also bind Cd and Zn in vitro, it is not transcriptionally induced by these ions and apparently does not protect against them (**Butt et al., 1984; Winge et al., 1985**). *S. cerevisiae* contains a single Cu-MT gene which is present in the CUP1 locus located on chromosome VIII (**Fogel & Welch, 1982; Karin et al., 1984**). Copper-resistant *S. cerevisiae* strains (CUP1r) contain 2-14 or more copies of the CUP1 locus, which are randomly repeated, and may grow on media containing $\geq$ 2 mm copper (**Fogel, Welch & Karin, 1983; Karin et al., 1984**). Copper-sensitive strains ($cup1^s$) do not grow at concentrations $\geq$ 0.15 mM (**Butt & Ecker, 1987**). Continuous subculture of S. cerevisiae in the presence of increasing copper concentrations can select for hyper-resistant strains (**White & Gadd, 1986**) which appear disomic for chromosome VIII. The disomic chromosomes exhibit differential patterns of CUP1 gene amplification which indicates that the copper resistance mechanism involves not only amplification of the CUP1 locus on the chromosomes but also disomy or aneuploidy of chromosome VIII (**Fogel et al., 1983; Butt & Ecker, 1987**). The DNA fragments of the CUP1 locus that confer copper resistance have been cloned (**Fogel & Welch, 1982;Henderson, et al, 1985**) and has shown evidence that the CUP1 locus was present as multiple copies in CUP1 strains which was obtained after restriction

enzyme analysis of the cloned DNA (**Butt & Ecker, 1987; Fogel et al., 1988**). The basic repeating unit 'CUP1 locus' is composed of 2-0 kb DNA fragments containing, with respect to restriction enzymes, a unique Xba I site and two sites for Kpn I and Sau3A. The full nucleotide sequence of the CUP1 locus has been determined **(Karin et al., 1984)**. Copper resistance in *S. Service* depends on amplification of the CUP1 locus (**Butt & Ecker, 1987; Fogel et al., 1988**). In simple terms, the gene amplification model suggests that because of the proposed homology of DNA sequences at the junction of CUP1 repeats, one or more units are looped out. If the loop is replicated, the copy number increases (amplification) but if the loop is degraded, the copy number decreases (deamplification) (**Fogel et al., 1983; Butt & Ecker, 1987; Fogel et al., 1988**). For more precise accounts of this model, readers should consult the detailed reviews available (**Hamer, et al, 1985; Thiele & Hamer, 1986; Hamer, 1986; Butt & Ecker, 1987; Fogel et al., 1988**). It should be noted that Cu-MT is not directly involved in Cu^{2+} uptake in *S. cerevisiae* (**Lin & Kosman, 1990**).

The mechanism by which copper ions induce transcription of the Cu-MT gene has received detailed attention (**Thiele & Hamer, 198&6; Butt & Ecker, 1987; Thiele, 1988; Furst et al., 1988; Furst & Hamer, 1989; Culotta et al., 1989; Welch et al., 1989; Hu, et al, 1990**). It is now known that a trans-acting regulatory protein, encoded by the ACE1 locus (**Thiele, 1988; Furst et al., 1988**) and also known as the CUP2 locus (Welch et al., 1989; Buchman et al., 1989) activates transcription of the Cu-MT gene in response to excess copper (or silver) ions (**Hu et al., 1990**). Although the ACE1 gene is constitutively expressed in the absence or presence of copper, the apoprotein cannot bind DNA (**Furst et al., 1988**). However, in the presence of Cu (I) [or Ag (I)], the amino-terminal portion of the ACE1 protein (amino acids 1-122) undergoes a conformational change which allows the protein specifically to bind to the CUP1 upstream activator sequence (UAS) (**Furst et al., 1988; Huibregtse, et al, 1989**). This metal-dependent DNA-binding domain is rich in cysteines and basic amino acids and bears structural similarities to yeast Cu-MT itself (**Furst et al., 1988; Furst & Hamer, 1989; Hu et al., 1990; Dameron et al., 1991**). It is proposed that the cysteine residues of ACE1 form a poly-nuclear Cu(i)-S core in which cysteine thiolates coordinate multiple Cu(i) ions and Cu(i) ions which are bound by multiple sulphur bonds (**Hu et al., 1990**). Wrapping the protein around this Cu(i)-S scaffold

would lead to the formation of positively charged loop structures that are postulated specifically to bind DNA and activate transcription (**Furst et al., 1988**). It therefore appears that the role of Cu in yeast Cu-MT regulation is to allow ACE1 to bind to the control sequences of the metallothionein gene (Fig. 2.3) (**Hu et al., 1990**); the Cu cluster organizes and stabilizes the conformation of the N-terminal domain of ACEl for specific DNA binding (**Dameron et al., 1991**).

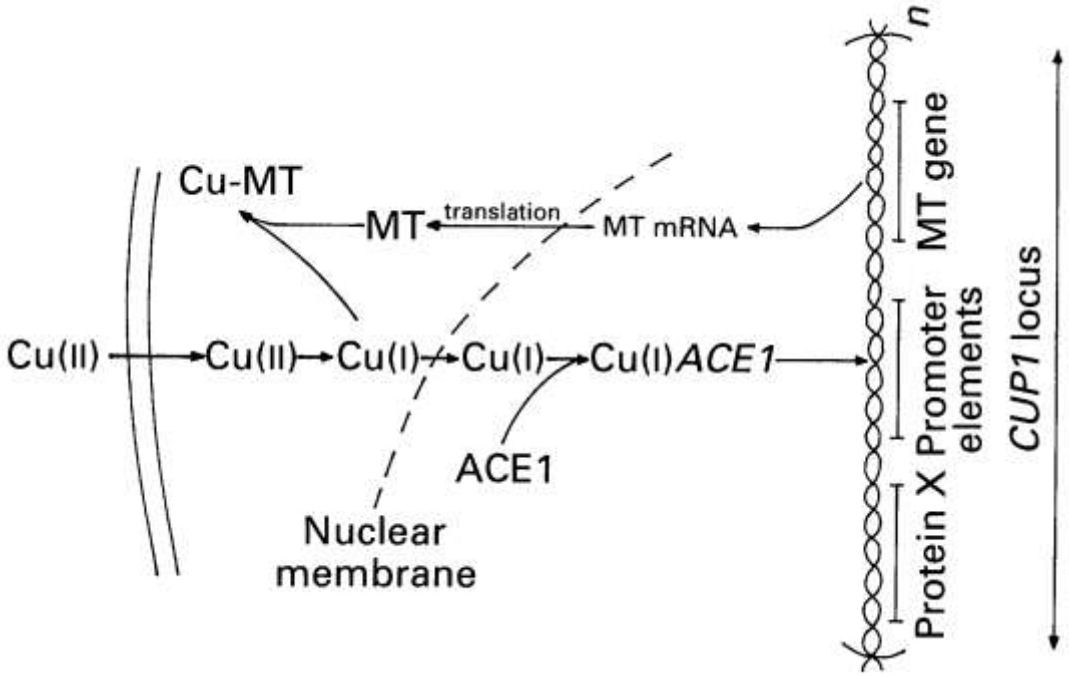


Fig. 2.3 Metalloregulation of the CUP1 locus in *Saccharomyces cerevisiae*.

The Cu-ACE1 metalloprotein complex interacts with upstream sequences from the CUP1 coding sequences and facilitates transcription of the MT gene with the CUP1 locus. Translation of the MT mRNA yields MT protein which acts to regulate the cytosolic concentration of copper ions (modified after **Mehra & Winge, 1991**).

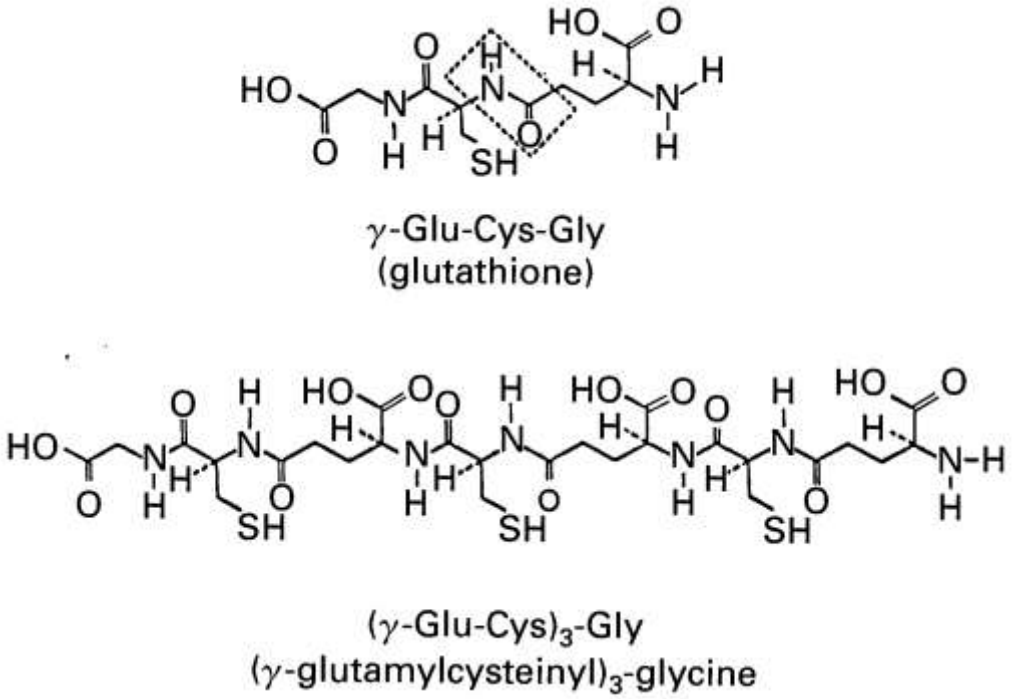


Fig. 2.4 Structures of glutathione and the metal-binding polypeptide, (γ-glutamylcysteinyl)3-glycine. Peptide bonds between glutamate and cysteine utilize the side chain, or γ-carboxylate of glutamate (outlined) rather than the az-carboxylate characteristic of proteins whose synthesis is ribosome dependent (modified after **Steffens, 1990**).

This metal-regulated system provides a powerful tool in biotechnology. Metal-regulated DNA sequences are efficient elements for heterologous gene expression and a variety of proteins have been expressed in the CUP1 expression system including human serum albumin, human ubiquitin gene, *E. coli galK* gene and human hepatitis virus antigen (**Gorman et al., 1986; Butt & Ecker, 1987**). Yeast Cu-MT may also be of potential in metal recovery since it can bind other metals besides Cu, e.g. Cd, Zn, Ag, Co and Au, although these metals do not induce MT synthesis (**Butt & Ecker, 1987**). The Cu-MT gene has also been used to transform brewing strains of *S. cerevisiae*, which are not very amenable to classical genetic studies and copper selection has proved a useful tool for the genetical manipulation of such strains and product improvement (**Butt et al., 1984; 1988; Henderson et al., 1985; Butt & Ecker, 1987**). When CUP1 was cloned into Escherichia coli, the bacterial cells acquired a new inducible ability to sequester toxic metals by metallothionein synthesis (**Berka et al., 1988**).

It should be mentioned that a Cd-resistant strain of *S. cerevisiae* synthesized a cytoplasmic Cd-binding protein when grown in Cd-containing medium (**Joho, et al, 1985 a; Joho, et al, 1985 b**). This protein (9kDa) exhibits the characteristics of a metallothionein, being rich in cysteine (18 mol%) and having a high Cd content (63 ,tg Cd (mg protein)$^{-1}$). There was a high similarity in amino acid composition between Cd and Cu-MT of *S. cerevisiae* (Inouhe et al., 1989). The Cd-MT of Cd- resistant *S. cerevisiae* is also encoded by the CUP1 gene and transcription of the CUP1 gene occurs in the absence of added metal ions unlike the inducible transcription in the Cu-resistant strain. Cadmium and copper further increase the rate of transcription of the CUP1 gene in the Cd-resistant strain (**Tohoyama et al., 1992**).

The similarity in structure between glutathione and y-glutamyl peptides indicates that biosynthesis of these peptides shares common features with that of glutathione (**Steffens, 1990**) and in fact they may be viewed as linear polymers of the y-glutamyl-cysteine (y-Glu-Cys) portion of glutathione (**Grill et al., 1989**). Because of the repetitive y-glutamic acid bonds, they cannot be regarded as primary gene products and must be formed by a ribosome- independent enzyme reaction (**Steffens, 1990**) (Fig. 2.4). Linkage of glutathione metabolism with that of y- glutamyl peptides has been kinetically demonstrated in vivo (**Grill, et al, 1987; Steffens, 1990**) and also by using mutants of S. pombe which lacked the glutathione biosynthetic enzymes, y-glutamylcysteine synthetase and glutathione synthetase. These were unable to synthesise glutathione or (y-Glu-Cys)n-Gly peptides and were hypersensitive to Cd (Mutoh & Hayashi, 1988). This work provided the first genetical evidence that y-glutamyl peptides were essential for Cd-detoxification and clearly demonstrated biochemical linkage with glutathione metabolism. It is now known that y-glutamyl peptides are synthesized by the enzyme y-glutamyl- cysteine dipeptidyl transpeptidase ('phytochelatin synthase'). This catalyses the transfer of the y- glutamylcysteine dipeptide moiety of glutathione to an acceptor glutathione molecule or a growing chain of (y-Glu-Cys)n-Gly oligomers, thus synthesizing metal-binding y-glutamyl peptides (**Grill et al., 1989**). The primary reaction can be expressed:

$$(\gamma\text{-Glu-Cys})\,\mathrm{Gly} + (\gamma\mathrm{Glu\text{-}Cys})_n\mathrm{Gly} \rightarrow (\gamma\text{-Glu-Cys})_{n+1}\mathrm{Gly} + \mathrm{Gly}.$$

Cd^{2+} is the best activator of the enzyme followed by Ag^{+}, Bi^{3+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+} and Au^{+} and it appears that the reaction product (y-glutamyl peptide) chelates the

activating metal ions thus terminating the reaction (**Grill et al., 1989; Loeffler et al., 1989**). In cell-free systems of *S. pombe*, an additional pathway has also been discovered which involves yEC polymerization from $(yEC)_n$ and glutathione to $(yEC)_{n+1}$ followed by addition of glycine with glutathione synthetase (**Hayashi et al., 1991**).

The originally discovered two y-glutamyl peptides in *S. pombe*, cadystin A and B (n = 2 and 3, respectively), were synthesized in response to Cd^{2+} (**Murasugi, et al 1981**) though other metals including Cu^{2+}, Pb^{2+}, Zn^{2+} and Ag^{+} were also effective (**Grill et al., 1987; Hayashi, et al., 1988**), a situation analogous to that in vascular plants (**Hayashi et al., 1988; Rauser, 1990**). However, the greatest amount of peptide synthesis occurs with Cd and it seems larger peptides bind Cd more strongly than smaller ones (**Hayashi et al., 1986, 1988**). When *S. pombe* was exposed to Cu(ii), a Cu-y-glutamyl peptide was synthesized similar in structure to the Cd-y-glutamyl peptide (Reese et al., 1988). However, only one Cu-y-glutamyl peptide complex which did not contain labile sulphur was observed; the metal was bound as Cu(i) (**Reese et al., 1988**).

Cadmium induced (but not copper-induced) y- glutamyl peptides contain labile sulphur. Cadmium stimulates the production of S^{2-} in *S. pombe* and *Candida glabrata* and a portion of this may be incorporated within the Cd-y-glutamyl peptide complexes (**Mehra et al., 1988; Reese & Winge, 1988; Winge et al., 1989**). The sulphide content is dependent on growth conditions and incubation time and concentrations of S^{2-}, ranging from 0.1 to 1.5 mol (mol peptide)-1, have been demonstrated (**Winge et al., 1989**). Incorporation of sulphide enhances the stability and metal content of the peptide complex (the molar sulphide: cadmium ratios ranged from < 0 06 in low sulphide forms to 0.8 in high sulphide forms) and this enhanced stability may increase the efficacy of the complex in maintaining a low intracellular concentration of Cd^{2+} (**Winge et al., 1989**). Complexes with a S: Cd ratio of 0.7 contain a 20 A diameter CdS crystallite coated with the y-glutamyl peptides. These complexes are quantum particles exhibiting size-dependent electronic states (**Dameron et al., 1991**), a feature of relevance to solid state physics since the crystallites exhibit properties analogous to those of semiconductor clusters (**Dameron et al., 1989**). The extracellular and cell- associated CdS colloid formed by interaction of Cd^{2+} with S^{2-} may also contribute to Cd^{2+} detoxification.

Candida (Torulopsis) glabrata is unique in expressing both metallothionein and y-glutamyl peptide synthesis for metal detoxification in a metal-specific manner (**Mehra et al., 1988, 1992a, b**). *C. glabrata* synthesizes two distinct MT polypeptides in response to copper which exhibit repeats of the Cys-X-Cys sequence, common in mammalian MT, and coordinates copper in Cu(i)-thiolate clusters. The cysteine content of the two molecules is 30 mol 0 *(S. cerevisiae* Cu-MT = 22 mol %; *N. crassa* Cu-MT = 28 mol %) with the copper content being between 10 and 13 mol per mol protein for each (Mehra et al., 1988). Two metallothionein genes (MT-I and MT-Il) from *C. glabrata* have been cloned and sequenced. The former is present as a single copy but multiple (3-9) and tandemly arranged copies of one MT-II gene are present in different strains. *C. glabrata* strains hyper-resistant to Cu showed further stable chromosomal amplification (>30 copies) of the MT-II gene (**Mehra, Garey & Winge, 1990**). Both genes were inducible by Ag but not by Cd, the later reducing amounts of both MT-I and MT-II mRNAs (**Mehra et al., 1989**). One MT-II gene, designated MT-II_a, was amplified in several wild-type strains of *C. glabrata* while another MT-II_b, occurs as a single copy (**Mehra et al., 1990**). The amplified MT-II_a gene contains autonomously replicating sequences (ARS) (**Mehra et al., 1992 b**). The Cd-y-glutamyl peptide of *C. glabrata* has n = 2 whereas peptides with n = 3 and 4 are more common in *S. pombe* and plants (**Grill et al., 1987; Reese et al., 1988**). Thus, *C. glabrata* can utilize two types of regulatory metal-binding molecules and the affinity and/or coordination preference of the molecules may determine whether MT or y-glutamyl peptides are synthesized (**Mehra et al., 1988, 1992a, b**).

The fungal vacuole is highly important organelle with functions including macromolecular degradation, storage of metabolites and cytosolic ion and pH homeostasis (**Wiemken, Schellenberg & Urech, 1979; Davis, 1986; Wada et al., 1987; Jones & Gadd, 1990**). It is often considered analogous to the mammalian lysosome because hydrolytic enzymes are contained in an acidic compartment although it has greater similarity to vacuoles of plant cells (**Matile, 1978; Achstetter & Wolf, 1975; Klionsky, et al, 1990**).

A vacuolar ATPase has been identified in several fungi, notably *S. cerevisiae, S. carlsbergensis* and *N. crassa* (**Okorokov et al., 1985; Bowman & Bowman, 1986; Anraku et al., 1989**) and is differentiated from mitochondrial and plasma membrane ATPases by means of pH optima, subunit composition and structure, and inhibitor

sensitivity (**Klionsky et al., 1990**). The vacuolar ATPase utilizes the energy arising from ATP hydrolysis to pump H^+ into the vacuole resulting in the generation of an electrochemical proton gradient of about 180 mV across the vacuolar membrane (tonoplast) **(Kakinuma, et al, 1981; Bowman & Bowman, 1986**). In the presence of an energy source, a pH gradient of 0-5-1V5 units can be generated across the tonoplast (vacuolar pH generally ~pH 6) (**Jones & Gadd, 1990**) and a membrane potential of- ~75 mV has been recorded for *S. cerevisiae* and 25-40 mV for *N. crassa* (**Klionsky et al., 1990**). The electrochemical proton gradient energizes transport of monovalent and divalent cations as well as other substances including basic amino acids into the vacuole (**Jones & Gadd, 1990; (Klionsky et al., 1990**). The main mechanism of transport appears to be proton antiport, although there is some evidence of a pyrophosphatase activity in *S. carlsbergensis* which may be responsible for a PP_i-dependent pH gradient (**Klionsky et al., 1990**). The gradients of pH and electrical potential difference across the tonoplast may be regulated through the interactions of a membrane potential-dependent cation channel (**Wada et al., 1987; Bertyl & Slayman, 1990**), chloride transport systems (**Wada, et al, 1986**) and the vacuolar ATPase itself (**Anraku et al., 1989**).

The fungal vacuole has an important role in the regulation of the cytosolic concentrations of metal ions, both for essential metabolic functions and the detoxification of potentially-toxic metal ions. Many ions, including inorganic phosphate (P_i) and monovalent and divalent metal cations, are preferentially located in vacuoles (**Ohsumi, et al, 1988**). Ratios of [total ion]: [cytosolic ion] may be 4 for K+, 4-28 for Mg^{2+}, > 70 for Mn^{2+} and 10 for Ca^{2+} (**Lichko, et al, 1982; Jones & Gadd, 1990**). The mechanism of ion transport across the tonoplast is by H^+ antiporters and in *S. carlsbergensis* apparent Km values for the transport of Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Pi are 004, 03, 08, 0-055-017 and 15 mm, respectively. It is assumed that transport proceeds until cytosolic concentrations of ions approach these values (**Okorokov et al., 1985**). These ionic substrates can stimulate vacuolar ATPase activity (**Okorokov, 1985**) although it is noteworthy that activity is inhibited by free Mg^{2+} at 04-05 mm (**Borst-Pauwels & Peters, 1981**). Localization of metal ions in the vacuole enables low cytosolic concentrations of, for example, Ca^{2+} to be maintained (**Ohsumi & Anraku, 1983; Eilam et al., 1985 a, b; Okorokov et al., 1985; Eilam & Chernichovsky, 1987; Cornelius & Nakashima, 1987; Ohsumi et al., 1988**).

Cytosolic concentrations of Mg^{2+} and Mn^{2+} may remain relatively constant, even under considerable environmental perturbations (**Lichko et al., 1982; Kihn, et al, 1988**). There is further evidence for compartmentation of Zn^{2+}, Co^{2+} and iron in yeast vacuoles, which may contribute to tolerant behaviour (**White & Gadd, 1986, 1987a; Raguzzi, et al, 1988**). Mutants of *S. cerevisiae* defective in vacuolar function were sensitive to Ca^{2+} and other toxic metals . Polyphosphates, the only inorganic macromolecular anion found in the vacuole, have an important role in maintaining ionic compartmentation and their biosynthesis accompanies vacuolar accumulation of Mg^{2+} or Mn^{2+} (**Okorokov et al., 1977, et al, 1980; Lichko et al., 1982; Okorokov et al., 1983 a, b; Miller, 1984; Kihn et al., 1988**). The significance of polyphosphate granules as a store for divalent cations has long been appreciated (**Doonan et al., 1979; Roomans, 1980**). In *S. cerevisiae*, vacuolar inclusions of Ca^{2+}-polyphosphate complexes vary with the amount of available polyphosphate **(Ohsumi et al., 1988**) although this was not the sole mechanism of Ca^{2+} 'trapping' in the vacuole (**Ohsumi & Anraku, 1983**). Vacuolar Zn^{2+} has also been associated with polyphosphate bodies in yeast (**Bilinski & Miller, 1983**) while intravacuolar deposits have also been observed in yeast grown in the presence of thorium (**Gadd & White, 1989b**). In the absence of an energy source, precipitation of Mn^{2+} within yeast cells has been demonstrated using Electron Spin Resonance (ESR) spectroscopy (**Kihn et al., 1988**).

2.6 Metal transformations

Fungi, as well as other microorganisms, can effect chemical transformations of metals by oxidation, reduction, methylation and dealkylation (**Gadd, 1992 a**). Although in contrast to bacteria detailed information is not available in several areas for fungi. Some enzymatic metal transformations may be involved in survival since certain transformed metal species are less toxic and/or more volatile than the original species. Plasmid-mediated reduction of Hg^{2+} to Hg° is a well-characterized bacterial metal resistance system and there is only a limited amount of evidence for an analogous process in filamentous fungi and yeasts (**Vonk & Sijpesteijn, 1973; Brunker & Bott, 1974; Yannai, et al, 1991**). Other reductions carried out by fungi include Ag+ to metallic Ag° which is deposited in and around cells (**Kierans et al., 1990**) and the reduction of Cu (ii) to Cu (i) by cell wall associated materials in *Debaryomyces hansenii* (**Wakatsuki, et al, 1988; Wakatsuki et al., 1991 a, b**). A copper-reducing enzyme, copper reductase, catalyses Cu (II) reduction of Cu (I) with NADH or

NADPH as e- donor (**Wakatsuki et al., 1991 a, b**). The location of copper reductase appears to be at the cell wall and this process may have a role in regulation of copper uptake (**Wakatsuki et al., 1991 b**). Fungal reductions of metalloids include reduction of tellurite to elemental tellurium, which can appear as a black deposit, located in the endoplasmic reticulum (**Corfield & Smith, 1970; Smith, 1974**) and the reduction of selenate or selenite to form amorphous selenium which imparts a red colour to fungal colonies owing to deposition primarily in walls and membranes (**Konetzka, 1977**).

Methylation of Hg and other metals and metalloids can be catalyzed by several fungi and may be viewed as a detoxification mechanism since methylated species are usually more volatile and may be lost from the environment (**Landner, 1971; Yannai et al., 1991; Gadd, 1992a, b**). There is a general agreement that methylation of Hg^{2+} involves methy- cobalamin (vitamin B12) (**Craig, 1986; Thayer, 1988**).

Biomethylation of As by transfer of carbonium ions (CH_3^+) from S-adenosylmethionine (SAM) has also been demonstrated **(Thayer, 1984, 1989; Andreae, 1986**). Original work by **Challenger (1945)** first demonstrated that trimethylarsine could be produced by several fungi from arsenite. Biomethylation of Se appears to be by an analogous mechanism to that of arsenic and several fungi can produce organoselenium compounds, e.g. dimethylselenide from inorganic selenium ones (**Chau & Wong, 1986; Thayer, 1988; Thompson-Eagle, et al, 1989**). Certain fungi may be capable of producing volatile dimethyl telluride from tellurium salts (**Konetzka, 1977; Chau & Wong, 1986**). Apart from these examples, fungal involvement in the methylation of other metals and metalloids has not been examined in detail. Organometallic compounds (which contain at least one metal-carbon bond) arise in the environment naturally, by abiotic and biotic means, and because of accidental and deliberate introduction. The later mainly results from the use of several organometallic and organometalloid compounds as biocides, e.g. organoarsenicals, organomercurials and organotins (**Craig, 1986; Thayer, 1988; Cooney & Wuertz, 1989**). Degradation involves the breaking of the metal-C bonds though detailed information about such processes is rather sparse for fungi. It should be noted that metal-C bonds can be disrupted by physico-chemical means, although fungal activity may enhance the conditions necessary for abiotic attack by, e.g. alteration of pH or metabolite excretion. Organotin degradation involves sequential removal of organic groups from the tin atom, a process generally resulting in a decrease in toxicity

(Blunden & Chapman, 1986). Degradation of tributyltin oxide and tributyltin naphthenate, used as wood preservatives, can be achieved by fungal action. Breakdown products include mono- and dibutyltins (**Barug, 1981; Orsler & Holland, 1982**). Organomercurials may be detoxified by organomercury lyase, the resulting Hg^{2+} being then reduced to Hg^{0} by mercuric reductase (**Tezuka & Takasaki, 1988**) a similar mechanism that found in bacteria. Trimethyl lead can be degraded by *Phaeolus schweinitzii*, a process with implications for fungal- treatment of wastes (**Macaskie & Dean, 1990b**).

2.7 Accumulation of metals by macrofungi

Elevated concentrations of heavy metals and radio nuclides often occur in the fruiting bodies of basidiomycete fungi when growing in polluted environments (**Brown & Hall, 1990**). This phenomenon is, as yet, poorly understood at the physiological level but is of significance because of the widespread consumption of edible wild fungi in many countries. In general, higher concentrations of Pb, Cd, Zn and Hg are found in macrofungi from urban or industrial areas although there are differences in uptake abilities between different species and different metals (**Tyler, 1980; Bressa, et al, 1988; Lepsova & Mejstrik, 1989; Brown & Hall, 1990**). Cadmium appears to be accumulated to quite high values in macrofungi, averaging around 5 µg g^{-1} d.wt though concentrations of up to 40 µg g^{-1} d. wt have been recorded (**Byrne, et al, 1976**). Average concentrations of As, Cu, Hg, Mn, Se and Zn in a range of up to 27 species were 1, 50, 2, 3, 30, 15 and 114 µg g^{-1}d. wt. respectively (**Byrne et al., 1976**). However, a wide range of accumulation values can occur depending on the species and location of growth. Caps of *Laccaria amethystina* exhibited total As concentrations of 100-200 µg g^{-1} d. wt. (**Stijve, et al, 1990; Byrne et al., 1990**). Dimethylarsinic acid was a major arsenic compound in cap extracts, providing tentative evidence for biomethylation of this element (**Byrne et al., 1990**). Accumulation of 110Ag and 203Hg was studied in *Agaricus bisporus* and concentration factors (metal concentration in mushroom:metal concentration in substrate) were found to be up to 40 and 37 respectively, with the highest Ag and Hg contents recorded being 167 and 75 g g^{-1} d.wt respectively. Silver-protein complexes were isolated which possibly corresponded with metallothioneins or analogous metal-binding proteins **(Byrne et al, 1990**).

2.8 Biotechnological aspects of fungal metal accumulation

Fungi, in common with other microbial groups, can accumulate metals and radionuclides from their external environment by means of the physico- chemical and biological mechanisms already discussed. Living and dead cells are capable of metal/ radionuclide removal from solution as well as excreted or derived products, e.g. cell wall constituents, pigments, polysaccharides, proteins and siderophores (**Gadd, 1988; Pighi, et al, 1989; Siegel, et al, 1990**). The removal of radionuclides, metal or organometal(loid) species, compounds or particulates from solution by microbial biomass is now often referred to as 'biosorption', particularly where predominant interactions are physico-chemical. Biosorption is an area of increasing biotechnological interest since the removal of potentially toxic and/or valuable metals and radionuclides from contaminated effluents can be used to detoxify them prior to environmental discharge. Furthermore, appropriate treatment of loaded biomass can enable recovery of valuable metals for recycling or further containment of highly toxic and/or radioactive species. Detailed reviews of this research area are available (Table 2.2) (**Gadd, 1988, 1990a, 1992 a, b**).

Although the basic features of metal accumulation are common to most microbial groups, fungi possess a number of unique attributes which reflect their morphological and physiological diversity. The majority of fungi exhibit a filamentous or hyphal growth form which enables efficient colonization of substrate.

Table 2.2 Some examples of metal and actinide accumulation by fungi

Organism	Element	Uptake (% dry weight)
Phoma sp.	Silver	2
Penicillium sp.	Uranium	8–17
Rhizopus arrhizus	Copper	1·6
	Cadmium	3
	Lead	10·4
	Uranium	19·5
	Thorium	18·5
	Thorium†	11·6
	Silver	5·4
	Mercury	5·8
Aspergillus niger	Thorium	18·5
	Thorium†	13·8
	Uranium	21·5
Saccharomyces cerevisiae	Uranium	10–15
	Thorium†	12
	Zinc	0·5
Yeast (14 strains)	Silver	0·05–1
Yeast (4 strains)	Copper	0·05–0·2

As described previously, a variety of mechanisms occur in fungal cells for the removal of metals and radionuclides from solution. These mechanisms range from physico-chemical interactions, such as adsorption, to processes dependent on cell metabolism, such as intracellular accumulation or extracellular precipitation of metals as a result of excreted metabolites. It is therefore assumed that there may be considerable differences in the mechanisms involved, depending on whether organisms are living or dead and, if living, whether growth takes place.

Metal uptake by living fungi can often be divided into two main phases. The first, which can also occur with dead cells, is often rapid, metabolism-independent binding to cell walls and other external surfaces; the second is energy-dependent intracellular uptake across the cell membrane (**Tsezos, et al, 1986; Gadd, 1986a, b, 1988; Huang et al., 1990**). In some cases, particularly where toxicity is evident, intracellular uptake may not be linked with metabolism but may be a consequence of increased membrane permeability and resultant exposure of metal-binding sites within the cell. These phases of uptake may not be clearly seen in all organisms. For certain radionuclides, e.g. actinides, it appears that accumulation may primarily be a result of physico-chemical interactions with cell walls with little or no intracellular uptake whereas with others, e.g. caesium, most accumulation is intracellular. In growing cells, either or

both phases of uptake may be obscured by additional aspects of metabolism, e.g. extracellular products which may form complex or precipitate metals outside the cells.

Various treatments may be employed to increase the capacity of fungal biomass for metal and/or radionuclide adsorption. Certain killing treatments may increase the adsorption capacity and powdering of dried biomass exposes additional binding sites (**Tobin et al., 1984**). Detergent treatment has also been used to increase the capacity of fungal biomass for adsorption, since this can result in disruption of cell components, such as membranes, and expose a wider variety of potential binding sites. Detergent treatment of *Penicillium spinulosum* greatly improved removal of Cu^{2+} from solution especially at high Cu^{2+} concentrations (**Ross & Townsley, 1986**). Detergents also increased uptake of thorium by *Saccharomyces cerevisiae* and *Rhizopus arrhizus*, although the effect was not as significant as for *P. spinulosum* (**Gadd & White, 1989c**). However, the conditions for thorium uptake (in 1 M HNO_3) were markedly different. It was likely that the biomass was already substantially permeabilized prior to detergent treatment. In *S. cerevisiae,* uranium uptake was increased by HCHO and $HgCl_2$ (**Strandberg et al., 1981**) again probably because of increased cell permeability.

In some circumstances, fungi can adsorb insoluble metal compounds, e.g. sulphides, and this may represent another area of potential biotechnological application. *Aspergillus niger* oxidized copper, lead and zinc sulphides to sulphates, the sulphide particles in the medium being adsorbed on to mycelial surfaces (**Wainwright et al, 1986**). *Mucor flavus* could adsorb lead sulphide, zinc dust and $Fe(OH)_3$ ('ochre') from acid mine drainage (**Singleton et. al., 1990**). *A. niger, Fusarium solani* and *P. notatum* could also remove ochre from solution but not as efficiently as *M. flavus* (**Wainwright, et al, 1986**). Intact, fresh mycelium was best for adsorption at 25°C; the presence of a carbon source was unnecessary. When *M. flavus* was grown with PbS for 2-5 d, it converted small particles of the compound to a fine and even suspension which was then completely adsorbed after 7 d (**Wainwright, et al, 1986**). Magnetite-loaded fungal biomass was susceptible to a magnetic field which has implications for removal of biomass from solution (**Wainwright et al., 1990; Dauer & Dunlop, 1991**).

Metabolism-dependent intracellular uptake or transport of metal ions into cells may be a slower process than adsorption and is inhibited by low temperatures, the absence

of an energy source (e.g. glucose) and by glucose analogues, metabolic inhibitors and uncouplers **(Ross, 1977; Norris & Kelly, 1977, 1979; Borst-Pauwels, 1981; Gadd, 1986a, b; Starling & Ross, 1990**). In certain fungi, especially yeasts, greater amounts of metal may be accumulated by such a process, in conjunction with internal sequestration than by metabolism-independent processes (**Ross, 1977; Norris & Kelly, 1977, 1979**). Many metals are essential for fungal growth and metabolism, e.g. Cu, Fe, Zn, Co and Mn, and all organisms must have the ability to accumulate these intracellularly from low external concentrations so that physiological needs are satisfied. However, at the relatively high concentrations of metals frequently used in uptake studies, energy-dependent intracellular uptake may be difficult to characterize and may not be as significant a component of total uptake as general biosorption. This is particularly true for filamentous fungi (**Gadd & White, 1985; Gadd, et al, 1987**). For such organisms, most published work suggests that general metabolism-independent processes such as adsorption and complexation make up the majority of total metal uptake by the biomass (**Duddridge & Wainwright, 1980; Ross & Townsley, 1986**). For uranium and thorium, it appears that little or none may enter cells (**Shumate & Strandberg, 1985**) although little detailed work on living cells has been carried out. However, yeasts may exhibit intracellular uptake of thorium (**Gadd & White, 1989b**). These organisms as well as other fungi may also precipitate metals around the cells as a result of metabolic processes and are capable of synthesizing intracellular metal-binding proteins.

In actively growing fungal cultures, the phases of adsorption and intracellular uptake may be obscured by changes in the physiology and morphology of the fungus and the physical and chemical properties of the growth medium (**Gadd, 1986a**). A frequently observed phenomenon is that metal uptake by growing batch cultures is maximal during the lag period or early stages of growth and declines as the culture reaches the stationary phase. This has been shown for copper and *Aurobasidium pullulans* (**Gadd & Griffiths, 1980b**), *Debaryomyces hansenii* (**Wakatsuki et al., 1979**) and *P. spinulosum, A. niger* and *Trichoderma viride* (**Townsley & Ross, 1985, 1986; Townsley, Ross & Atkins, 1986a**). Changes in the kinetics of Cu^{2+} uptake by *A. pullulans* were related to the fall in medium pH during growth and the alleviation of Cu^{2+} toxicity at low pH (**Gadd & Griffiths, 1980b**). However, for several filamentous fungi reduction of pH of the medium was not the only factor responsible

for reduced Cu^{2+} uptake. A reduction could occur before any significant drop in the pH and in the media where the pH was maintained at 5.5 (**Townsley & Ross, 1985, 1986**). Explanations for this include alterations in cell wall composition during growth and/or the release of metabolites, e.g. citric acid, that can control metal availability (**Townsley & Ross, 1985, 1986**). Complexation may be involved in uptake and may partly explain the differences in uptake capacities observed between non-growing and growing fungal cultures (**Townsley & Ross, 1986**). *Candida utilis* exhibits cyclical accumulation of Zn^{2+} in batch culture with the highest uptake during the lag period and late exponential phase; it was suggested that there was regulation of Zn^{2+} uptake in this organism (**Failla & Weinberg, 1977**). At low (10 nM) external Mn^{2+} concentrations, intracellular [Mn^{2+}] remained relatively constant in batch cultures of *C. utilis* and there appeared to be a specific, high affinity Mn^{2+} transport system (**Parkin & Ross, 1986 a, b**).

As mentioned previously, many fungi have the ability to tolerate high concentrations of potentially toxic metals which may be useful when employing living cells in recovery systems. However, tolerance may be related to decreased intracellular uptake and/or impermeability of the cells toward metal ions (**Gadd, 1986 a**). A close connection between intracellular uptake and toxicity of Cu, Cd and Zn has been shown in *S. cerevisiae* (**Ross, 1977; Ross & Walsh, 1981; Gadd & Mowll, 1983; Mowll & Gadd, 1983; Joho etal., 1983, 1985a, b; Gadd etal., 1984b; White & Gadd, 1986***).* Melanized chlamydospores of *A. pullulans* are impermeable to heavy metals and more tolerant than hyaline cell types (**Gadd, 1984; Mowll & Gadd, 1984**). In contrast to the above examples, a Mn-resistant mutant of *S. cerevisiae* accumulated more manganese than the sensitive parental strain, probably as a result of an efficient internal sequestration system of greater efficiency in the resistant strain (**Bianchi et al., 1981 a, b**).

Where a reduction in intracellular uptake occurs at low external pH, a corresponding reduction in toxicity may also take place. *P. ochrochloron* can grow in saturated $CuS0_4$ at pH 0 3-2-0, but is sensitive to $\geq 4 \times 10^{-5}$ M near neutrality (**Gadd & White, 1985**). For *P. ochrochloron*, a constant rate of Cu2+ uptake occurs above approximately 16 mm at low pH, but at pH 6 and above uptake markedly increases and is concomitant with toxicity (**Gadd & White, 19**85).

2.9 Metal removal by inducing, excreted or derived biomolecules

Some metal-sequestering biomolecules may be induced by the presence of certain metals, e.g. metallothioneins and phytochelatins or by deprivation of an essential metal ion, e.g. Fe^{3+}, leading to siderophore production (**Raymond et al., 1984**). Other molecules with significant metal-binding abilities may be overproduced as a result of exposure to potentially toxic metal concentrations, e.g. fungal melanins (**Gadd, 1986a, 1988**). However, the majority of metal-binding biomolecules found in fungi are synthesized or excreted as a result of 'normal' growth and/or are important structural components, particularly in cell walls (**Macaskie & Dean, 1990a**). Metal-binding by such compounds may be fortuitous and relative efficiencies may largely depend on the metal species present and the chemical nature and reactivity of the metal binding ligands present (**Volesky, 1990; Tsezos, 1990; Tsezos et al., 1986**). It should be noted that the macromolecular composition of a given fungal species can be altered by cultural and genetic manipulations and there is diversity in chemical composition, particularly in relation to cell walls. As described earlier, many fungi have a high chitin content in cell walls and this polymer of N-acetyl glucosamine is a highly effective metal and radio- nuclide biosorbent (**Tsezos, 1986, 1990; Macaskie & Dean, 1990a**). For uranium, rapid coordination to the amine nitrogen of chitin and simultaneous adsorption in the cell wall chitin structure are followed by slower precipitation of uranyl hydroxide. Accumulation of hydrolysis products continues until a final equilibrium is achieved. Chitosan and other chitin derivatives also have significant biosorptive capability (**Tsezos, 1986**). Other biomolecules of probable interest from fungi include phenolic polymers, melanins, mannans, phosphomannans, other polysaccharides, proteins (including metallothioneins and other metal-binding proteins) and externally-excreted metal-binding molecules, including siderophores, and citric and oxalic acids (**Gadd, 1986 a, 1990 a)**.

2.10 Metal recovery

Biotechnological exploitation of metal accumulation by microbial biomass, including fungi, may depend on the ease of metal recovery for subsequent reclamation, containment and biosorbent regeneration (**Tsezos, 1984, 1986, 1990; et al 1989; Volesky, 1990; Gadd & White, 1992**). The means of metal recovery may depend on the ease of removal from the biomass which in turn can depend on the metal species involved and the mechanism of accumulation. Metabolism-independent biosorption is frequently reversible by non-destructive methods whereas energy-dependent

intracellular accumulation and compartmentation/binding to induced proteins etc. are often irreversible and require destructive recovery (**Gadd, 1988**). The later may be achieved by incineration or dissolution in strong acids or alkalis (**Brierley et al., 1985**). Most work has concentrated on non-destructive desorption which should ideally be highly efficient, economical and result in minimal damage to the biosorbent. Dilute mineral acids (~ 0 1 M), e.g. HNO_3, HCI or H_2SO_4, can be effective for the removal of heavy metals and radionuclides from fungal biomass (**Tsezos et al., 1987**) although at higher concentrations (> 1 M) or with prolonged exposure there may be irreversible damage which reduces the potential of the biomass to be reused (**Tsezos, 1984**). Organic complexing agents may be effective, although relatively expensive, desorbers without affecting integrity of the biomass. EDTA treatment may also be useful in distinguishing surface complex formation from intracellular, unexchangeable metal (**Galun et al., 1983; Tsezos et al., 1987**).

Carbonates and/or bicarbonates are efficient desorption agents with potential for cheap, nondestructive metal recovery. Of several elution systems examined for uranium desorption from *R. arrhizus*, $NaHCO_3$ exhibited> 90 % efficiency of removal and high uranium concentration factors (**Tsezos, 1984**). The solid: liquid ratios can exceed 120:1 (mg:ml) for a 1 M $NaHCO_3$ solution with eluate uranium concentrations of at least 1-98 x 104 mg 1-1 (**Tsezos, 1984**). The solid:liquid (S/L) ratio, i.e. the ratio of weight of loaded biomass to the eluant volume required for complete recovery, needs to be maximized in any recovery process in order to yield an eluate of minimal volume yet containing the highest possible metal concentration (**Tsezos, 1990**).

2.11 Biosorption of meals and radionuclides by fungal biomass

In a recent study by **Mishra and Malik** (2012) the effectiveness of fungal isolates, *Aspergillus lentulus* (FJ 172995) for concurrent removal of heavy metals like chromium, copper and lead from industrial effluent was examined. They inferred that Cr, Cu, Pb, and Ni tolerant *Aspergillus lentulus* accumulated a significant amount of each metal. The removal metals from synthetic solutions showed the trend like Pb^{2+} (100%) > Cr^{3+} (*79%) > Cu^{2+} (78%) > Ni^{2+} (42%) after 5 days. When the same fungal strain was used to treat the multiple metal contamining electroplating effluent, the metal concentrations declined by 71%, 56%, and 100% for Cr, Cu and Pb respectively within 11 days.**Congeevaram et al 2007** concluded in another study that mainly pH was attributed to organism specific physiology of biosorption. Many

species of fungi have been reviewed by **Wang and Chen** (2009) whose biomass adsorbed considerable amount of heavy metals (Table 2.3).

Table 2.3 Biosorption by fungal biomass (mg g^{-1})

Fungal species	**Metal ions**
Aspergillus niger, Mucor rouxii & Rhizopus arrhizus	Au
Penicillium spp.	Ag, Cu, Cd, Pb
Penicillium, Aspergillus, Trichoderma, Rhizopus, Mucor, Fusarium spp	Pb, Cu, Cd, Zn Th, U, Cs, La
Phanerochete chrysoporium	Cd, Pb, Cu

Source: Wang & Chen (2009)

Radionuclide uptake has been examined in a range of filamentous fungi and yeasts, mainly from the perspective of environmental biotechnology and while the mechanism(s) involved in uptake may vary between different elements and species, actinide accumulation appears mainly to comprise metabolism-independent biosorption (**Tobin et al., 1984**). The main site of actinide uptake is the cell wall (**Weidemann et al., 1981; Tsezos et al., 1986; Volesky, 1990**), although permeabilization of cells with carbonates or detergents can increase uptake, indicating that intracellular sites are also capable of binding metals (**Gadd & White, 1989c**). The mechanism of biosorption varies between elements. Both adsorption and precipitation of hydrolysis products occurs with uranium (**Tsezos & Volesky, 1982 a**) while coordination with cell wall nitrogen was the main mechanism for thorium (**Tsezos & Volesky, 1982 b, Tsezos, 1983**). Precipitation or crystallization of radionuclides within or on cell walls may also be involved in some circumstances (**Galun et al., 1987**).

In *S. cerevisiae,* uranium was deposited as a layer of needle like fibrils on the cell walls reaching up to 50% of the dry weight of individual cells (**Strandberg et al., 1981**). Such precipitation has also been observed for thorium (**Tsezos, 1986; Gadd & White, 1989 a, b**). Under some conditions thorium may also accumulate intracellularly in *S. cerevisiae* with preferential localization in the vacuole (**Gadd & White, 1989c**).

Biosorption of uranium and thorium can be affected by the external pH. Initial rates of uranium uptake by yeast increase above pH 2.5 (**Strandberg et al., 1981**). At pH values below 2.5, the predominant species is UO_2^{2+} but, at greater pH values hydrolysis products include $(UO_2)_2(OH)_2^{2+}$, $UO_2(OH)+$ and $(UO_2)_3(OH)^{5+}$. The resulting reduction in solubility favours biosorption (**Tsezos & Volesky, 1982 a, 1983**). Similar phenomena occur with thorium where at pH values below 2, Th^{4+} is the main species present. At higher pH values $Th(OH)_2^{2+}$ and other products are formed which are absorbed more efficiently than Th^{4+} (**Tsezos & Volesky, 1982b**). Most previous studies on thorium biosorption have been conducted at pH ranges above 2. This is representative of many natural waters but certain industrial process streams containing actinide elements are extremely acidic (pH < 1). Furthermore, other cations like Fe^{3+}, which may be present in waste liquors, can interfere with actinide uptake. Consequently, if biomass is used to remove actinides from these waste streams, it must be capable of performing at pH values <1 and in the presence of potentially competing chemical components. Despite these difficulties, biomass from several fungal species removes thorium from solution in 1 M HNO_3, pH 0-1 (**Gadd & White, 1989 a, b; White & Gadd, 1987**). Thorium uptake was altered by the biomass concentration, the uptake per unit biomass being reduced at high biomass concentrations. Thorium uptake was also increased by detergent pretreatment and in the case of filamentous fungi varied with culture conditions. This implies that the characteristics of thorium uptake by fungal biomass can be manipulated to achieve optimal performance by these or similar means (**Gadd & White, 1989b**). Strains of fungi which gave the best performance as thorium biosorbents in batch culture under acidic conditions were examined in several bioreactor configurations (**White & Gadd, 1987**). Static or stirred bed bioreactors removed thorium unsatisfactorily probably because of poor mixing. However, an air-lift reactor removed approximately 90-95% of the thorium supplied over extended time periods. This type of reactor

provided efficient circulation and effective contact between the thorium solution and the fungal biomass. Of the species tested, *A. niger* and *R. arrhizus*, used as mycelial pellets, were the most effective with loading capacities of approximately 116 and 138 mg g^{-1}d.wt at an inflow concentration of 3 mm thorium (as nitrate). The efficiency of thorium biosorption by *A. niger* was markedly reduced by other inorganic solutes while thorium uptake by *R. arrhizus* was relatively unaffected. Air-lift bioreactors containing biomass of *R. arrhizus* could remove thorium from acidic solution (1 M HNO_3) over a wide range of initial concentrations (01-3 mM) (**White & Gadd, 1987**). Thus, biosorption using fungal biomass is a technically feasible method for the removal of thorium from acidic solutions similar to those encountered in industrial process streams. However, the practicality of this approach is very much dependent on economic considerations. The availability of waste biomass or cheap growth substrates may favour such a process while others, such as the need for handling and transport of a bulky biosorbent or the need for on-site propagation, would mitigate against it. The balance of these factors may only be determined in individual cases by detailed costing.

Das et al., (2008) made a review on biosorption of heavy metals by various microbes of which filamentous soil fungi made a crucial role as biosorbent.

A summarized version of the biosorptive capacity of various metals by fungal species are given in Table 2.4

Table 2.4 The biosorptive capacity of various fungal organisms

Biomass type	**Metal**	**Biosorption capacity (mg/g)**	**Reference**

Aspergillus sydoni	Cr(VI)	1.76	Kumar et al. (2008)
Aspergillus niger	Cr(VI)	3.1	Mungasavalli et al. (2007)
Aspergillus niger	Pb	32.60	Dursun (2006)
Aspergillus niger	Cu	15.6	Dursun et al. (2003a)
Aspergillus niger	Pb	34.4	Dursun et al. (2003a)
Aspergillus niger	Pb	93	Spanelova et al. (2003)
Aspergillus niger	Cu	9.53	Dursun et al. (2003b)
Aspergillus niger	Pb	2.25	Kapoor et al. (1999)
Aspergillus niger	Cd	1.31	Kapoor et al. (1999)
Aspergillus niger	Cu	0.75	Kapoor et al. (1999)
Aspergillus niger	Ni	1.75	Kapoor et al. (1999)
Aspergillus niger	Cu	23.62	Mukhopadhyay et al. (2007)
Aspergillus niger	Cu	28.7	Dursun (2006)
Aspergillus niger	Cr(VI)	117.33	Khambhaty et al. (2009)
Aspergillus terreus	Pb	201.1	Kogej and Pavko (2001)
Aspergillus terreus	Cu	160–180	Gulati et al. (2002)
Candida sp.	Cu	4.8	Donmez and Aksu (1999)
Kluyveromyces marxianus	Cu	6.44	Donmez and Aksu (1999)
Lentinus sajor caju	Cr(VI)	191.24	Bayramoglu et al. (2005)
Mucor hiemalis	Cr(VI)	53.5	Tewari et al. (2005)

Mucor rouxii	Cd	20.31	Yan and Viraraghavan (2008)
Mucor rouxii	Zn	53.85	Yan and Viraraghavan (2008)
Mucor rouxii	Pb	53.75	Yan and Viraraghavan (2008)
Mucor rouxii	Ni	20.49	Yan and Viraraghavan (2008)
Neurospora crassa	Pb	49.1	Kiran et al. (2005)
Neurospora crassa	Cu	12.3	Kiran et al. (2005)
Penicillium simplicissium	Cd	52.50	Fan et al. (2008)
Penicillium simplicissium	Zn	65.60	Fan et al. (2008)
Penicillium simplicissium	Pb	76.90	Fan et al. (2008)
Penicillium canescens	Cd	102.7	Say et al. (2003b)
Penicillium canescens	Pb	213.2	Say et al. (2003b)
Penicillium canescens	Hg	54.8	Say et al. (2003b)
Penicillium chrysogenum	Ni	82.5	Su et al. (2006)
Penicillium chrysogenum	Cd	210.2	Deng and Ting (2005b)
Penicillium chrysogenum	Cu	108.3	Deng and Ting (2005b)
Penicillium chrysogenum	Cu	92	Deng and Ting (2005a)
Penicillium chrysogenum	Pb	204	Deng and Ting (2005a)
Penicillium chrysogenum	Ni	55	Deng and Ting (2005a)

Penicillium chrysogenum	Ni	260	Tan et al. (2004)
Penicillium chrysogenum	Cr(III)	18.6	Tan and Cheng (2003)
Penicillium chrysogenum	Ni	13.2	Tan and Cheng (2003)
Penicillium chrysogenum	Zn	6.8	Tan and Cheng (2003)
Penicillium chrysogenum	Pb	96	Skowronski et al. (2001)
Penicillium chrysogenum	Cd	21.5	Skowronski et al. (2001)
Penicillium chrysogenum	Zn	13	Skowronski et al. (2001)
Penicillium chrysogenum	Cu	11.7	Skowronski et al. (2001)
Penicillium italicum	Cu	0.4–2	Ahluwalia and Goyal (2007)
Penicillium italicum	Zn	0.2	Ahluwalia and Goyal (2007)
Penicillium purpurogenum	Cr(VI)	36.5	Say et al. (2004)
Penicillium purpurogenum	Cd	110.4	Say et al. (2003a)
Penicillium purpurogenum	Pb	252.8	Say et al. (2003a)
Penicillium purpurogenum	Hg	70.4	Say et al. (2003a)
Penicillium purpurogenum	As	35.6	Say et al. (2003a)
Phanerochaete chrysosporium	Pb	419.4	Kogej and Pavko (2001)
Phanerochaete chrysosporium	Cu	20.23	Say et al. (2001)
Rhizopus nigricans	Pb	403.2	Say et al. (2001)
Rhizopus arrhizus	Cu	10.8	Dursun et al. (2003b)

Rhizopus arrhizus	Cr(VI)	78	Aksu and Balibek (2007)
Saccharomyces cerevisiae	Pb	211.2	Say et al. (2001)
Saccharomyces cerevisiae	Cu	7.11	Donmez and Aksu (1999)
Saccharomyces cerevisiae	Pb	79.2	Al-Saraj et al. (1999)
Saccharomyces cerevisiae	Cu	6.4	Al-Saraj et al. (1999)
Saccharomyces cerevisiae	Zn	23.4	Al-Saraj et al. (1999)
Saccharomyces cerevisiae	Hg	64.2	Al-Saraj et al. (1999)
Saccharomyces cerevisiae	Co	9.9	Al-Saraj et al. (1999)
Saccharomyces cerevisiae	Ni	8	Al-Saraj et al. (1999)
Saccharomyces cerevisiae	Cd	35.5–58.4	Park et al. (2003)
Saccharomyces cerevisiae	Cr(VI)	32.6	Ozer and Ozer (2003)
Saccharomyces cerevisiae	Ni	46.3	Ozer and Ozer (2003)
Saccharomyces cerevisiae	Pb	270.3	Ozer and Ozer (2003)

AIMS AND OBJECTIVES

During the last decades, extensive attention has been paid on the management of environmental pollution caused by hazardous materials such as heavy metal and complex organic molecules. Decontamination of such toxicants from the soil, water around the industrial area, waste dumps and similar contaminated sites has been a challenge for a long time. A number of methods have been developed for the removal of heavy metals from liquid wastes/contaminated site lechates such as precipitation, evaporation, electroplating, ion exchange, membrane processes etc. All these methods have several disadvantages such as unpredictable metal ion removal, high reagent requirement, generation of toxic sludge etc. Microbial treatment specially the use of fungi and bacteria provide an innovative biotechnological approach for much better cleanup process of contaminated sites/materials/environment.

The present study shall therefore be targeted at identifying the mechanism by which the mycoflora develop tolerance against the toxic heavy metals, so that the same could be applied in removing the metal contaminants from the soil by the way of bioremediation. In general fungi shall naturally be viewed with greater potentialities because of their aggressive growth, greater biomass production and extensive hypal richness in soil.

These prime objectives are as followings:

- To understand the nature of toxicity induced by various toxic metals on soil fungi;
- To study the role of soil component on bioavailability in fungal system;
- To evaluate the metal–metal interaction and its biotic effect with respect to fungal system;
- To understand the mechanism of metal tolerance and removal by selected fungi.

MATERIALS AND METHODS

4.1 Sampling of soil

Soil samples were collected from four pre-selected contaminated sites along with a control (comparatively non-contaminated) site in and around Kolkata city during 2005-2007. The sites are as follows:

(i) Waste dumping site of Dhapa areas of Kolkata;

(ii) Tannery waste dump sites along Eastern Metropolitan Bypass areas of Kolkata;

(iii) Contaminated site within the area of Central Bus Terminus, Esplanade Area, Kolkata ;

(iv) Waste dump sites within the premises of Calcutta Medical College Hospital, Kolkata;

(v) Victoria Memorial Monument premises, Kolkata as non-contaminated control site.

Each site has known metal contaminants as per previous report. 100gms of surface soil was collected aseptically in poly bag and were stored at -20^{o} C before further analysis.

4.2 Isolation of metal resistant microorganisms

Fungal strains were isolated from soil using conventional dilution plate culture techniques. One gm of pre-weighted soil was suspended in 10ml of sterilized distilled water and then serially diluted to 10^{-3} dilution and 1ml of such dilutant was plated in sterile petridish containing pre-coated media. The soil dilutant was spread over the plate and incubated at 28± 2^{o}C for six days. Three fungal media viz. Potato Dextrose Agar, Malt agar and Czapek dox Agar were used for this purpose.

4.3 Media compositions

Potato Dextrose (PD) Agar was prepared using 250gms of potato boiled in 100ml of distilled water for 30 minutes and the filtrate was mixed with 2gms of dextrose and 20 gms of agar powder per liter.

Malt Agar medium was prepared with mixture of malt extract and agar powder 15gms per liter. Identically Czapekdox (CD) medium was prepared (glucose -2gm, $NaNO_3$ – 2.5gm, KCl -0.5gm, $MgSO_4$,$7H_2O$ -0.5gm, $FeSO_4$ – 0.5gm, $ZnSO_4$, $7H_2O$ – 0.5gm, KH_2PO_4 – 1gm and agar powder 15gms per liter) (**APHA, 1992**).

4.4 Standard plate counts

Heterotrophic plate counts were done by Pour Plate Method after incubation for 48 hours in plate count agar (**APHA, 1992**). Samples were serial-diluted with sterilized distilled water for getting appropriate counts. All microbiological operations were done aseptically. Finally to isolate the preliminary metal resistant fungal strains isolated from the sampling sites ,medium in respective plates were added with 50 ppm of metals like arsenic, cadmium and chromium.

4.5 Isolation of pure cultures

In nature, microbial populations do not segregate themselves by genomic or specific identities but exist in coherent mixture of all existing types. In the laboratory this mixed population needs to be separated into pure cultures. As the pure cultures contain only one specific type of organism, these are very much suitable for study of their properties.

By the streak-plate dilution technique *i.e.* by spreading a loopful of culture over the surface of a agar plate and incubating, pure cultures were obtained by identifying and culturing distinct single colonies with sufficient distance from other colonies (**Trivedy and Goel, 1984).**

4.6 Maintenance of cultures

Media used in preservation, regular maintenance and in experiments (if not mentioned otherwise) are as mentioned above.

Fungal cultures were incubated at 28±2°C (as this temperature is prevalent in this country) (if not stated otherwise) and then after proper growth preserved under refrigeration of 4°C in appropriate nutrient media slants and stabs as stock cultures. Sub-culturing was done at regular intervals to maintain purity of cultures.

The fungal strains were isolated in metal amended slants for further studies. Morphological, physiological and biochemical characteristics of the isolated fungal species was given in table 4.1

Table 4.1 Morphological and biochemical characteristics of the isolated fungal species.

Sl. No.	Parameters
1	Colony colour
2	Conidial shape
3	Vesicle shape
4	Sterigmata number and position
5	Conidiospore colour
6	Identification of fungal strains
7	Optimization of pH and temperature on heavymetal removal
8	Heavymetal tolerance assay
9	Antibiotic sensitivity assay
10	Cross metal resistance assay
11	Metal biosorption studies
12	Assay of metallothionein proteins

4.7 Selection of tolerant strains

For determining metal tolerance limit of these fungal strains, the strains were allowed to grow in Czapek dox agar media incorporating different metal concentration. After incubating for appropriate periods, tolerant strains were detected and identified by visible prominence of growth *i. e* colony formation and size.

4. 8 Heavy metal analysis of contaminated soil

Each soil sample (1gm) was taken in the digestion tube (100ml) with addition of 10ml of HNO_3, $HClO_4$ (1:2) solution and heated in sand bath until clear solution was developed. The solutions were filtered through Whatman No1 filter paper and volume was made up to 50ml by adding distilled water. Each soil sample was digested in triplicate for analysis of contaminated heavy metal content separately. Blank (control) samples contained 10ml of HNO_3, $HClO_4$ (1:2) solution were also digested and those were diluted in the same way for comparison. Finally all metal analysis (Arsenic, Chromium and Cadmium) were performed by using Atomic Absorption Spectrophotometer with flow injection hydride generation (Perkin Elmer Analyst 400) (**APHA, 1998**).

4.9 Identification of fungi

Isolated fungal culture strains were identified on basis of macroscopic (colony character, morphology, colour, texture, shape, diameter and appearance of colony) and microscopic (septation in mycelium, presence of specific reproductive structures, shape and structure of conidia and conidiospore etc) characteristic using fungal identification manuals (**Thorn and Raper, 1945; Domsch et al., 1980 Ellis and Ellis, 1992; Zafar et al., 2006**).

4.10 Growth optimization of media, pH and temperature

Isolated strains were grown in sterilized Czapekdox broth medium for establishment of the optimum temperature, pH that supports the exuberant growth of those species. The pH of the medium was adjusted using dilute HCl or NaOH. The temperature ranges were varied from 25°C- 35°C, and pH was from 3 to 9.

The fungal strains were inoculated into a series of 250ml conical flasks containing varied concentration of each pre-selected metal (arsenic, chromium and cadmium) in CDB medium. The pH was varied from 3 to 9 (3, 5, 7 and 9). The pH of the medium was adjusted using dilute (N/10) HCl or NaOH. For optimization of temperature against each pH the representative culture was incubated at different temperatures (25 to 35^{o}C). Fungal biomass of each flask was harvested and weight was measured separately. The residual heavy metal of CD broth medium was also examined. Based upon the heavy metal removal and biomass data, the optimal pH and temperature were determined (**Pujol et al., 1997**).

4.11 Antibiotic sensitivity test

Antibiotic (antifungal) sensitivity of each metal tolerant fungal strains was also measured using the same cup assay method described earlier (**Pujol et al., 1997**). The antibiotics used for this assay were Nystatin, Griseofulvin and Kanamycin.

4.12 Heavy metal tolerance assessment

To explore the tolerance of the isolates to the heavy metals, the fungal strains were exposed to varying concentrations of heavy metal in Czapek dox broth. To each freshly prepared growth medium, selected metal was amended with concentration which varied between 50ppm to 5000ppm. After 6 days of incubation, the extents of colony growth (in diameter) was compared with the control (i.e. growth of fungi in medium without metal). All the experiments were carried out in triplicate (**Congeevaram et al., 2007**).

4.13 Cross metal resistance assay

Cross heavy metal resistance of the fungal isolates was determined by conventional cup assay method (**Dhar et al., 2004)**. Selected metal tolerant fungal strains were grown separately in Czapekdox agar medium supplemented with diverse concentration (sub-lethal to lethal) of each heavy metal separately. Then cups were prepared where various other metallic solutions (50ppm to 500ppm) were poured. For each test triplicates were made and incubated for 6 days. Zone of inhibition of fungal

growth around the cup was examined and measured in terms of MIC in each case separately.

4.14 Metal toxicity of the fungal isolates

The effect of heavy metals on the growth of the fungal isolates was determined separately by inoculating the isolates in Czapek dox broth medium supplemented with preselected metal concentration (sub-lethal concentration) of each heavy metal against each fungal isolates were done separately. These inoculated flasks were incubated in shaker-incubator at 28± 2^{o}C. Control were maintained and consisted of inoculated medium without the supplementation of heavy metal. The weight of fungal mycelium were taken at regular intervals (3, 6, 9 and 12 days) for study the growth responses against the metal concentrations **(Dugal et al., 2012)**.

4.15 Metal removal by selected strains

To study metal removal capacity of selected fungi, the strains were grown in 50 ml. Czapek Dox broth (pH 7) containing 20 ppm, 100ppm and 1000ppm of respective metal solution (arsenic, chromium and cadmium). The cultures were incubated in 28±2°C in a shaker incubator. A control set was maintained side by side. The removal percentage was determined in 3, 6 and 9 days interval. The inocula were 2 ml. of fresh spore suspension of inoculum strength: 4-5x 10^3 c.f.u./ml. For determination of residual metal in the filtrate, the filtrate (passed through 0.22µ Milipore filter) was taken out and residual metal estimation was done by AAS(Perkin Elmer model Analyst 400) (**Ahmed et al., 2006**).

4.16 Metal biosorption by fungal biomass

2gms of each wet weight from the fungal biomass thus harvested in previous experiment were washed with clean distilled water and then homogenized for 15 min in a blender. The biomass was then digested with HNO_3, HCl mixture (1:2) solution in digestion flask as mentioned earlier and further cell wall bound heavy metals (arsenic, chromium and cadmium) were measured separately using Atomic Absorption Spectrometer(AAS). Control set was maintained using non metal tolerant species. The metal absorption capacity of chitosan was also measured for comparison in the same way (**Ahmed et al 2006**).

The metal adsorption by the dead fungal biomass was also studied using 6-7 days grown 2gms of oven dried fungal mat in 50ml of different concentration of metal solution with continuous shaking. After 3days the fungal biomass was harvested through centrifugation. The bioadsorption capacity dead fungal biomass was measured by AAS after digestion (**Ahmed et al 2006**).

4.17 Metal adsorption by Chitosan

To study the bioadsorption of metals by chitosan, 20ppm and 100ppm of metal concentration were prepared for chromium, cadmium and arsenic respectively**.** The metal solutions were prepared form standard solutions of Chromium (1000 ppm), Arsenic (1000 ppm) and Cadmium (1000 ppm) (Merck Germany). 50 ml of solution of each metal concentration were taken and 500 mg of chitosan was added (Sigma-Aldrich). Then the mixture was continuously stirred using magnetic stirrer for 6 hours at room temperature (30 °C). After that the solution was filtered and the filtrate was measured by Atomic Absorption Spectroscopy assisted with FIAS (Perkin Elmer Model Analyst 400). The test was performed in varying pH to analyse the relation of metal accumulation by chitosan against different pH **(Sewandi et al., 2011).**

4.18 Assay of metallothionein protein

Spore suspension of wild non metal tolerant and metal tolerant fungal strains (test organisms) were inoculated in Czapekdox broth medium supplemented with sub-lethal concentration of respective heavy metal and allowed to incubate in a shaker at 28+2°C for 96 hours. The mycelium was then harvested and washed thrice with distilled water under aseptic conditions. The excess water was blotted off and preserved at -20°C for preparation of cell free extract of each strain separately. For each experiment triplicate was run in aseptic way. Metallothionein was measured according to the method of **Virarengo et al. (1997)**. In the preparation of sample, mycelium of both wild (non metal tolerant) and metal tolerant strain were homogenized separately in a mixture of 0.5 mol l^{-1} sucrose, 0.02 mol l^{-1} tris-HCl buffer (pH 8.6) with added $6x10^{-6}$ mol l^{-1} leupeptine, $5x10^{-3}$ mol l^{-1} phenyl methyl sulphonyl fluoride (PMSF) as antiproteolytic agent and 0.01% β-mercaptoethanol as reducing agent. The homogenate was then centrifuged at 30000xg for 20mins to obtain the supernatant containing metallothionein. The collected supernatant was then

treated with ethanol- chloroform as described by **Kimura et al., (1979)**. 1.05 ml of cold (-20^{0}C) absolute ethanol and 80µl of chloroform were added to aliquot of 1 ml of supernatant. The samples were then centrifuged at 6000x g for 10 mins at 0-4^0 C. The collected supernatant was combined with 1 mg RNA and 40µl 37% HCl and subsequently with three volume of cold ethanol. The samples were maintained at –20^0 C for 1 h and centrifuged in a swinging rotor at 6000x g for 10 min. The pellets containing metallothionein was then washed with 87 % ethanol and 1 % chloroforms in homogenizing buffer, centrifuged at 6000x g for 10 min and dried under nitrogen stream. The pellets were re-suspended in 150 µl NaCl (0.25 mol l^{-1}) and subsequently 150µl HCl (1N) containing EDTA (0.004 mol l^{-1}) and 4.2 ml NaCl (2 mol l^{-1}) containing 5,5-dithiobis-2-nitrobenzoic acid (DTMB) (4.3x 10^{-4} mol l^{-1}) buffered with 0.2 mol l^{-1} Na-Phosphate (pH 8) were added . The mixture was finally centrifuged at 3000g for 5 mins. The absorbance of supernatant was measured spectrophotometrically at 412 nm. The metallothionein concentration was estimated considering reduced glutathione (GSH) as reference standard **(Pal et al., 2005).**

4.19 Scanning Electron Microscopic(SEM) study

The mycelium of metal tolerant fungal strains and their respective wild strains were washed with buffer and dehydrated in a series of ethanol water solution (30,50, 70 and 90% ethanol, 5 minutes each) and dried at a critical point under a CO_2 atmosphere for 30 minutes. Then mounting was made on aluminium stubs, and mycelium was coated with 90A^o thick gold palladium coating in a polaron Sc 7640 Sputter coate for 30 min. Coated mycelium were viewed at 15KV with Scanning Electron Microscopy (IB2 Ion Coater HITACHI S-530) (**Mishra et al., 2013**).

RESULTS

Urban environment particularly soil is highly contaminated with toxic metals and organic pollutants. Mycoflora (fungi) of such contaminated soil plays a crucial role in soil decontamination. In the present study a detailed analysis of soil of contaminated sites were made, followed by isolation and characterisation of tolerant fungal strains. These strains will be useful for soil decontamination in near future.

5.1 Soil analysis

The soil samples were collected from different waste dumping sites along with the non-polluted sites around Kolkata. In general the concentration of heavy metal is high in waste dumping sites. The soil samples were collected in three replicates from five different sites. The pH of the soil was neutral to alkaline. The fungal growth and metal absorption of fungi is dependent on pH of the medium. As there was no waste dumping on sampling site 5, it was treated as control zone. Thus except sampling site 5 all the other sampling sites contained high concentration of Chromium and Cadmium. Chromium content was highest (50-5400 ppm) in sampling site 1 and lowest (10-20 ppm) in sampling site 5. Arsenic was found in only sampling site 1 but it was in significant amount. As per World Health Organisation (WHO) guideline the maximum permissible limit of Arsenic in drinking water is 10µg/L, whereas the sampling site 1 contains 1-2.5 ppm of arsenic. All the five sampling sites contain high amount of Lead which ranges from highest 8.5- 230 ppm (Sampling site 1) to lowest 1.0-1.12ppm (Sampling site 5). All the five sampling sites also contained high amount of Nickel. Among them sampling site 1 contained the highest amount (35-57 ppm). The texture of the soil was loamy to clay loamy, only the soil of sampling site 4 was sandy loamy. The cation content of the soil was from moderate to high. The maximum amount of Ca^{++} is 3.0 Meq/L and maximum amount of Mg^{++} is 9.0 Meq/L. As the sampling site 1 contains the highest amount of heavy metal it indicates that this dumping site received a large amount of waste material. Thus, these results indicated an increased content of potentially toxic metal in the soil, which might exert selection pressure on microbial community including fungi. Long term exposure of microbial community in these heavy metal contaminated soils might have built some tolerant mechanism among those microorganisms. As soil is a heterogeneous system an array

of reactions can occur. Thus the tolerance and absorption capacity of heavy metals by microbes including bacteria and fungi depends on a number of factors which include soil pH, EC, cations, anions, texture, and other chemical characters. The physicochemical characters of the soils of five sampling sites are given in table 5.1.

Table 5.1 Soil characteristics of the sampling sites

Sl. No.	Soil Parameters	Dhapa Dumping site (Site 1)	Tannery waste, EM Bypass (Site 2)	Central Bus Terminus, Esplanade (Site 3)	Calcutta Medical College & Hospital (Site 4)	Victoria Memorial (Site 5)
1	pH	7.3-7.7	6.8-7.2	7.5-8.0	7.2-7.5	7.0-7.3
2	EC (dSm^{-4})	1.70	1.50	1.85	1.70	2.0
3	Ca^{++} (Meq/L)	1.7	1.3	2.0	3.0	2.0
4	Mg^{++} (Meq/L)	5.5	4.5	7.0	9.0	7.0
5	Hardness (mg/gm)	120	130	150	160	80
6	Texture	Loamy	Clay loam	Loamy	Sandy Loam	Clay Loam
7	Cr (ppm)	50-5400	150-6400	30-50	20-50	10-20
8	Cd (ppm)	1.0-6.5	2.0-8.5	1.5-2.5	1.0-2.5	0.5-0.9
9	As (ppm)	1.0-2.5	BDL	BDL	BDL	BDL
10	Pb (ppm)	8.5-230	5.7-8.9	2.3-3.5	2.8-4.0	1.0-1.2
11	Ni (ppm)	35-57	5.7-8.9	2.3-3.5	2.8-4.0	1.0-2.1

5.2 Isolation of Fungal strains from contaminated Soil

Almost **30** different fungal strains were isolated from soil samples of five different sites of Kolkata. The strains were characterised considering different Morphological & Biochemical characters. The detailed morphological characters and biochemical characters are given in Table 5.2.

Table 5.2 Morphological and biochemical characteristics of the isolated fungal species

Sl. No	Parameters	Isolated Fungal strains									
		AsA1	**AsA2**	**AsA3**	**AsA4**	**AsA5**	**AsB1**	**AsB2**	**AsB3**	**AsB4**	**AsB5**
1	Colony colour	Dark brown	Brown	Black	Green	White	Pale green	Green	Black	Black	Black
2	Conidial shape	Globous	Spherical	Club shape	Globous	Banana shape	Club shape	Globous	Spherical	Club shape	Club shape
3	Vesicle shape	Globous	Lanceolate	Globous	Globous	septate	Globous	Globous	Globous	Globous	Globous
4	Sterigmata number and position	Many, Entire surface	Upper part of vesicle	2/3 part of vesicle	Many, Entire surface	-	2/3 part of vesicle	Many, Entire surface	Upper part of vesicle	2/3 part of vesicle	2/3 part of vesicle
5	Conidiospore colour	Brown	Brown	Black	Brown	Black	Black	Brown	Black	Black	Black
6	Identification of fungal strains	*Aspergillus Sp.1*	*Aspergillus Sp.2*	*Aspergillus Sp.3*	*Penicillin Sp.1*	*Fusarium Sp.1*	*Penicillin Sp.2*	*Aspergillus Sp.4*	*Aspergillus niger*	*Aspergillus Sp.5*	*Aspergillus Sp.6*
7	Optimization of pH	6.5	7.0	6.8	7.0	7.2	6.5	7.0	6.8	7.0	7.2
8	Optimization of temp. (^{o}C)	28	30	28	30	32	30	28	30	25	32
9	Heavy metal tolerance assay	As	As	As	As	As	As	As	As	As	As
10	Antibiotic sensitivity assay	+	+	+	+	-	-	-	+	+	-
11	Cross metal resistance assay	Cr, Cd	Cr, Cd	Cr, Cd	Cr, Cd	Cr, Cd	Cr, Cd	Cr, Cd	Cr, Cd	Cr, Cd	Cr, Cd
12	Metal biosorption studies	yes	Yes	Yes	yes	Yes	Yes	yes	Yes	Yes	Yes
	Strain No	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**

Table 5.2 Cont….

Sl. No.	Parameters	Isolated Fungal strains									
		AsC1	**AsC2**	**AsC3**	**AsC4**	**AsC5**	**CrTW 1**	**CrTW 2**	**CrTW 3**	**CrTW 4**	**CrTW 5**
1	Colony colour	Pale green	Black	Black	Green	Grey	Dark brown	Green	Green	Brown	Black
2	Conidial shape	Globous	Spherical	Club shape	Globous	Elongated	Club shape	Globous	Spherical	Club shape	Club shape
3	Vesicle shape	Globous	Lanceolate	Globous	Globous	-	Globous	Globous	Globous	Globous	Globous
4	Sterigmata number and position	Many, Entire surface	Upper part of vesicle	2/3 part of vesicle	Entire surface	-	Upper part of vesicle	Many, Entire surface	Upper part of vesicle	2/3 part of vesicle	1/2 part of vesicle
5	Conidiospore colour	Brown	Black	Black	Brown	Dark	Black	Brown	Greenish	Black	Black
6	Identification of fungal strains	*Aspergillus flavus*	*Aspergillus Sp.7*	*Aspergillus Sp.8*	*Penicillium Sp.3*	*Fusarium Sp.2*	*Aspergillus niger*	*Aspergillus Sp.8*	*Penicillium Sp.4*	*Aspergillus Sp.8*	*Aspergillus Sp.9*
7	Optimization of pH	6.5	7.0	6.8	7.0	7.2	6.5	7.0	6.8	7.0	7.2
8	Optimization of temperature	30	30	28	30	32	30	28	30	25	32
9	Heavy metal tolerance assay	As	As	As	As	As	As	As	As	As	As
10	Antibiotic sensitivity assay	+	+	+	+	-	-	-	+	+	-
11	Cross metal resistance assay	Cr, Cd	Cr, Cd	Cr, Cd	Cr, Cd	Cr, Cd	As, Cd	As, Cd	As, Cd	As, Cd	As, Cd
12	Metal biosorption studies	yes	Yes	Yes	yes	Yes	Yes	yes	Yes	Yes	Yes
	Strain No	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**

Table 5.2 Cont….

Sl. No	Parameters	Isolated Fungal strains									
		CrEsp 1	**CrEsp 2**	**CrEsp 3**	**CrM1**	**CrM2**	**CdEP**	**CdEP 1**	**CdEP2**	**CdEP 3**	**CdEP 4**
1	Colony colour	Dark brown	Brown	Black	White	Green	Brown	Green	Black	Black	Black
2	Conidial shape	Globo us	Spheri cal	Club shape	Banana shape	Globous	Club shape	Globo us	Spherical	Club shape	Club shape
3	Vesicle shape	Globo us	Lance olate	Globo us	Septate	Globous	Globous	Globo us	Globous	Globo us	Globo us
4	Sterigmata number and position	Entire surface	Upper part of vesicle	1/2 part of vesicle	-	Many, Entire surface	2/3 part of vesicle	Entire surface	Upper part of vesicle	2/3 part of vesicle	2/3 part of vesicle
5	Conidiospore colour	Brown	Deep brown	Black	Grey	Brown	Deep brown	Brown	Black	Black	Black
6	Identification of fungal strains	*Aspergillus Sp.10*	*Aspergillus Sp.11*	*Aspergillus Sp.12*	*Fusarium Sp.3*	*Penicillium Sp.5*	*Aspergillus flavus*	*Aspergillus Sp.13*	*Aspergillus niger*	*Aspergillus Sp.14*	*Aspergillus Sp.15*
7	Optimization of pH	6.5	7.0	6.8	7.2	7.0	7.0	7.0	6.8	7.0	7.2
8	Optimization of temperature	28	30	28	32	30	30	28	30	25	32
9	Heavy metal tolerance assay	Cr	Cr	Cr	Cr	Cr	Cd	Cd	Cd	Cd	Cd
10	Antibiotic sensitivity assay	+	+	+	-	+	-	-	+	+	-
11	Cross metal resistance assay	As, Cd	As, Cd	As, Cd	As, Cd	As, Cd	Cr, As	Cr, As	Cr, As	Cr, As	Cr, As
12	Metal biosorption studies	yes	Yes	Yes	Yes	yes	Yes	yes	Yes	Yes	Yes
	Strain No	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

Among the aforementioned thirty strains, five strains were selected depending on the metal tolerance capacity for further detailed study.

5.3 Identification of selected strain

Amongst the five fungal strains selected, two strains were selected against arsenic, two strains against chromium and one strain against cadmium. Two arsenic tolerant strains AsC1 (isolated from Dhapa soil) and AsB3 were common on these four sites, so we have carried out all our experiments with these two strains. The strains were identified as *Aspergillus flavus* (AsC1) and *Aspergillus niger* (AsB3) considering their colony colour, colony shape, conidiospore colour, and microscopic characters etc. Two strains CrTW1 and CrTW3 were selected against chromium which were identified as *Aspergillus niger* and *Penicillium* sp. respectively. One strain CdEP was selected against cadmium which was identified as *Aspergillus flavus.* (Table 5.3; Fig 5.1- 5.5)

Table 5.3 Morphological characters of selected strain

Strain Id	Strain Name	Colony Character
AsC1	*Aspergillus flavus*	Pale green colony, brown spore
AsB3	*Aspergillus niger*	Black colony, Black spore
CrTW1	*Aspergillus niger*	Dark brown colony, Black spore
CrTW3	*Penicillium* sp.	Green colony, Greenish spore
CdEP	*Aspergillus flavus*	Brown colony, Deep brown spore

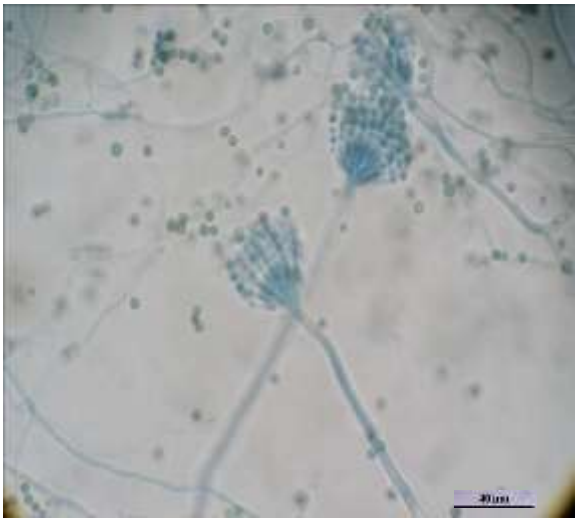


Fig. 5.1 Microphotograph of AsC1

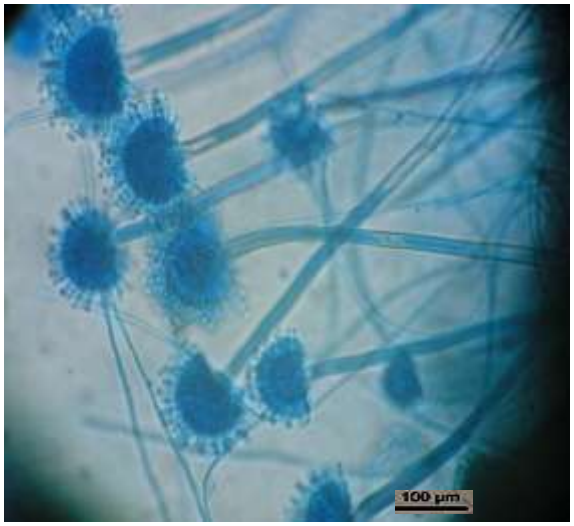


Fig. 5.2 Microphotograph of AsB3

Fig. 5.3 Microphotograph of CrTW1

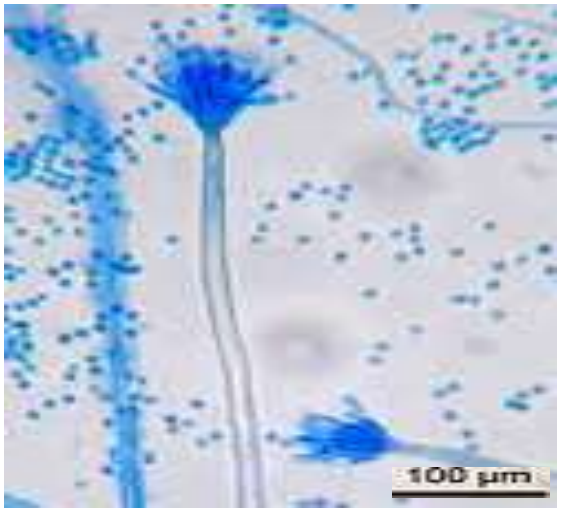


Fig. 5.4 Microphotograph of CrTW3

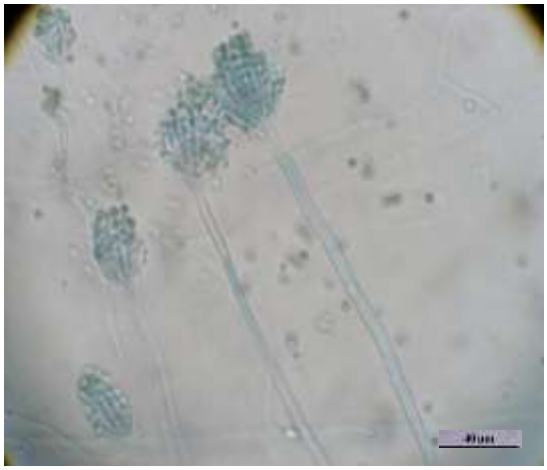


Fig. 5.5 Microphotograph of CdEP

5.4 Optimization of pH and Temperature on growth of selected fungal strains

Temperature and pH are two important factors for optimum growth of fungal strains. The growth of the studied fungal strains were measured againstdifferent pH (pH 3-9) in medium.With the increase in pH from 3 to 7 the biomass yield was found to enhance in all the cases and being the maximum at pH 7. Further increase in pH caused a decrease in biomass (Table 5.4). Thus, all the fungal strains showed optimum growth at neutral pH. The pH content of the soil of the sampling sites were also more over neutral.

Table- 5.4 Effect of pH for growth of isolated fungal strains (in terms of mycelia weight after 6days of incubation)

Strain Name	**Mycelial weight(g) (Mean ±SD)**			
	pH 3	**pH 5**	**pH 7**	**pH 9**
AsC1 (*Aspergillus flavus*)	1.3±0.2	1.57±0.3	1.89±0.52	1.26±0.63
AsB3 *(Aspergillus niger)*	1.5±0.78	1.87±0.54	2.1±0.21	1.7±0.45
CrTW1 *(Aspergillus niger)*	1.22±0.18	1.60±0.28	1.76±0.25	1.44±0.22
CrTW3 *(Penicillium* sp.)	1.25±0.4	1.62±0.34	1.78±0.36	1.5±0.24
CdEP (*Aspergillus flavus)*	1.35±0.4	1.66±0.32	1.80±0.25	1.56±0.42

Similarly to optimise the temperature for maximum mycelial growth, the fungal strains were incubated in different temperature (25, 30 & 35 °C) and in suitable medium. The maximum mycelial weight was obtained at 30°C temperature in all the cases (Table 5.5). The effects of temperature on fungi are related to the chemical reactions within the fungal cells. For optimum growth, temperature must be in a range that allows the most efficient progression of the chemical reactions necessary for growth. As temperatures progress above the optimum temperature, the chemical reactions occur less efficiently, and growth slows. Temperature 30°C and pH 7, both

these physical conditions showed maximum growth in terms of mycelial weight so all further experiments were carried out maintaining these growth conditions.

Table-5.5 Effect of temperature for growth of isolated fungal strains (in terms of mycelial weight after 6days of incubation)

Strain Name	**Mycelial weight(g) (Mean ±SD)**		
	Temperature-25°C	**Temperature-30°C**	**Temperature-35°C**
AsC1 (*Aspergillus flavus*)	1.15±0.25	1.95±0.45	1.01±0.32
AsB3 *(Aspergillus niger)*	1.3±0.20	2.01±0.35	1.69±0.56
CrTW1 *(Aspergillus niger)*	1.26±0.12	1.68±0.24	1.56±0.25
CrTW3 *(Penicillium* sp.)	1.16±0.18	1.72±0.36	1.58±0.16
CdEP (*Aspergillus flavus)*	1.22±0.14	1.86±0.33	1.70±0.45

5.5 Antibiotic sensitivity test

An antibiotic sensitivity (or susceptibility) test was done to choose the antibiotic that would be most effective against the specific types of microorganism. Some types of bacteria or fungus are resistant to certain antibiotics because of differences in their genetic materials. The objective of laboratory testing with antibiotics was to obtain reproducible indications of the susceptibility of the fungal isolates to an agent *in vitro*. This is also important from clinical point of view.

Three different fungal antibiotics (Nystatin, Griseofulvin and Kanamycin) were used to evaluate the antibiotic sensitivity of these five fungal strains (Table 5.6). AsB3 was found to be more resistant towards Griseofulvin and Kanamycin than AsC1, while against Nystatin both the strains showed similar range of tolerance. Similarly the chromium resistant fungal strains CrTW1 and CrTW3 were also found more resistant to antibiotic Griseofulvin and Kanamycin. CdEP strain was more susceptible to all

the three antibiotics. The antibiotic Nystatin was more effective to all the tested fungal strains than Griseofulvin and Kanamycin .

Table: 5.6 Antibiotic sensitivity test with the fungal strains

Strains	Antibiotics					
	Nystatin		Griseofulvin		Kanamycin	
	Maximum growing conc. (ppm)	Minimum inhibitory conc. (MIC) (ppm)	Maximum growing conc.(ppm)	Minimum inhibitory conc. (MIC) (ppm)	Maximum growing conc.(ppm)	Minimum inhibitory conc. (MIC) (ppm)
AsC1	60	80	60	80	60	80
AsB3	60	80	80	100	80	100
CrTW1	40	60	80	100	80	100
CrTW3	20	40	80	100	80	100
CdEP	60	80	60	80	40	60

5.6 Metal Tolerance Level

Fungal survival in presence of the toxic metals mainly depends on intrinsic biochemical and structural properties, physiological and/or genetical adaptation, including morphological changes, and environmental modification of metal speciation, availability and toxicity. The relative importance of each often becomes difficult to determine. Metal resistance means the ability of an organism to survive metal toxicity by means of a mechanism produced in direct response to the metal species concerned and metal tolerance may be defined as the ability of an organism to survive metal toxicity by means of intrinsic proper ties and/or environmental modification of toxicity. Microbes can tolerate high metal concentration either by binding the metal on their cell wall or by accumulating it in the cytosol.

In this study all the five fungus were isolated from urban contaminated sites, thus they can tolerate high amount of heavy metals. As from the result of maximum tolerance

level it was revealed that the minimum metal tolerance concentration is 1000 ppm and the maximum tolerance level is 2000 ppm. Among the two arsenic tolerant fungi AsC1 can tolerate higher amount of arsenic up to 2000 ppm. Whereas AsB3 can tolerate arsenic up to 1000 ppm. Both the two strains CrTW1 and CrTW3 can tolerate chromium up to 1300 ppm. Whereas CdEP stain can tolerate cadmium up to 1200 ppm. (Table 5.7)

Table 5.7 Metal Tolerance Level (MTL) study

Sl. No.	Strains	Metals	Concentration in ppm
1	AsC1	Arsenic	2000
2	AsB3	Arsenic	1000
3	CrTW1	Chromium	1300
4	CrTW3	Chromium	1300
5	CdEP	Cadmium	1200

5.7 Cross Metal Tolerance

As mentioned previously, many fungi have the ability to tolerate high concentrations of potentially toxic metals which may be useful when employing living cells in recovery systems. Further these metals were tested for cross metal tolerance. Each strain showed high tolerance level for other metals too. AsC1 which showed highest tolerance level of 2500 ppm for arsenic, also showed high tolerance level for chromium (1000 ppm) followed by lead, cadmium and zinc. It showed less tolerance level for mercury. Among all the metals every strain showed less tolerance level for mercury. Apart from arsenic, AsB3 strain showed highest tolerance level for chromium, followed by zinc, lead, and cadmium. For CdEP strain apart from cadmium it showed highest tolerance level for chromium followed by zinc, arsenic, and lead. Both the strain CrTW1 and CrTW3 showed highest tolerance level for zinc, followed by arsenic and cadmium. These two strains CrTW1 and CrTW3 showed less tolerance level for other metals apart from chromium in comparison to other fungal

strains. As all heavy metals have similar toxic mechanism thus multiple tolerances are common phenomena among heavy metal tolerant fungi. In this study the fungal isolates thus showed multiple tolerance capacity (Table 5.8, Fig 5.6, 5.7).

Table 5. 8 Cross metal resistance

Strains	MIC in ppm					
	As	Cd	Pb	Hg	Zn	Cr
AsC1	2500	500	700	55	650	1000
AsB3	1200	450	625	60	750	900
CdEp	1000	1500	450	50	1000	1050
CrTw1	500	350	250	60	1200	1500
CrTw3	450	300	200	60	1100	1150

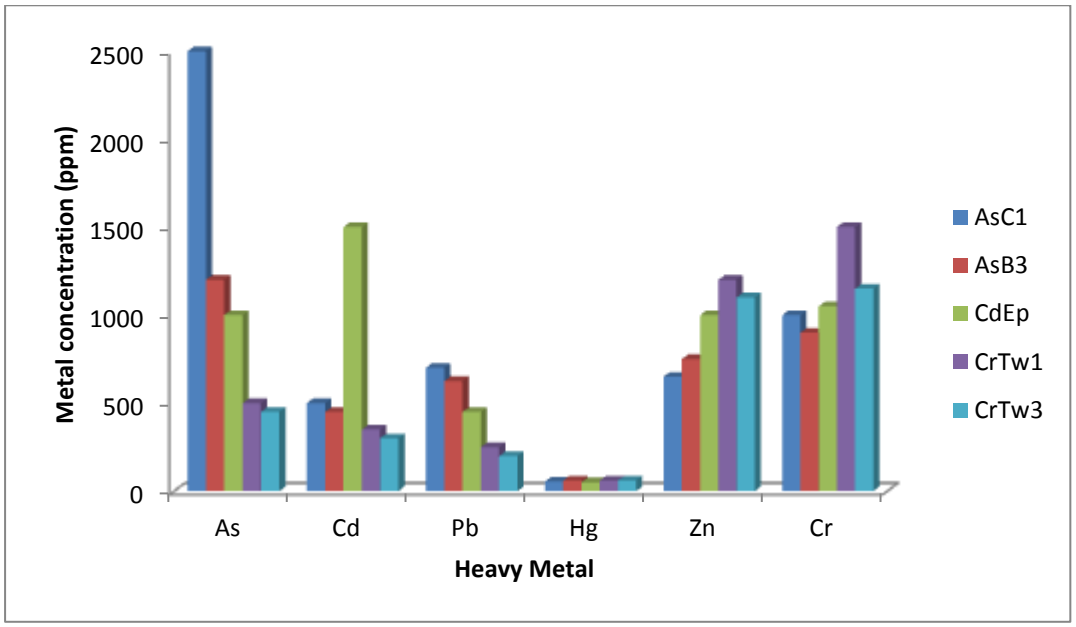


Fig. 5.6 Cross metal tolerance by fungal strains

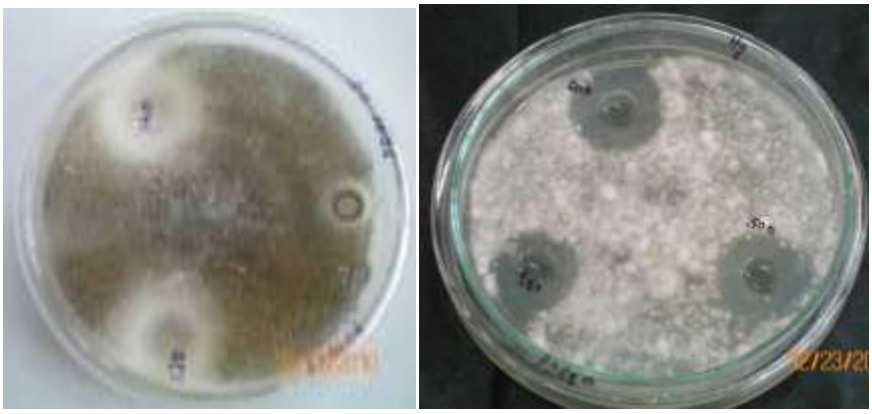


Fig. 5.7 Inhibition zone by metal

5.8 Growth response of fungal strain in different metal concentration

The growth response of AsC1 was tested against different doses of arsenic and one set was kept as control. The growth in the control set was higher than in any arsenic treated set. Among different arsenic treated dose which ranges from 1000 ppm to 2500 ppm the strain showed highest growth rate against 2000 ppm. But at 2500 ppm dose its growth was ceased. In relation to incubation time, the growth of the strain decreased with incubation time. It reveals that the metal stress increased with incubation time. But in case of the control set the growth became stable (Fig. 5.8).

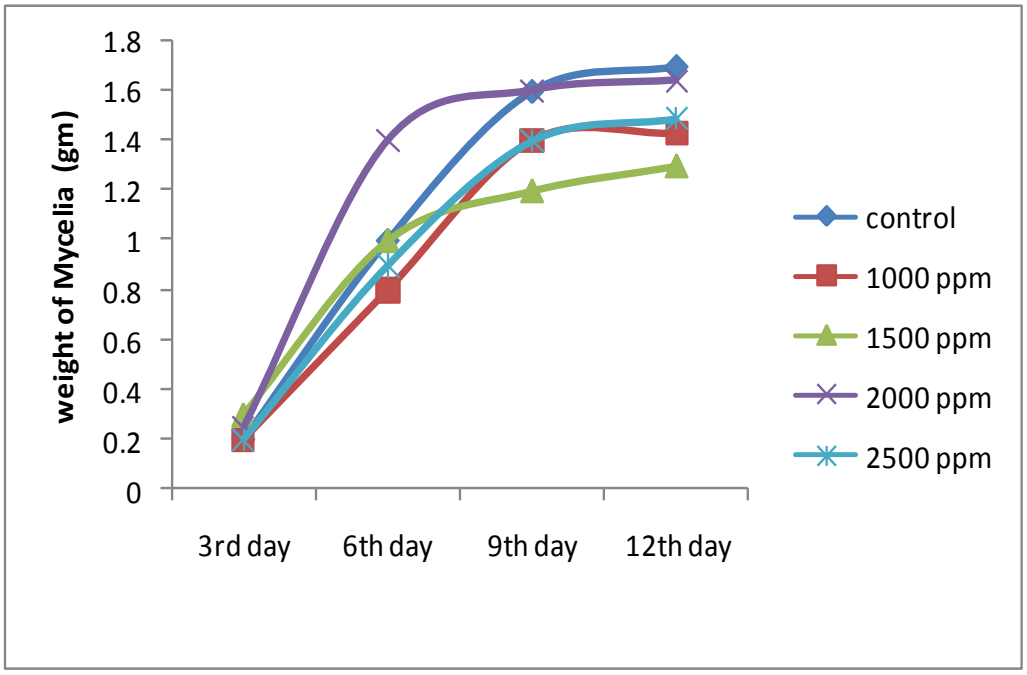


Fig. 5.8 Growth response of AsC1 in different doses of arsenic

The strain AsB3 showed maximum growth against 1500 ppm and minimum growth against 2000 ppm arsenic. The growth of the strain decreased with incubation time. The growth rate was ceased at 12th day in metal treated media. Thus the metal dose imposing stress on the fungal strain at 12th day at 2500ppm. The growth in the control set was normal with incubation time (5.9).

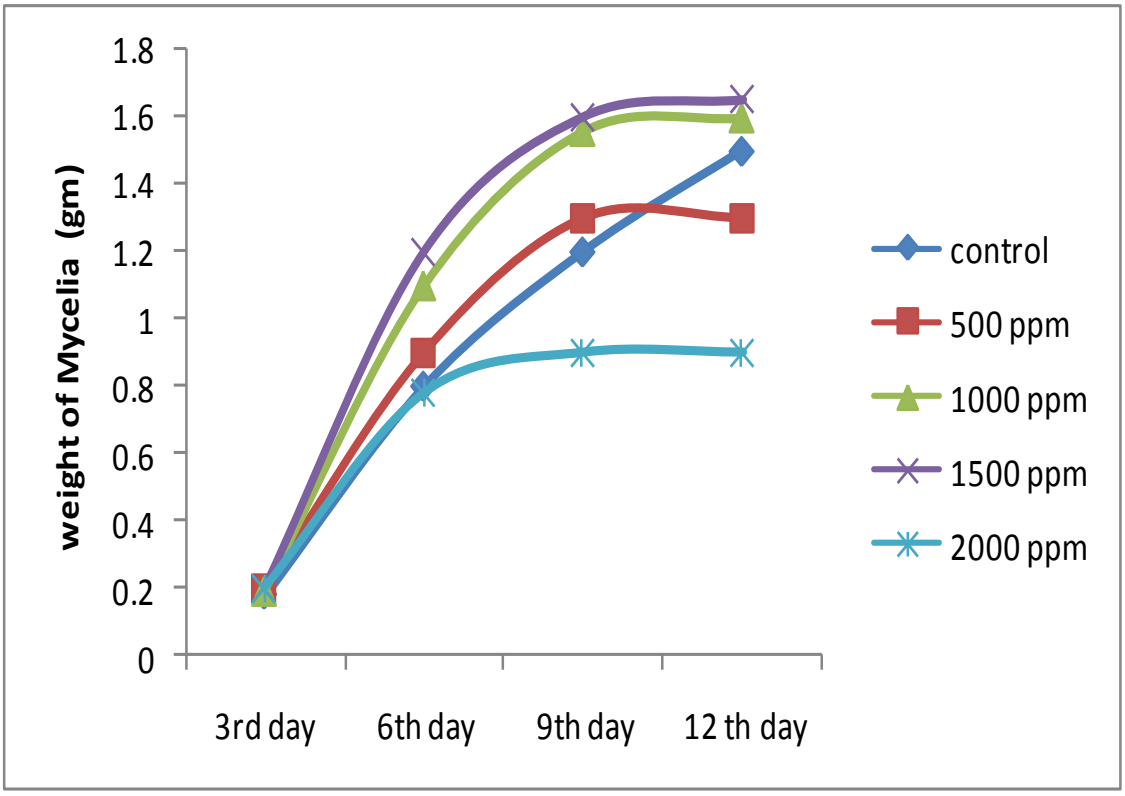


Fig. 5.9 Growth response of AsB3 in different doses of arsenic

The strain CrTW1 showed highest growth against 1500 ppm and showed lowest growth at 2000 ppm. The growth of the strain first increased up to 9th day against all the treated doses of chromium but after that the growth decreased at 12th day of incubation time. The growth in the control set became stagnant after the 9th day.

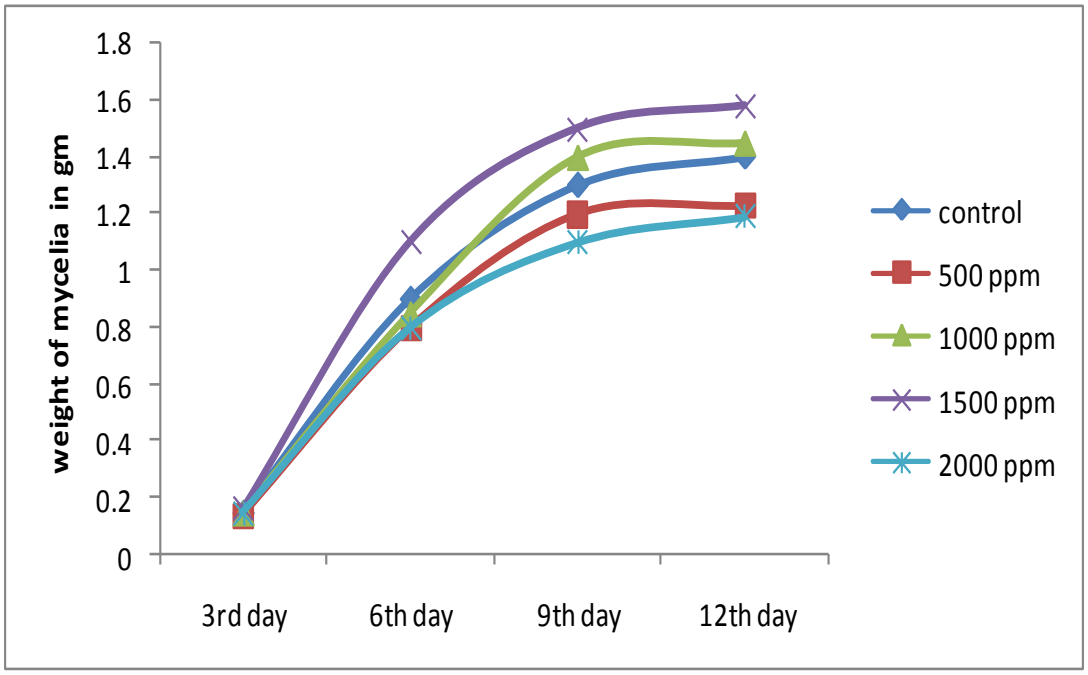


Fig. 5.10 Growth response of CrTW1 in different doses of chromium

The strain CrTW3 showed highest growth at 1500 ppm and lowest growth at 2000 ppm. The strain showed highest growth at 9th day after that its growth was ceased. In the control set the growth however became stable after 9th day (Fig. 5.11).

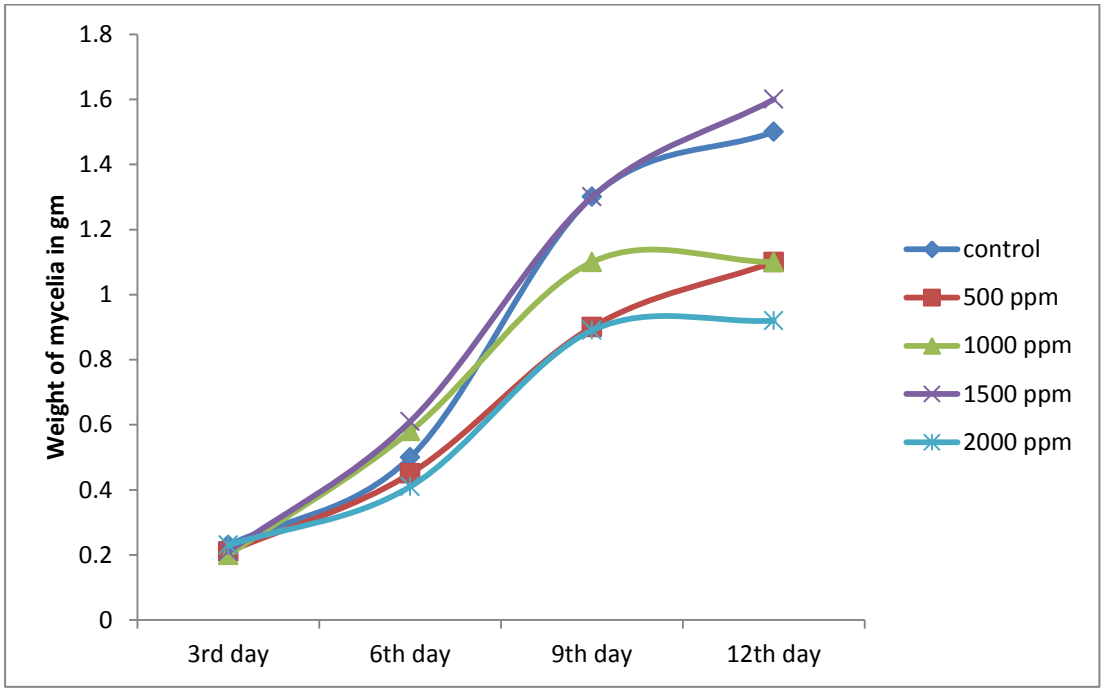


Fig. 5.11 Growth response of CrTW3 in different doses of chromium

The strain CdEP showed highest growth for 1000 ppm and minimum growth for 2000 ppm. The growth ceased after the 9th day. The growth was however normal for thecontrol set (Fig. 5.12).

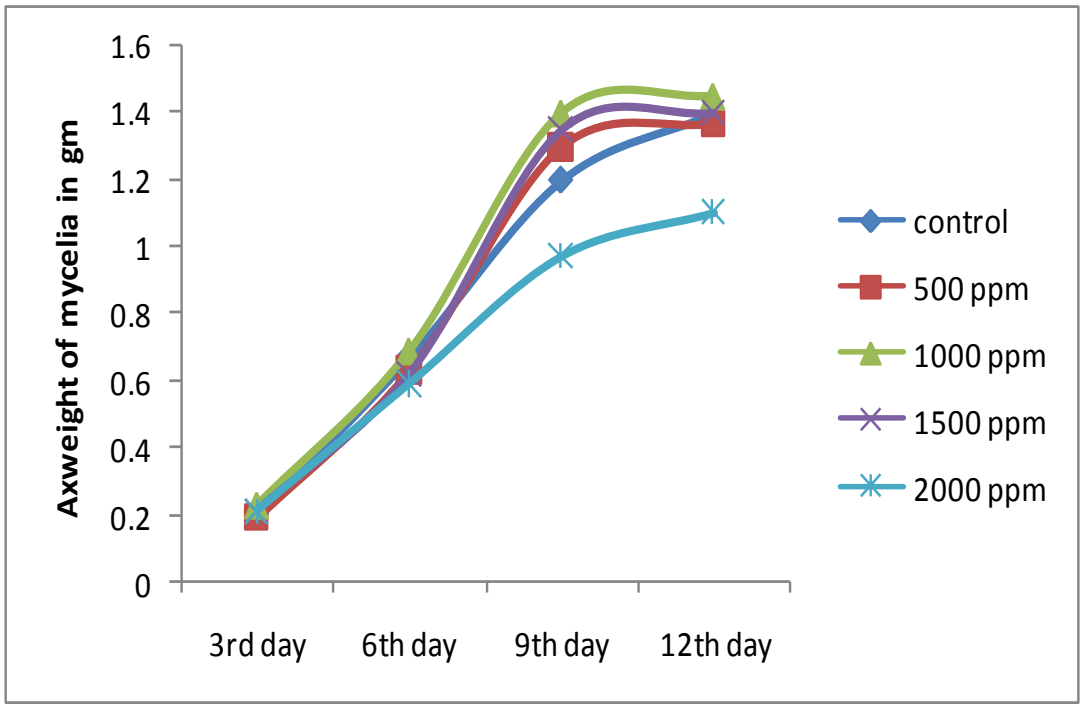


Fig. 5.12 Growth response of CdEP in different doses of cadmium

5.9 Test for metal removal *in vitro*

To study the metal removal capacity, the selected fungal strains were grown in Czapekdox broth (25ml) with respective metal concentration 20, 100 and 1000 ppm for 3, 6 and 9 days and with continuous shaking incubation at 30°C at pH 7. Fungal biomass was harvested by filtration through 0.22µ Millipore filter and the residual metal concentrations of the broth was measured and the percentage of metal removal was then calculated.

The strain AsC1 showed highest metal removal capacity at 100ppm. The metal removal capacity decreased at 1000 ppm. The metal removal capacity of AsC1 strain increased with incubation time but goes on saturation after a certain time (Table 5.9; Fig. 5.13). The percentage of arsenic removal increased in AsB3 strain with increasing incubation time. The strain AsB3 showed highest arsenic removal against

100 ppm dose of treated arsenic. The percentage of arsenic removal decreased at 1000 ppm dose of treated arsenic (Table 5.10; Fig. 5.14).

Thus for both the strain (AsC1 and AsB3) the arsenic stress occured at 1000 ppm of arsenic treated concentration. Both the strain showed their maximum removal (70%-75%) efficiency when grown in 100ppm concentration of arsenic. In case of both the strain the maximum removal efficacy was obtained at the 6^{th} day of incubation. With further increase in incubation time (i.e from 6^{th} day to 9^{th} day), saturation was observed in removal efficacy. The results also depicted that AsC1 is capable of removing more arsenic than AsB3 strain (Table 5.9 & 5.10; Fig. 5.13 & 5.14).

Table 5.9 Arsenic removal by AsC1 strain *(Aspergillus flavus)*

As in media	Days of incubation	Removal %	SD
20ppm	3rd day	65.0	2.2
	6th day	70.0	4.3
	9th day	70.1	3.6
100ppm	3rd day	73.1	3.5
	6th day	76.0	4.2
	9th day	76.1	2.9
1000ppm	3rd day	50.0	6.4
	6th day	55.0	3.8
	9th day	55.2	5.7

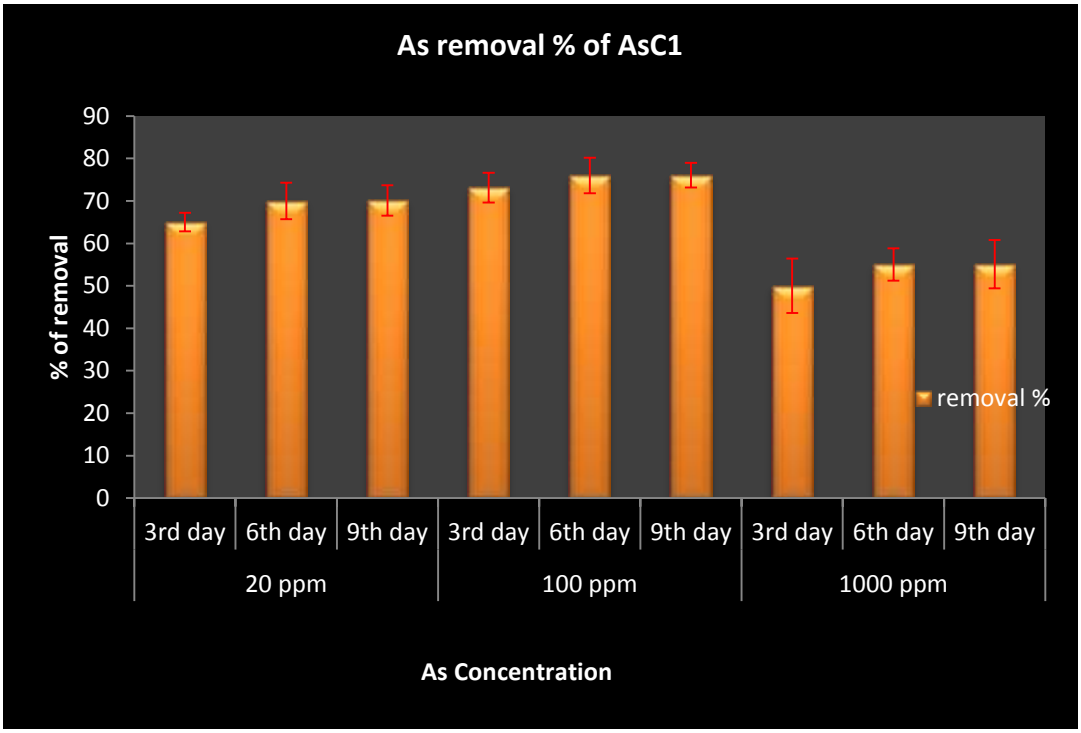


Fig. 5.13 Arsenic removal by AsC1 strain

Table 5.10 Arsenic removal by AsB3 strain (*Aspergillus niger*)

As in media	Days of incubation	removal %	SD
20ppm	3rd day	60	5.3
	6th day	65	3.2
	9th day	65.2	6.2
100ppm	3rd day	65	2.5
	6th day	70	5.5
	9th day	70.3	4.7
1000ppm	3rd day	50	4.4
	6th day	54	6.6
	9th day	55	4.8

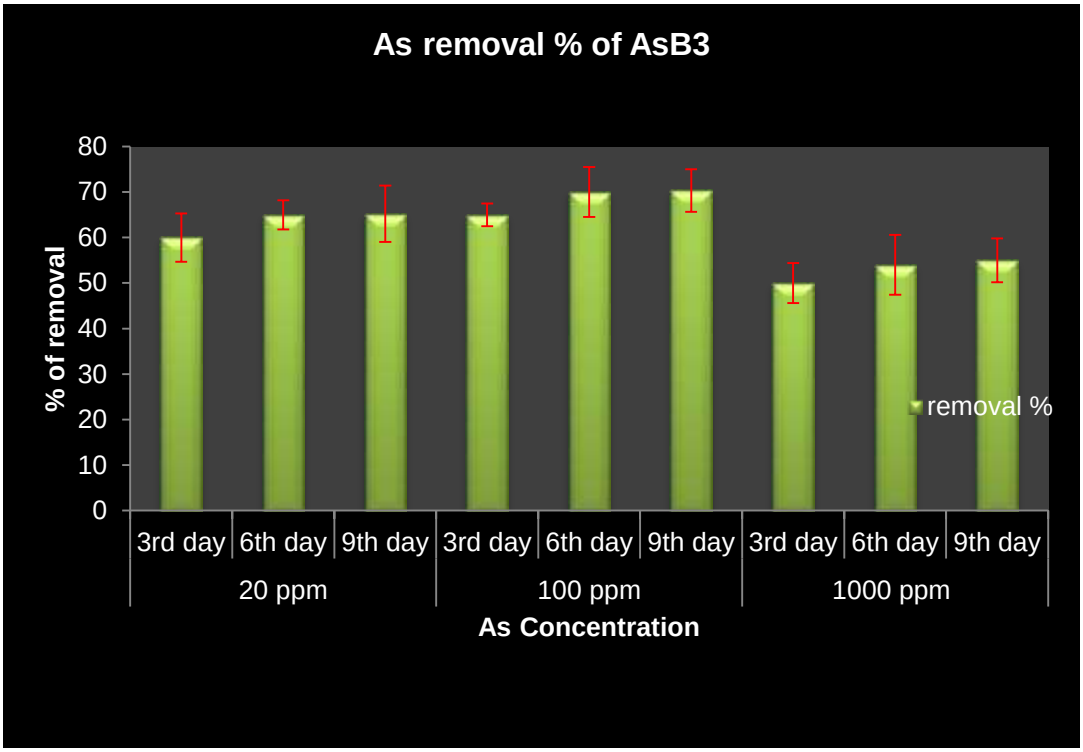


Fig. 5.14 Arsenic removal by AsB3 strain

The strain CrTW1 showed increased metal removal capacity against 20 ppm dose of treated chromium concentration. With further increase in treated dose of Chromium the metal removal capacity decreased. It exhibited highest metal removal capacity (76.2%) against 20 ppm of treated concentration at 9th day of incubation. The metal removal capacity also increased with increasing incubation time but after the 6th day it reached a saturation point and further increase did not occur with increase in incubation time (Table 5.11; Fig. 5.15).

The other chromium tolerant strain CrTW3 also showed maximum metal removal capacity at 20 ppm of treated chromium concentration. The metal removal capacity increased (51.4%) and came to a stable point at 9th day of incubation against 20 ppm of treated chromium dose. The metal removal ability of the strain decreased at 1000 ppm of treated concentration at the 3rd day. But after that the percentage of metal removal slowly increased and again came to a saturation point at 9th day of incubation. In case of every treated concentration there is a significant increase in metal removal percentage from the 3rd day of incubation to the 6th day of incubation. (Table 5.12; Fig. 5.16)

Thus both the strain showed highest metal removal capacity at 20 ppm of chromium concentration and at the 9th day of incubation. Chromium removal percentage decreased at 1000 ppm of treated concentration for both the strain and their metal removal capacity also got saturated after the 6th day (Table 5.11 & 5.12; Fig. 5.15 & 5.16).

Table 5.11 Chromium removal (%) of CrTW1 strain (*Aspergillus niger*)

Cr in media	Days of incubation	removal %	SD
20ppm	3rd day	63.5	3.2
	6th day	74.8	2.3
	9th day	76.2	2.6
100ppm	3rd day	44.6	4.5
	6th day	62.4	5.1
	9th day	65.5	2.9
1000ppm	3rd day	36.2	5.6
	6th day	47.6	4.8
	9th day	48.4	4.7

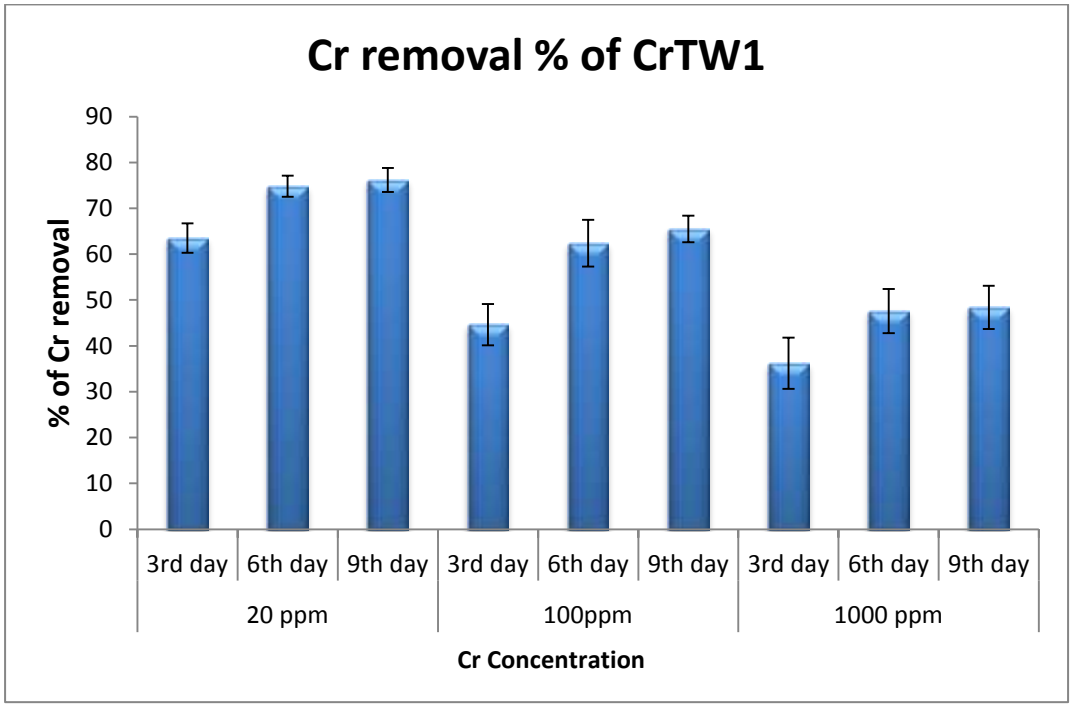


Fig. 5.15 Chromium removal by CrTW1 strain

Table 5.12 Chromium removal by CrTW3 strain (*Penicillium* sp.)

Cr in media	Days of incubation	removal %	SD
20ppm	3rd day	40.6	6.2
	6th day	48.8	7.2
	9th day	51.4	4.6
100ppm	3rd day	25.8	4.5
	6th day	28.5	5.8
	9th day	29.2	6.9
1000ppm	3rd day	16.4	5.6
	6th day	22.6	8.4
	9th day	22.2	7.2

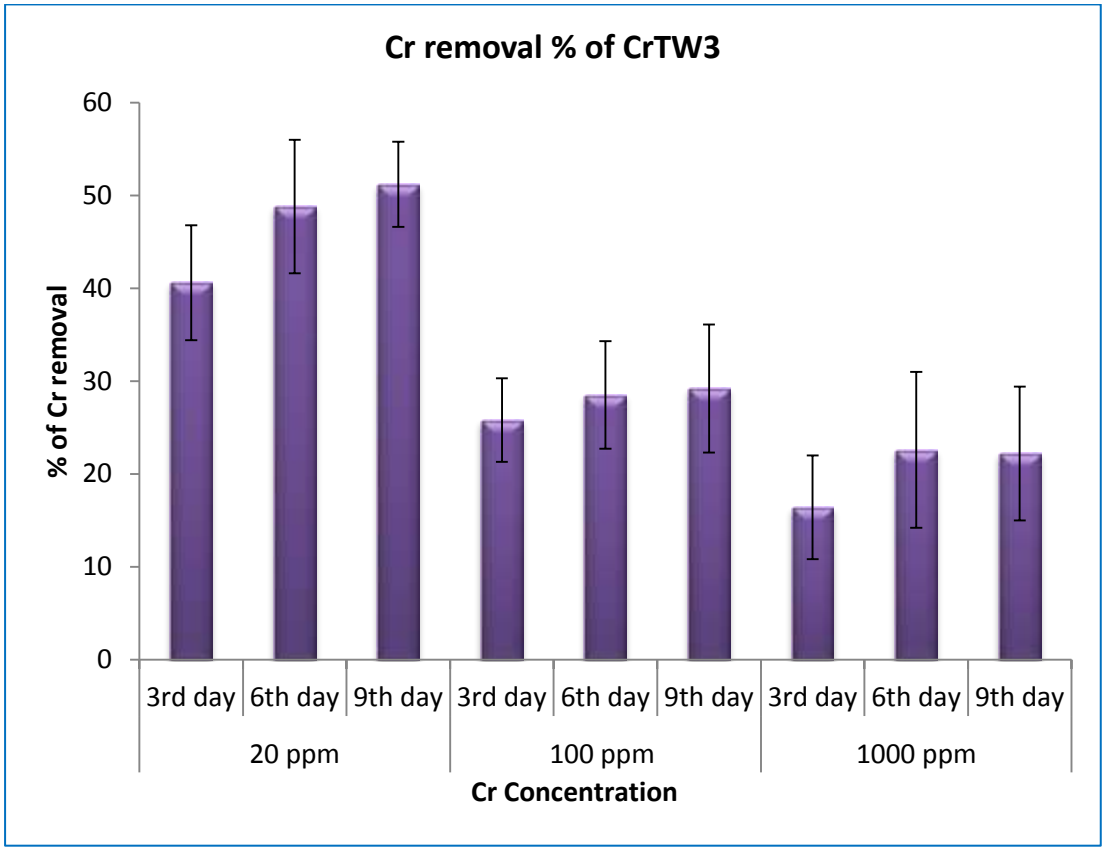


Fig. 5.16 Chromium removal by CrTW3 strain

The fungal strain CdEP used for removal of cadmium from different concentration of cadmium containing medium in different incubation time at *in vitro* condition. It had demonstrated utmost (67.2%) cadmium removal percentage against 20 ppm of treated cadmium concentration at 9th day of incubation time. It exhibits decreased metal removal percentage at 1000ppm of treated concentration. A significant increase in metal removal percentage from the 3rd day of incubation to the 6th day of incubation took place but after that with the increase in incubation time the metal removal capacity attained saturation (Table 5.13; Fig. 5.17).

Table 5.13 Cadmium removal by CdEP strain (*Aspergillus flavus)*

Cd in media	Days of incubation	removal %	SD
20ppm	3rd day	53.6	6.2
	6th day	64.9	4.3
	9th day	67.2	2.6
100ppm	3rd day	42.6	5.5
	6th day	52.5	4.1
	9th day	55.5	3.9
1000ppm	3rd day	33.2	3.6
	6th day	46.6	4.2
	9th day	48.2	7.4

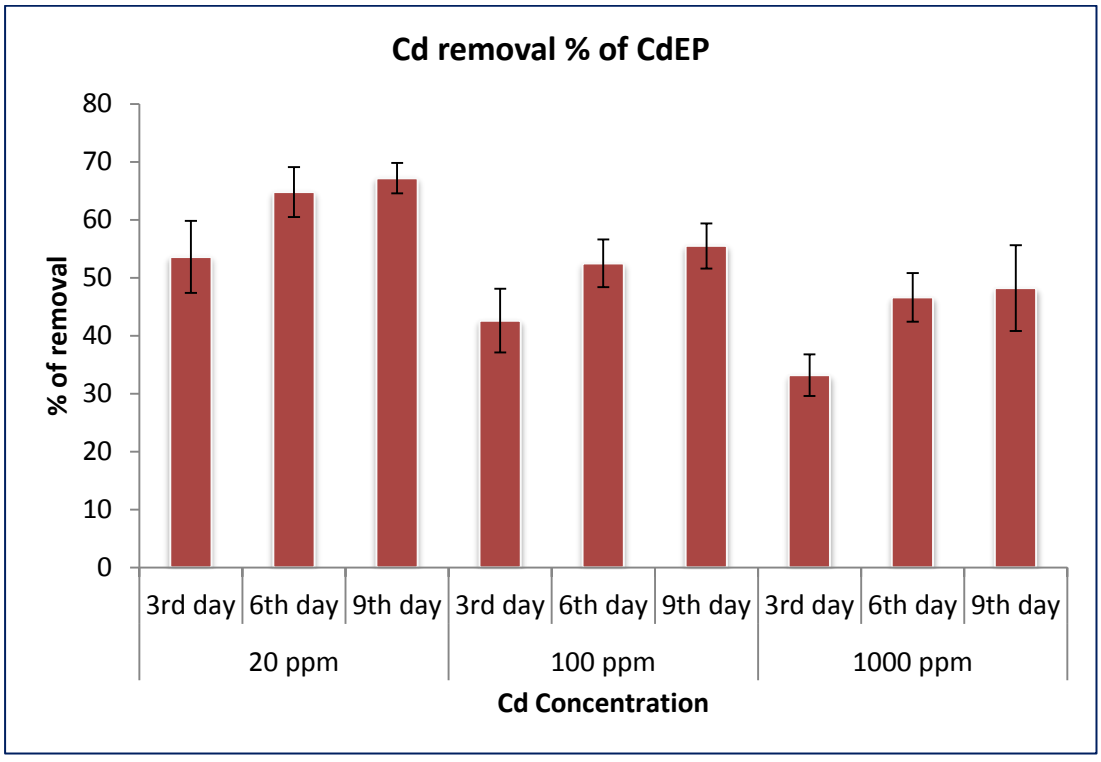


Fig. 5.17 Cadmium removal by CdEP strain

5.10 Metal accumulation by live fungal mycelium

The bioaccumulation of metal was measured in the fungal mycelium after treatment with different concentrations (20, 100 and 1000ppm) of metal with different incubation period (3, 6 and 9 days). The live fungal mat could also accumulate metal from the metal containing broth.

The bioaccumulation capacity of AsC1 strain increased with incubation time up to the 6th day. After 6th day the accumulation capacity became stable and did not increase significantly. It also increased with the increase in treated dose of arsenic (Fig. 5.18).

The bioaccumulation capacity of AsB3 strain increased with the increase in arsenic dose. The strain showed highest accumulation capacity after 6th day of incubation and after that the accumulation capacity ceased (Fig. 5.19).

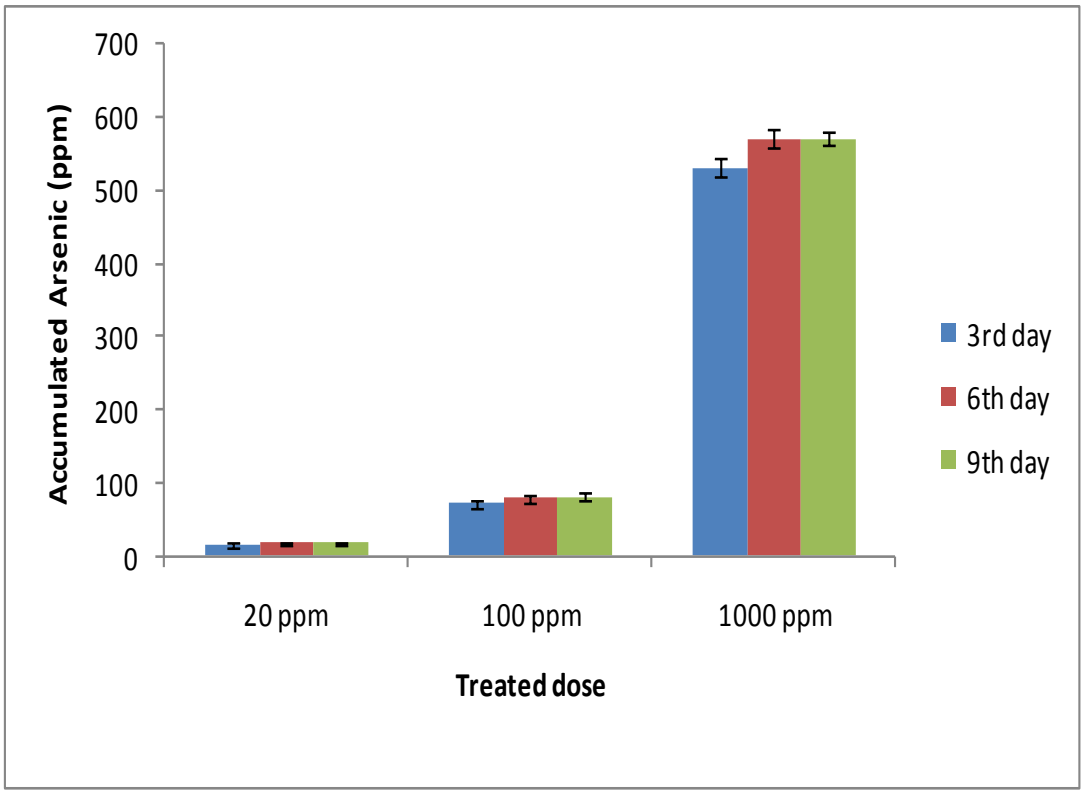


Fig. 5.18 Accumulation of Arsenic by live fungal mycelium of AsC1

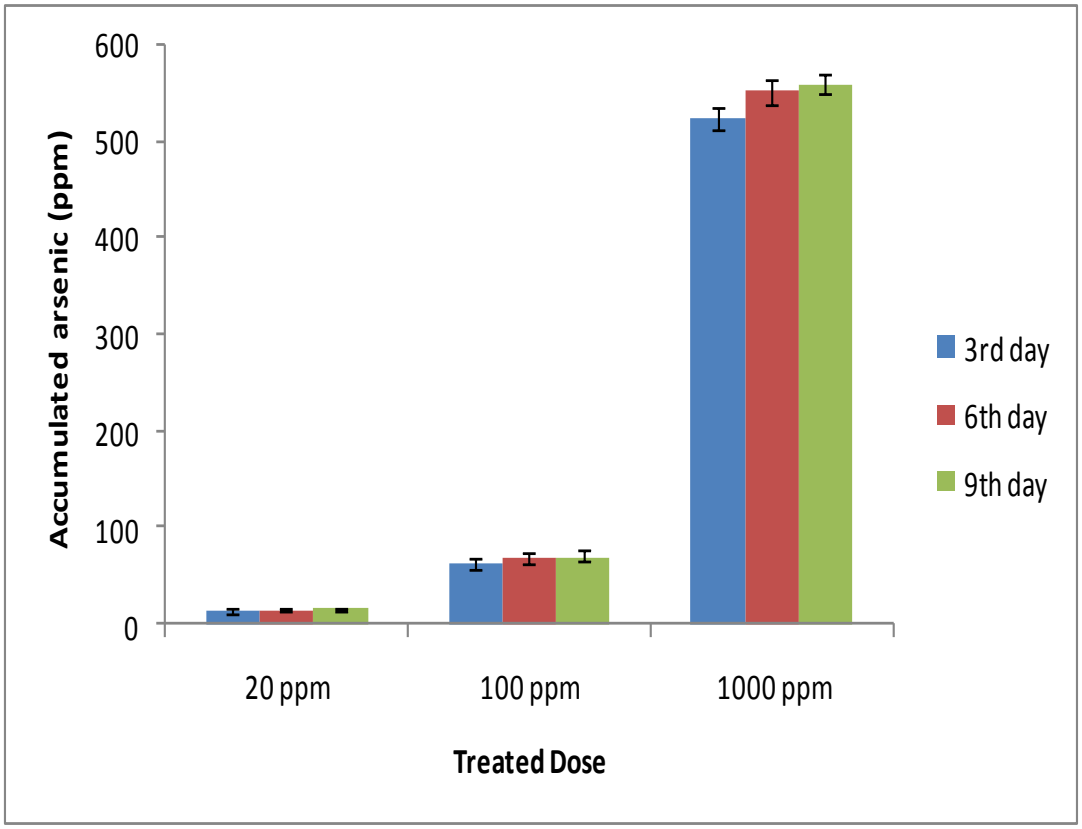


Fig. 5.19 Accumulation of Arsenic by live fungal mycelium of AsB3

The bioaccumulation capacity of the strain CrTW1 increased with the increase in chromium dose. The accumulation capacity increased till the 6th day of incubation and after that the accumulation capacity ceased at 9th day of incubation (Fig. 5.20).

The strain CrTW3 exhibited highest level of accumulation at the 6th day of incubation and at 9th day the accumulation capacity came to a stable point. The accumulation capacity increased with increasing metal dose (Fig. 5.21).

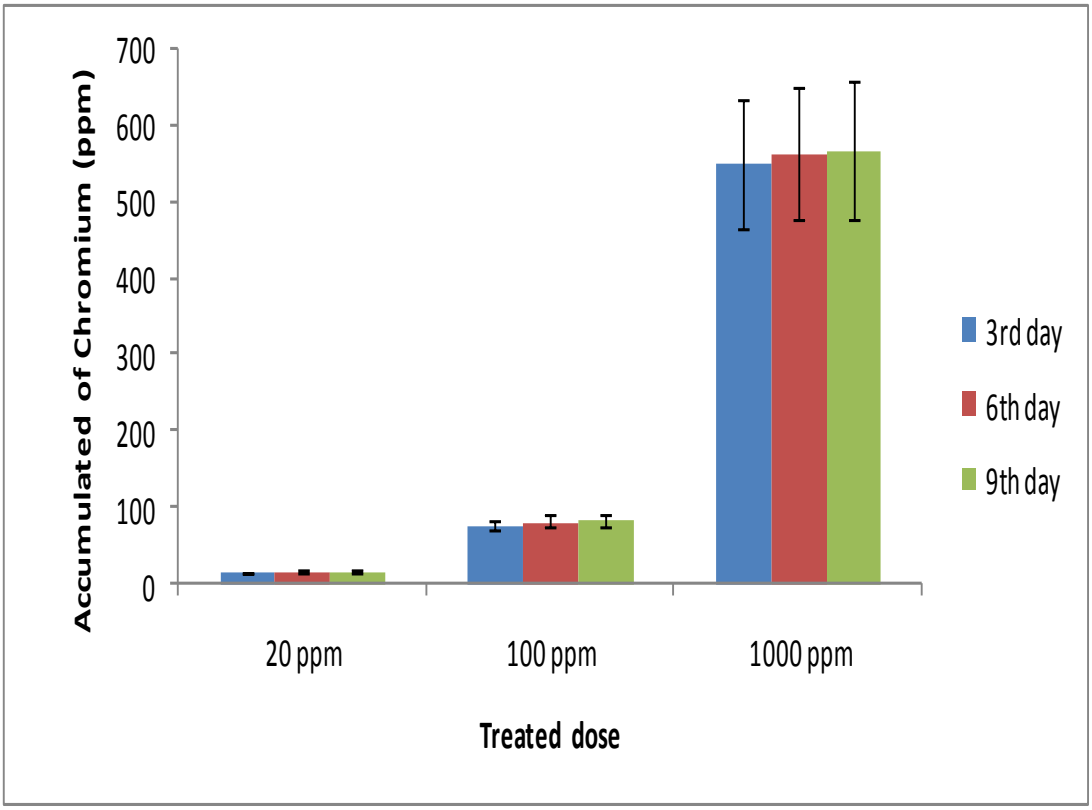


Fig. 5.20 Accumulation of Chromium by live fungal mycelium of CrTW1

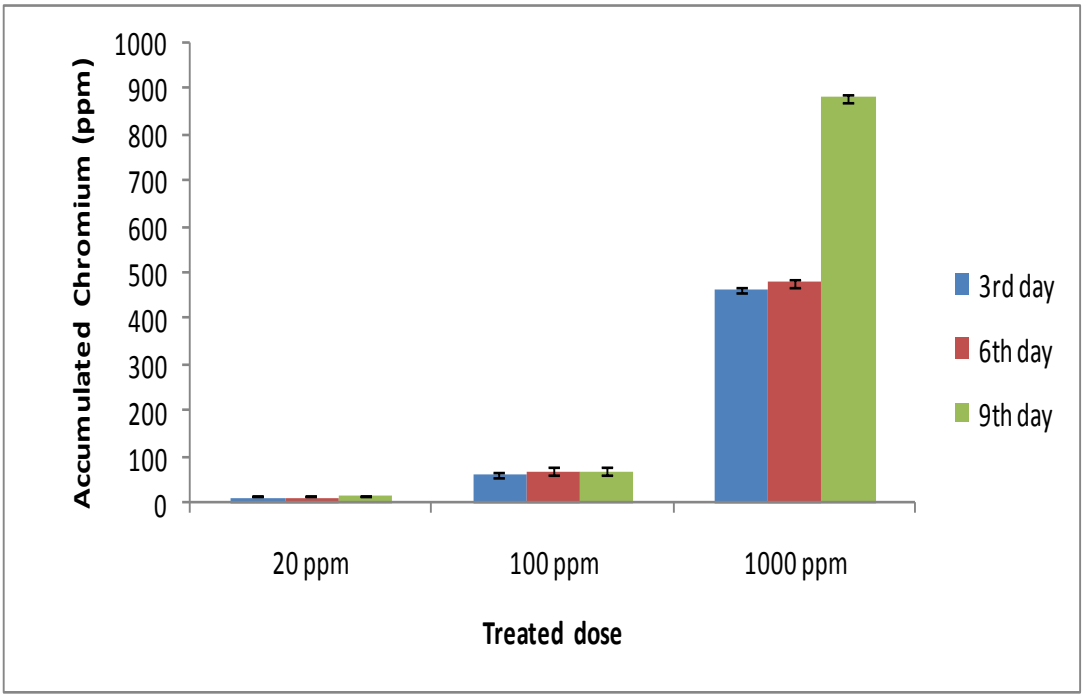


Fig. 5.21 Accumulation of Chromium by live fungal mycelium of CrTW3

The bioaccumulation of the cadmium by the fungal strain CdEP was measured applying different metal concentration against incubation time. The strain CdEP

showed maximum accumulation capacity at the 6th day of incubation. The accumulation capacity increased with increasing cadmium concentration (Fig. 5.22).

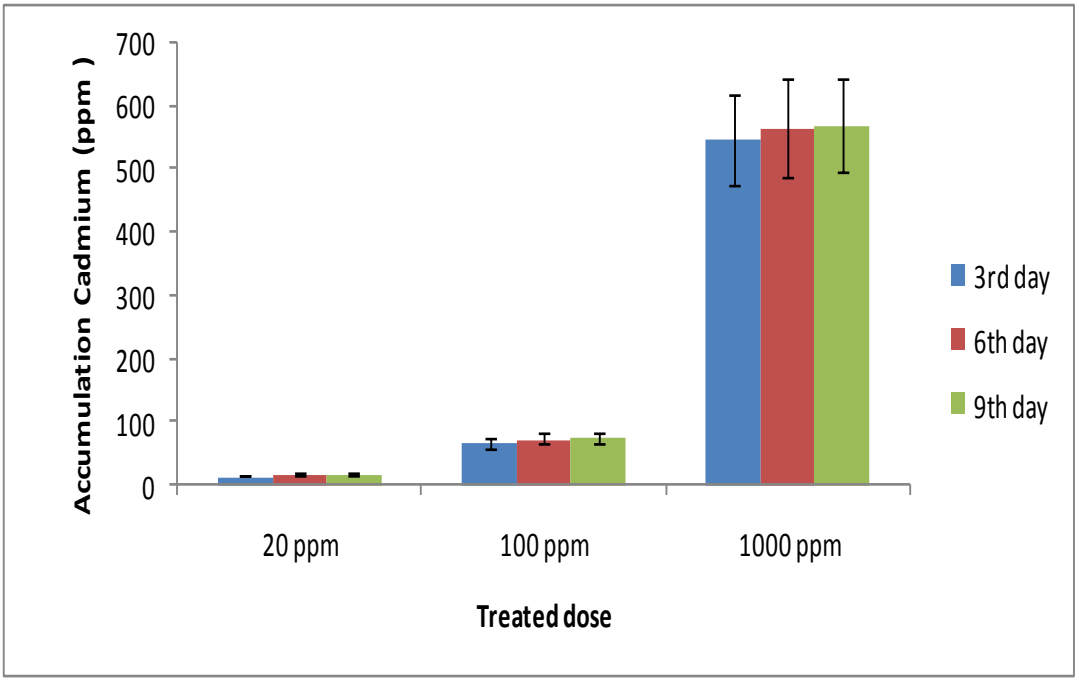


Fig. 5.22 Accumulation of Cadmium by live fungal mycelium of CdEP

In actively growing fungal cultures, the phases of adsorption and intracellular uptake may be hidden by changes in the physiology and morphology of the fungus and the physical and chemical properties of the growth medium. A frequently observed phenomenon is that the metal uptake by growing batch cultures is maximal during the lag period or early stages of growth and declines as the culture reaches the stationary phase. Thus in this study when all the strain reached at the 9th day of incubation, the accumulation capacity declined.

5.11 Metal uptake by dead fungal mycelium

The metal binding capacity of the fungal biomass permits the removal of toxic metals through biosorption onto dead and inactive fungal biomass. Adsorptive pollutants like metals and dyes can be removed by living microorganisms, but can also be removed by dead biological materials. In this study the dead mycelial mass of all the strains were treated with three different doses of metal concentration (20,100 and 1000ppm)

and the accumulation was observed after the 3rd day of incubation with continuous shaking (Fig. 5.23).

Among the two strains AsC1 and AsB3, AsC1 exhibited higher arsenic accumulation. The proportion of adsorption of AsC1 strain decreased with increase in treated dose of arsenic.

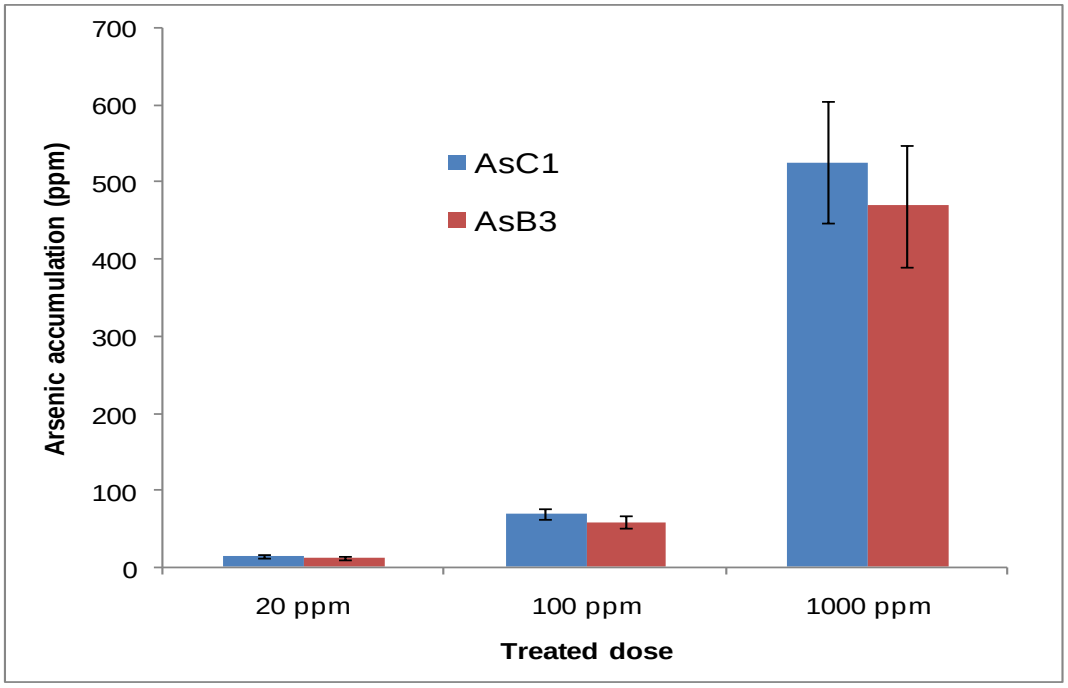


Fig. 5.23 Arsenic accumulation in dead mycelium of AsC1 and AsB3 strain

Between the two chromium tolerant strains CrTW1 exhibited proportionate adsorption in dead mycelia with a consistently increasing rate while comparing with CrTW3. (Fig. 5.24). The proportionate adsorption of both the strains decreased with the increase in chromium treated dose.

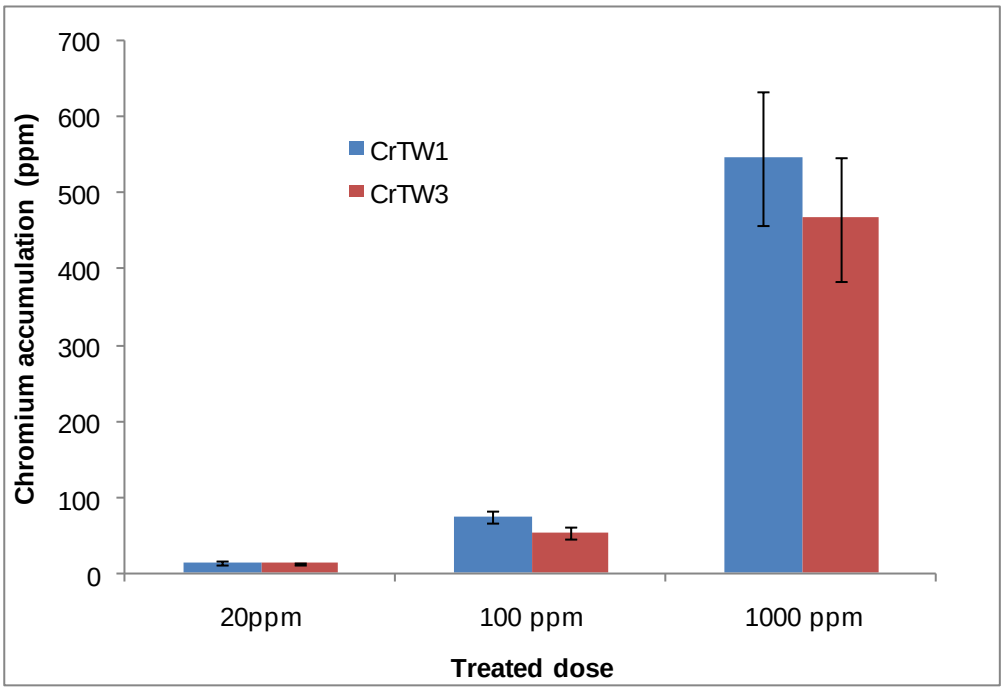


Fig. 5.24 Accumulation of chromium by dead mycelium of CrTW1 and CrTW3 strain

The strain CdEP showed maximum proportionate adsorption capacity against 20 ppm dose of cadmium. But the proportionate adsorption decreased against 100 ppm and 1000 ppm of treated cadmium concentration. (Fig. 5.25).

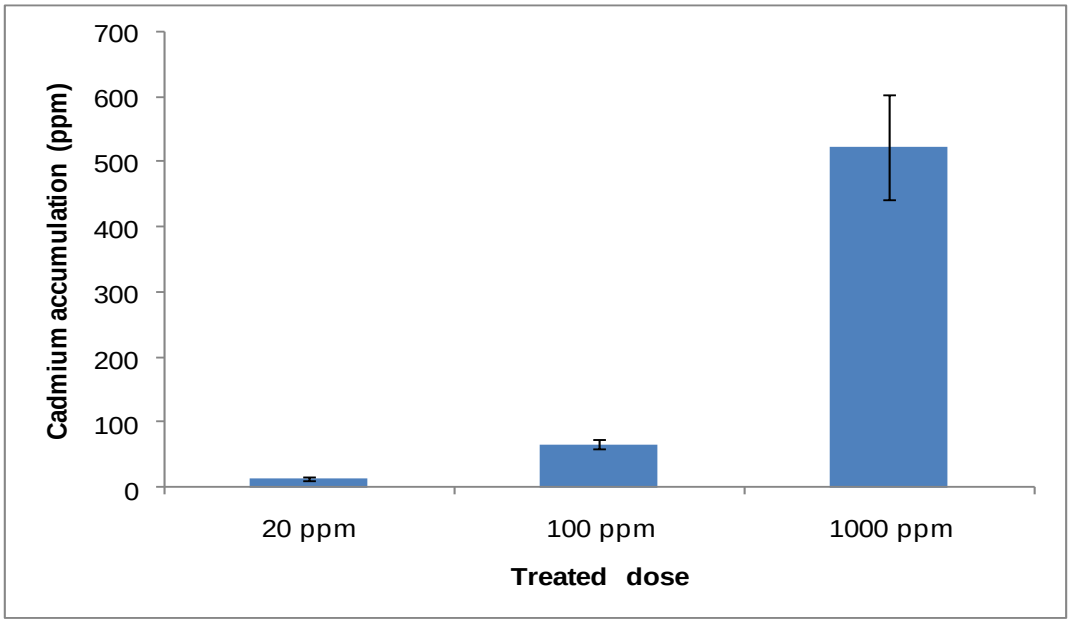


Fig. 5.25 Accumulation of Cadmium by dead mycelium of CdEP strain

All the fungal strains showed more or less maximum accumulation when exposed to 20 ppm of metal concentration. The accumulation capacity decreased with increase in

treatment dose and at certain dose it became saturated. The complex phenomenon of bioaccumulation was based on active metabolic transport, where as biosorption by dead biomass (or by some molecules and/or their active groups) was passive and occurred primarily due to the 'affinity' between the biosorbent and adsorbate.

5.12 Metal adsorption by chitosan

The chitosan used in this work was derived from crab shells. Two concentrations (20 ppm and 100 ppm) of each metal had been studied for chitosan. The pH was varied to analyse the relation between pH and metal adsorption by chitosan (Table 14, 15, 16).

Table 5.14 Adsorption of Arsenic in Chitosan

Total chromium in original solution	Total As after addition of chitosan	pH	Total chromium in original solution	Total As after addition of chitosan
20 ppm	11.74 ppm	6	100 ppm	67.12 ppm
20 ppm	15.98 ppm	4	100 ppm	83.32 ppm
20 ppm	18.17 ppm	2	100 ppm	93.39 ppm

Table 5.15 Adsorption of Chromium in Chitosan

Total chromium in original solution	Total Cr after addition of chitosan	pH	Total chromium in original solution	Total Cr after addition of chitosan
20 ppm	13.67 ppm	6	100 ppm	82.12 ppm
20 ppm	15.67 ppm	4	100 ppm	87.98 ppm
20 ppm	18.89 ppm	2	100 ppm	92.76 ppm

Table 5.16 Adsorption of Cadmium by Chitosan

Total chromium in original solution	Total Cd after addition of chitosan	pH	Total chromium in original solution	Total Cd after addition of chitosan
20 ppm	14.72 ppm	6	100 ppm	74.12 ppm
20 ppm	16.92 ppm	4	100 ppm	86.32 ppm
20 ppm	19.19 ppm	2	100 ppm	91.39 ppm

For all the metals, the metal adsorption increased with increasing pH. Minimum adsorption was observed at pH 2 for all the metal. For all the metal the adsorption capacity increased with increase in pH. At 20 ppm of treated concentration chitosan absorbed highest level of cadmium but at 100 ppm chitosan adsorbed highest level of arsenic.

5.13 Analysis of Metallothionein

The Metallothionein are naturally occurring metal-binding peptides which plays an important role in metal accumulation. Metallothioneins (MTs) and polychelatins are the main metal sequestering molecules used by cells to immobilise metal ions offering selective, high affinity binding sites. Over expression of this peptide indicates metal accumulation in the microbial cell. From this study it is depicted that after exposure to metal concentration the expression of metallothionein increased. In compare to control set the metallothionein increased in a significant amount. Hence it is proved that these cells not only bind metals in their cell wall but also accumulates metal intracellularly. The present study have further revealed that the metallothionein production was induced in presence of metal in all the five strains and hence it could be recommended as a biomarker in exposing heavy metals like arsenic, chromium and cadmium(Table 5.17; Fig. 5.26).

Table 5.17 Estimation of Metallothionein in metal treated and control strains

Sl. No	Strain ID	Strain Name	Metal Concentration	Metallothionein Concentration(µg/g)	
				Control	Metal treated
1	AsC1	*Aspergillus flavus*	2000	12.17	16.52
2	AsB3	*Aspergillus. niger*	1000	11.30	18.37
3	CrTW1	*Aspergillus. niger*	1300	10.09	17.34
4	CrTW3	*Penicillium* sp.	1300	12.34	16.98
5	CdEP	*Aspergillus. flavus*	1200	15.66	18.874

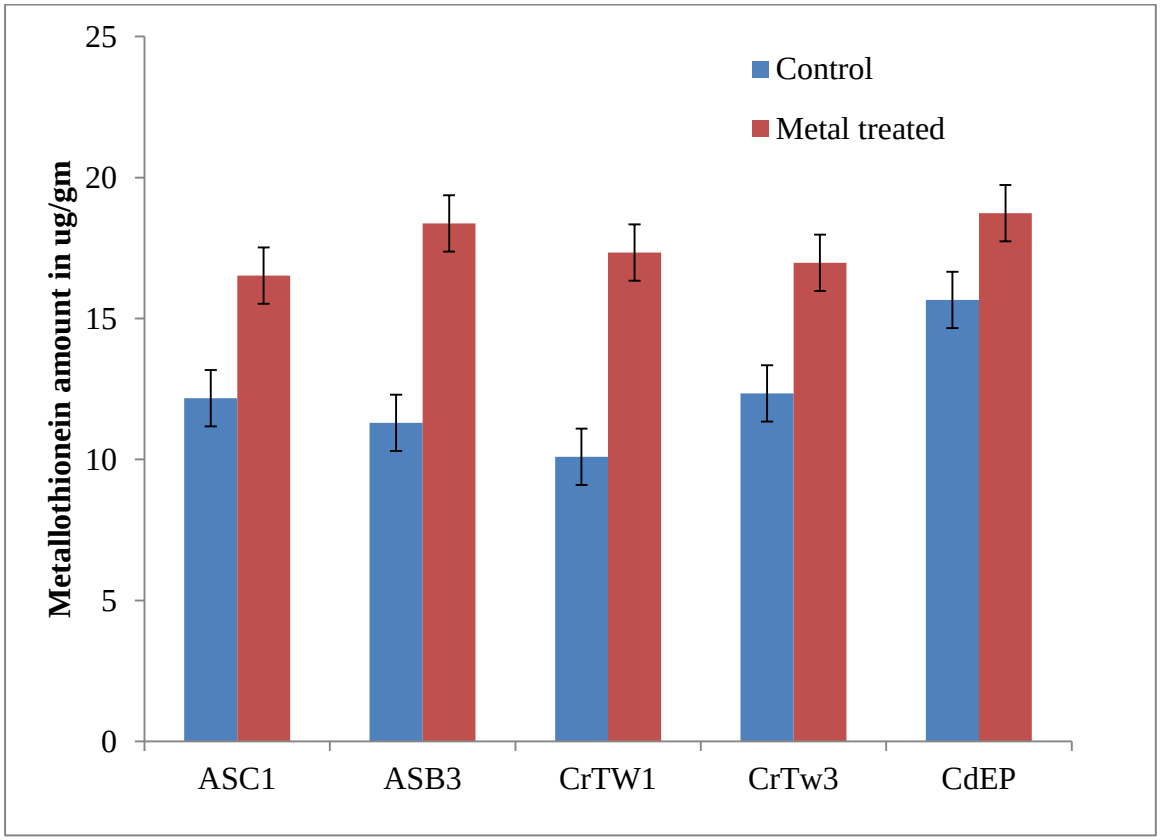
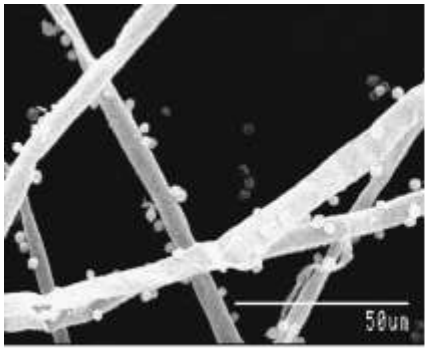


Fig. 5.26 Metallothionein activity of the studied fungal strains

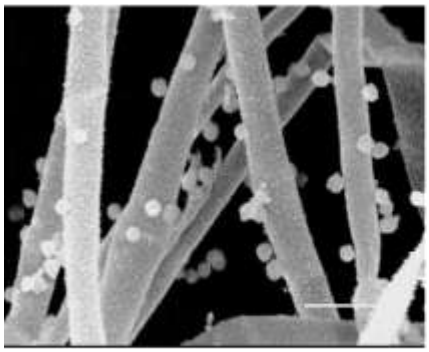
5.14 Scanning Electron Microscopy (SEM) study of the fungal isolates

Scanning electron micrographs were recorded using a software-controlled digital scanning electron microscope. The SEM images of the samples were recorded to study the surface morphology. The SEM images of the fungal strains with and without treatment of the metal concentration are shown in figures (Fig 5.27 – 5.31). An examination of the SEM micrographs revealed the presence of many pores and small openings on the biosorbent surface after metal treatment. The morphology was also damaged in many cases after metal treatment. A comparison of these micrographs before and after adsorption revealed the presence of significant changes in the morphology of the surface regarding the biosorbent.

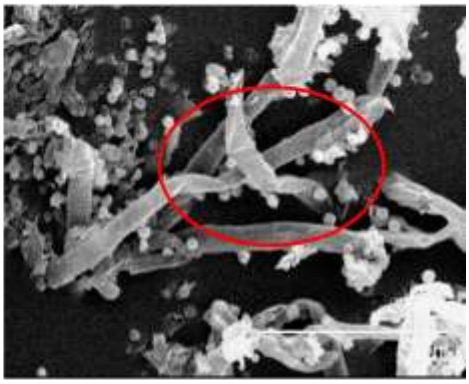
Scanning Electron Microscopy (SEM) study of the fungal strains treated with metal and those without metal showed significant morphological differences. From the figures it is revealed that all the strains were damaged due to metal exposure. The mycelial deformities, reduction of conidial vesicle and conidium and coiling of the hyphae were clearly found in metal treated ones comparing to the control one (Fig. 5.27 – 5.31). Breakage of hyphae, shrinkage of cell was also observed. The holes in the cell wall were a significant observation.



AsC1-Control-1000x

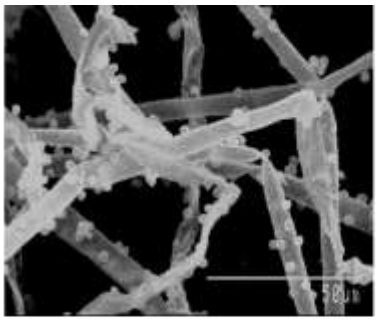


AsC1-Control-1500x

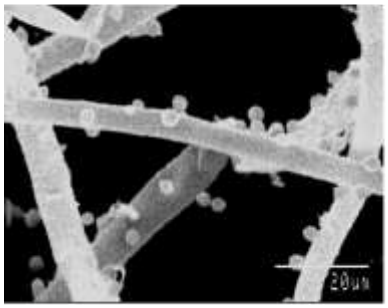


AsC1-2000-treated-1000x

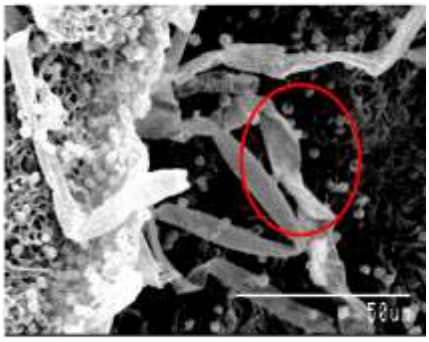
Fig. 5.27 Morphological changes in Arsenic treated fungal strain(AsC1) observed using Scanning Electron Microscopy (SEM)



AsB3-Control-1000x

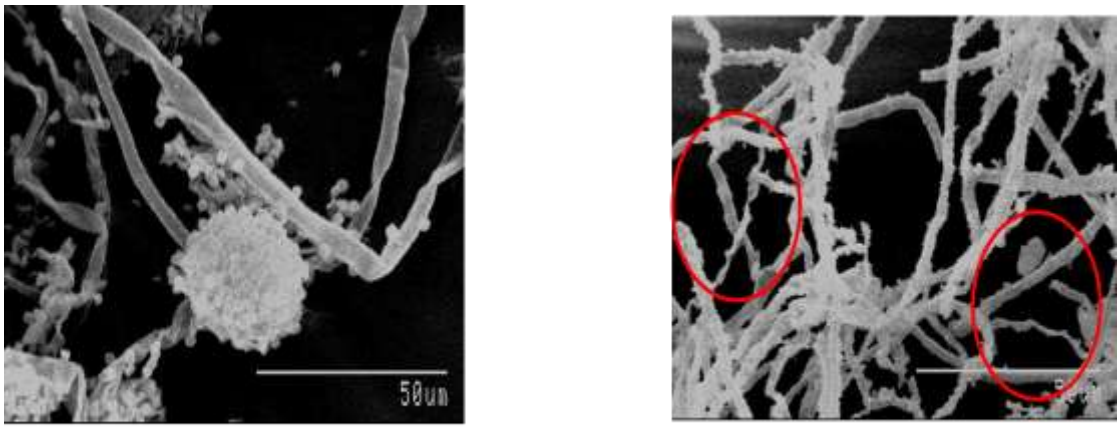


AsB3-Control-1500x



AsB3-1000-Treated-1000x

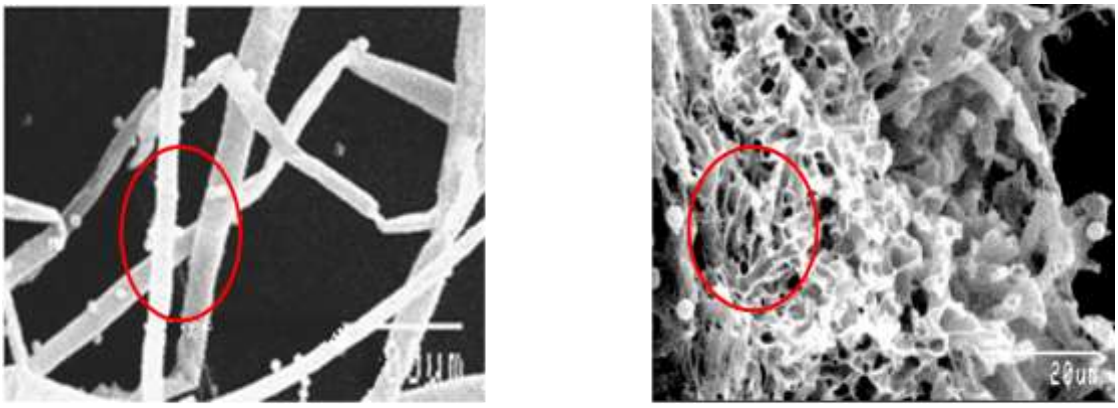
Fig. 5.28 Morphological changes in Arsenic treated fungal strain(AsB3) observed using Scanning Electron Microscopy (SEM)



CrTW1-Con-1000x

CrTW1-1000-Treated_1000x

Fig. 5.29 Morphological changes in Chromium treated fungal strain(CrTW1) observed using Scanning Electron Microscopy (SEM)



CrTW3-Con-1500x

CrTW3-2000-treated-1500x

Fig. 5.30 Morphological changes in Chromium treated fungal strain(CrTW3) observed using Scanning Electron Microscopy (SEM)

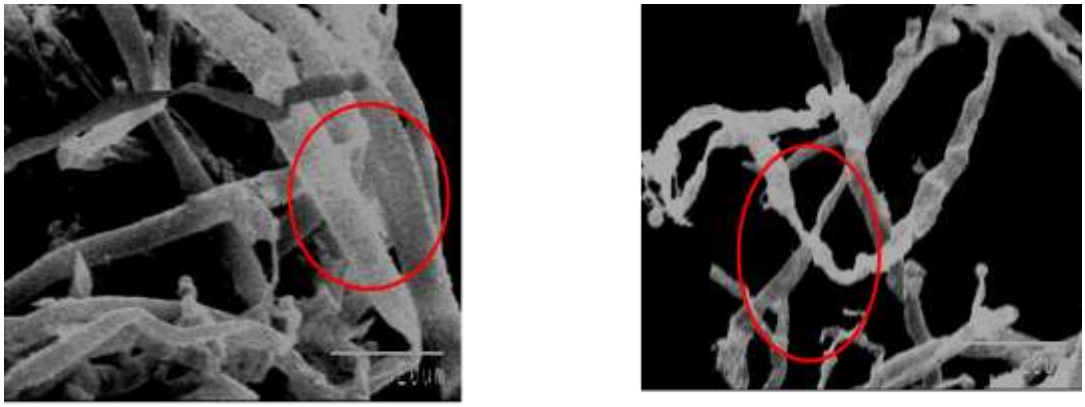


Fig. 5.31 Morphological changes in Cadmium treated fungal strain(CdEP) observed using Scanning Electron Microscopy (SEM)

DISCUSSION

Heavy metal toxicity is an alarming problem in the recent era. The study of the interaction between toxic metals and fungi has always remain a scientific interest. The observations on the ability of fungi to resist and adapt to toxic metals stimulated further work on the physiological, bio-chemical and genetical explanations for these phenomena (**Gadd, 1993**). In fungi the metal toxicity may depend on the strain and different stages of its growth. The detoxification mechanism of heavy metal also varies from strain to strain. The survival of fungi in the presence of metal depends on several factors like intrinsic biochemical and morphological properties, physiological and genetical adaptation.

Microbial populations in metal polluted environments adapt to toxic concentrations of heavy metals and become metal resistant (**Prasenjit and Sumathi, 2005**). Exposure of microorganisms to heavy metals can lead to physiological adaptation or the selection of mutants (**Donmez and Aksu, 2001; Italiano et al., 2009**) and such changes may be associated with increased metal accumulation and sorption capacity (**Villegas et al., 2008**). In this study five fungal strains were selected from a number of fungal isolates depending on their metal tolerance capacity from the urban contaminated soils around Kolkata city. The pH of the soil was neutral to alkaline. The fungal growth and metal absorption of fungi is dependent on pH of the medium. These soils contained significant amount heavy metals (Cd, As, Cr, Pb, Ni). Thus it was assumed that the fungal strains would be tolerant to high concentration of heavy metals. It has been reported that a number of fungi from all taxonomic groups may be found in metal polluted soils and they possessed the ability to survive in that environment by detoxification of the metal **(Ross, 1975; Gadd, 1986a; Baldi, Vaughan & Olson, 1990; Turnau, 1991).** Usually metal toxicity reduces the abundance of fungal population and thus only the metal tolerant species survive (**Babich & Stotzky, 1985; Duxbury, 1985).** In this study all the selected five fungal strains could resist and tolerate different heavy metals up to a certain range. The strain AsC1 could tolerate arsenic concentration up to 2000ppm and AsB3 strain could tolerate arsenic up to 1000 ppm. The strain CrTW1 and CrTW3 could tolerate chromium up to 1300ppm. The strain CdEP could tolerate cadmium up to 1200ppm.

These studies revealed that all the strains could tolerate high level of respective heavy metals. Thus they may possess some mechanism for metal tolerance.

The fungal strains were identified as *Aspergillus flavus* (AsC1), *Aspergillus niger* (AsB3), *Aspergillus niger* (CrTw1), *Penicillium* sp. (CrTw3) and *Aspergillus flavus* (CdEP) considering their morphological and microscopic characters. The suitable media, pH, incubation temperature and time were selected for the studied fungal strains according to their growth features. Temperature and pH are the two main environmental factors for fungal growth and sporulation (**Mehra and Jaitly, 1995**). The temperature can reach a point where growth stops and cell components begin to be actually damaged by the heat. Enzymes are proteins that change structurally when heated to their limit of tolerance. Likewise, membranes, which contain lipids, change in structure and their function of protecting and regulating the internal environment of the cell becomes compromised (**Kamil et. al, 2011**).

The antibiotic (Nystatin, Griseofulvin and Kanamycin) sensitivity test of the studied fungi shown resistance to certain variable concentrations. From MIC test the antibiotic Nystatin showed to be more effective to all the tested fungal strains than Griseofulvin and Kanamycin which have importance from clinical point of view. **Rambali et al, (2001)** studied the susceptibility testing of pathogenic fungi with intraconazole.

The fungal strains which are resistant to arsenic can also tolerate other heavy metals like Cd, Cr, Pb, Zn. This indicates that all the strains are positive for cross metal tolerance test and they posses multi metal tolerance activity. Thus the detoxification mechanism of the fungal strains are not metal specific but for only one metal it showed maximum amount of tolerance limit. In this study the specific metal was chosen for specific strains. Like arsenic was chosen for AsC1, AsB3; Chromium for CrTW1 and CrTW3; and Cadmium for CdEP. Metals are directly and/or indirectly involved in all aspects of fungal growth, metabolism and differentiation. While many metals are essential (e.g. K, Na, Mg, Ca, Mn, Fe, Cu, Zn, Co, Ni etc.) , many others have no apparent essential function (e.g. Rb, Cs, Al, Cd, Ag, Au, Hg, Pb etc.). However, all these elements can interact with fungal cells and can be accumulated by physico-chemical mechanisms and transport systems of varying specificity (**Gadd, 1988**).

The fungal strains could grow in respective metal containing media. All the strains were also grown without metal as control set. In the presence of metal the strains could grow up to a certain concentration but beyond that concentration their growth decreased. All the strains were grown in metal treated concentration up to 9^{th} day of incubation and after that their growth ceased, where as in the control set the growth achieved a stable state. Both the strain AsC1 and AsB3 showed highest growth at 1500ppm. The two strains which were treated against chromium also showed highest growth against 1500ppm. The strain CdEP showed highest growth against 1000ppm dose of cadmium concentration. Thus all the selected strains exhibited high growth rate against these metals up to a certain range, which proves that they can tolerate the toxic effect of the metal. The metal can bind the cell wall or may accumulate within the cytosol. The growth rate and biomass production of microorganisms is highly affected by metal ion concentration. Often, growth rate and biomass production is inversely proportional to the toxicity of the heavy metals. It decreases with increasing toxic metal concentration in the growth medium. **Acikel & Ersan (2010)** also reported that with the increase in Ni(II) ion concentrations in the range 0–50ppm, the specific growth rates of *R. delemar* decreased significantly. In tolerant strains, complex regulatory networks exist that ensures that the metabolic costs of the heavy metal response are kept at a minimum without compromising the detoxification process (**Bellion et al., 2006**). The change in growth parameters are also dependent on the nature of metal present. **Dursun et al. (2003b)** observed the growth of *Aspergillus niger* in presence of Cu(II), Pb(II), and Cr(VI) ions. Maximum biomass production by *A. niger* had been reported in absence of metal ions while the growth was inhibited by all concentrations of Cr(VI) tested. In this study the strains were tested against that particular metal which it can tolerate at its highest amount.

These fungal strains can bio-accumulate heavy metal from the respective medium. Bioaccumulation includes both bio-adsorption and bio-absorption. Bio-adsorption involves the attachment of metal on the cell wall. The wall is the first cellular site of interaction with metal species and metal removal from solution may be rapid although rates will depend on factors such as type of metal ion and biomass, concentration of metal and environmental factors.

The metal removal capacities of all the strain were high. The metal removal percentage ranged from 30% to 76%. The metal removal percentage of strain AsB3

ranged from 50% to 70%. The highest metal removal percentage showed by AsC1 was 76% and the lowest was 50%. The metal removing percentage for the strain CrTW1 ranged from 36% to 76.2% and for CrTW3 ranged from 22% to 51%. The metal removing capacity of CdEP ranged from 33% to 67%. Among all the five strains the strain CrTW3 showed lowest metal removing capacity. These results indicated that all the five fungal strain could accumulate metal from the metal treated broth thus resulting removal of the metal from the media. These kind of fungal strains thus can be effective for the remediation of metal contaminated water and soil. Metal tolerance property was reported from *Penicilium* genus (**Gadd et al, 1984a, Gadd and White, 1985**). The metal removal capacity may depend on a number of factors such as metal ion, environmental factor, and concentration of metal. **Ahmad et al. (2006)** studied the biosorption by metal tolerant *Aspergillus niger* and *Penicillium* sp. and concluded that fungi of metal contaminated soil have high level of metal tolerance and biosorption properties.

The bioaccumulation capacity was also tested with live fungal mycelium. These processes include both bio-adsorption and bio-absoprtion. All the strains can accumulate metal up to 6^{th} days of incubation after that the accumulation capacity become stagnant. Thus the strains can accumulate metal up to a certain time and showed tolerance but after that the strain succumbs to the toxicity.

After uptake of the metal the cell wall is the first space where the metal interacts. The metal biosorption in the fungal cell wall may be complex, involving different compositions and mechanisms. The uptake of metal by cell wall is variable depending on the wall structure. Chitin and chitosin have received attention as metal sorbing components of the cell wall. While studying the metal bio-adsorption capacity of the dead fungal mycelium in this case, it is found, that in all the fungal strains the metal accumulation by fungal mycellium decreased with increase in treated metal dose. Among the two strains AsC1 and AsB3, AsB3 strain showed highest arsenic accumulation. Among the chromium tolerant strains CrTW3 strain showed highest accumulation. The strain CdEP can accumulate significant amount of cadmium in the dead mycelium. Thus among the five fungal strains, AsB3 strain can accumulate highest metal in the cell wall.

Chitosan is a good source for adsorption of heavy metals. Chitosan is a biopolymer, which is extracted from crustacean shells or from fungal biomass. The high porosity

of this natural polymer results in novel binding properties for metal ion such as cadmium, copper, lead, mercury and chromium etc. Chitosan occur naturally in the environment in large quantities and run second in abundance to cellulose. It has an amine functional group which is strongly reactive with metal ions. Considerable research has been done on the uptake of metal cations by chitosan. The amine groups on chitosan bind metal cations at pH close to neutral. At low pH, chitosan is more protonated and therefore it is able to bind anions by electrostatic attraction (**Guibal, 2004**). In this study chitosan could adsorb significant amount of metal from metal treated solution. The adsorption capacity decreased with decrease in pH. At low pH i.e, pH-2 chitosan could adsorb lowest amount of all three metals. At low pH the amine group get protonated. That means chitosan get positively charged. Chromium, cadmium and arsenic ions are also positively charged. As a result repulsive forces occur between the metal ions and chitosan instead of attraction. Therefore at low pH arsenic, chromium and cadmium uptake will be reduced.

The metallothionein protein was estimated to analyse the bioaccumulation of metal. Metallothionein is a peptide which plays an important role in metal accumulation. MTs have been found throughout the animal kingdom, in higher plants, in eukaryotic microorganisms, and in many prokaryotes. MTs have been divided into three classes based on their structural similarities. Class I, II and III with multiple isoforms within each class. MT-I and MT-II are ubiquitously expressed and are stress inducible (**Thirumoorthy et al., 2007**). The amino acid sequences of MTs from many mammalian sources reveal that all contain approximately 61 amino acids of remarkably similar composition. More importantly, all contain 20 cysteine residues that remain invariant along the amino acid sequence. All cysteines are known to participate in the coordination of 7 mol of Cd or zinc (Zn) per mol of MT (**Kägi & Vallee, 1960; Klaassen, et al., 1999**). Coordination of these cysteine residues results in a high binding affinity for Zn and Cd (**Curtis et al., 1999**). The increase in metallothionein content reveals the accumulation of the metal. In this study all the five strains showed increased amount of metallothionein content in comparison to the control set. The strain CdEP showed highest amount of metallothionein followed by AsB3 strains. According to previous results we have seen that AsB3 and CdEP strain could accumulate high amount of metal among the five strains, thus the metallothionein level of these two strains were also high among the five strains.

The microbes which can resist metal go through a range of morphological and ultra structural changes. After intake of the metal, the toxic metal ion form complexes with cellular membrane. This causes the loss of its integrity and impairs its function. (**Yilmazer and Saracoglu, 2009).** It was reported that the morphology and physiology of the cell changes with increased concentration of metal **(de Siloniz et al., 2002).** In case of fungi, mycelia become short, dense, and broken in the metal treated strains in comparison to the control strains. Improved aggregation of the fungal hyphae can be one of the morphological strategies in response to toxic metals (**Gadd, 2007**). Due to aggregation of hyphae the exposed surface area reduces and facilitates high local concentrations of extracellular products (organic acids and siderophores), metal precipitating agents, polysaccharides and pigments with metal binding abilities (**Baldriah, 2003; Dutton and Enans, 1996**). Twisting and looping of individual hyphae and formation of intertwined hyphal strands in response to cadmium stress (**Lilly et al., 1992**) and decreased overall mycelial length in response to Cd and Cu stress has been reported (**Gadd, 2001**). Saccharomyces cerevisiae and Aureobasidium pullulans employ different mechanisms to overcome Pb toxicity as the former accumulates Pb inside the cells while the later displays extracellular sequestration. Scanning Electron Microscopy reveals that concomitant with the Pb(II) accumulation, the cell surface of *S. cerevisiae* became rough and the amounts of potassium, phosphorus and sulphur on the cell surface decreased (**Suh et al., 1998**). In Taiwan one study was carried out with effect of Cu (II) and Zn (II) on growth and cell morphology of *Thraustochytrids* isolated from fallen mangrove leaves (**Lin et al.2010**). *Schizochytrium* cells exposed to >32 ppm concentration showed formation of holes in the cell wall, shrinkage and general deformation of the cell structure as depicted by light and scanning electron micrograph while at very high concentrations (>250 ppm) cell lysis resulting in leakage of cellular content was observed. **Baratelli et al. (2007)** investigated morphological and ultra structural changes in *Paracoccus* sp. DE2007 strain isolated from Pb polluted site. The SEM image revealed that cells were more deformed and had a higher tendency to aggregate. The *Paracoccus* sp. DE2007 strain is able to capture lead extracellularly in its EPS envelopes, but does not bioaccumulate intracellularly. Still increase in number of intracellular vesicles was noticeable in presence of lead. This could be related to an increase in the synthesis of lipid compounds that play an important role as a carbon reserve in microorganisms under stress conditions such as the exposure to heavy metals. In this

study when the strains were exposed to high concentration of metal for a certain period of incubation time the Scanning Electron Microscopy study revealed morphological changes. The cells were deformed, the breakage of hyphae was observed. Formations of holes in the cell wall, shrinked and wrinkled surface were also depicted from the SEM analysis. Major holes in the cell wall were observed in *Penicillum* sp. (CrTW3) strain.

The five strains were identified mostly form *Aspergillius* genus and one from *Penicilllium* genus. AsC1 was identified as *Aspergillus flavus***,** AsB3 was identified as *Aspergillus niger,* CrTW1 as *Aspergillus niger***,** CrTw3 as *Penicillium* sp. and CdEP was identified as *Aspergillus flavus.* Previously several study reported various strains from *Aspergillus* and *Penicillium* genus as heavy metal tolerant fungi. **Liu et al. (2009)** reported *Aspergillus flavus* as copper and Zinc resistant strains which can tolerate copper up to 400 ppm and zinc up to 800 ppm. **Ezzouhri et al. (2009)** reported both *Aspergillus* and *Penicillium* strains as lead, copper and chromium resistant strains. **Parameswari et al. (2010)** reported *Aspergillus niger* as Cr and Ni resistant fungal strain. **Congeevaram et al. (2007)** isolated some strains, in which Cr-resistant *Aspergillus* sp. and *Micrococcus* sp. survived to a maximum level of 10000 ppm. **Valix et al. (2001)** achieved nickel tolerance of up to 2000 ppm by *Aspergillus foetidus* and *Penicillium simplicissimum* on solid media through step wise acclimatization. Thus it is evident from the previous study that *Aspergillus* and *Penicillium* genus had adapted to toxic effects of heavy metals and developed heavy metal resistant mechanism.

The use of microorganism in heavy metal remediation has attracted a special attention in recent decades. In the recent years extensive research and development has occurred on biological methods, which have been considered as an eco-friendly alternative for remediation of heavy metal contamination. Nevertheless, a large pool of these studies have investigated microbial systems such as fungi, bacteria, and algae as adsorbing agents for removal of heavy metals (**Munoz et al., 2006; Pena-Castro, 2004**). Briefly, biosorption studies involving easily available dead or live biomass are a suitable low-cost approach to treat heavy metal pollution. In this study all the strains can tolerate high amount of heavy metal. The strains can grow in the metal containing media up to a certain period of incubation time. Both the dead and the live mycelia of all the strains can accumulate metal from metal containing media.

Biosroption is a new and less expensive method for removal of heavy metals. Among biosorbents, attempts have been made to use fungal biomass for the removal of heavy metals. These strains could be helpful to build a metal removal strategy but more intensive study is needed on the mechanism of metal absorption by the cell wall and cytosol.

CONCLUSION

Heavy metal pollution has been a serious environmental threat of the recent era. Use of wastewater in agriculture has increased in the recent years due to inherent treatment capacity of soil and high contents of major micronutrients in it. However, wastewater, particularly from industries, contains high concentrations of heavy metals which enter into human beings and animals through food chain. Therefore, it is desirable to remove these heavy metals from wastewater through low cost technology before it is used in agriculture. Physico-chemical methods such as reverse osmosis, solvent extraction, lime coagulation ion exchange and chemical precipitation for removal of heavy metals from wastewater are very expensive and these do not remove heavy metals from wastewater up to the desired level. Microorganisms including fungi have been reported to exclude heavy metals from wastewater through bioaccumulation and biosorption at low cost and in an eco-friendly way. Microbial population in metal adapted environment build metal defence mechanism and adapt to toxic concentration of heavy metal and ultimately became metal resistant. Different species of *Aspergillus* have been reported as efficient chromium and nickel reducers (**Gopalan & Veeramani, 1994).**

Biological mechanisms implicated in fungal survival (as distinct from environmental modification of toxicity) include extracellular precipitation, complexation and crystallization, transformation of metal species by oxidation, reduction, methylation and dealkylation, biosorption to cell walls, pigments and extracellular polysaccharide, decreased transport or impermeability, efflux, intracellular compartmentation and precipitation and/or sequestration (**Ross, 1975; Gadd & Griffiths, 1978; Gadd, 1988, 1990a, b, 1992a-c; Brown & Hall, 1990; Mehra & Winge, 1991).** A particular organism may directly and/or indirectly rely on several survival strategies. For example, metallothionein synthesis is a mechanism of Cu^{2+} resistance in *Saccharomyces cerevisiae* yet Cu^{2+} binding or precipitation around the cell wall and intracellular transport are also components of the total cellular response (**Gadd & White, 1989a**). In this study detailed analysis have been done with the tolerance and bioaccumulation capacity of five fungal strains.

The major findings of these studies are:

- Five fungal strains were isolated namely AsC1, AsB3, CrTW1, CrTW3 and CdEP out of total screening 30 fungal isolates. All these strains could accumulate high amount of heavy metals. AsC1 and AsB3 were tested against arsenic, CdEP strain for cadmium tolerance, CrTW1 and CrTW3 for chromium.
- AsC1 and CdEP were identified as *Aspergillus flavus*, AsB3 and CrTW1 were identified as *Aspergillus niger* and CrTW3 was identified as *Penicillium* sp.
- The optimum pH and temperature has been analysed for the five strains. At pH 7 and at a temperature of 30ºC all the further experiments were carried out.
- From the antibiotic test it was found that the antibiotic Nystatin was more effective to all the tested fungal strains than Griseofulvin and Kanamycin.
- All the fungal strains could tolerate high level of metal concentration. AsC1 could tolerate arsenic up to 2000 ppm, AsB3 could tolerate arsenic upto 1000 ppm. CrTW1 and CrTW3 could tolerate chromium up to 1300 ppm. CdEP could tolerate cadmium upto 1200 ppm.
- All the fungal strains could accumulate other metals like lead, mercury, Zinc. The strains tested for arsenic could also tolerate cadmium and chromium with the other metals.
- The strain AsC1 showed highest growth against 2000 ppm dose of treated arsenic up to the 9^{th} day of incubation. The strain AsB3 showed highest growth against 1500 ppm dose of treated arsenic up to 9^{th} day. The strain CrTW1 and CrTW3 showed highest growth at 1500 ppm dose of treated chromium up to 9^{th} day. The strain CdEP showed highest growth against 1000 ppm dose of treated cadmium. All the strains showed growth up to the 9^{th} day and after that their growth ceased.
- The metal removal capacity of all the strains was high. The metal removal percentage ranged from 30% to 80%. The metal removal percentage of strain AsB3 ranged from 50% to 70%. The strain AsC1 showed highest metal removal percentage with 76% and lowest with 50%. The metal removing percentage for CrTW1 ranged from 36% to 76% and for CrTW3 ranged from 22% to 51%. The metal removing capacity of CdEP ranged from 33% to 67%.

- The metal bioaccumulation was also tested in the live mat of the fungal strains. All the strains could accumulate considerable concentration of metal. All the strains accumulated metal up to the 6^{th} day of incubation and after that the accumulation capacity became stagnant.
- For all the fungal strains the metal accumulation by the cell wall decreased with increase in treated metal dose. Among the two strains AsC1 and AsB3, AsB3 strain showed maximum metal accumulation by the cell wall. Among the chromium tolerant strains CrTW3 strain showed maximum metal accumulation by the cell wall. The strain CdEP can also accumulate cosiderably high level of metal in the cell wall. The metal absorption capacity by chitosan was also tested. Chitosan could adsorb metal to a significant level, but the metal accumulation capacity decreased with decrease in pH. At low pH2 chitosan adsorbed least amount of metal.
- The metallothionin expression also increased upon metal exposure. The content of metallothionin increased to a significant level in comparison to the control set for every metal.
- The morphological changes have been observed in the five fungal strains by Scanning Electron Microscopy. Shrinkage of cell, breakage of hyphae, mycelial deformities, reduction of conidial vessels were clearly shown in metal treated fungal strains.

Filamentous fungi are employed in fermentation industries to generate diverse metabolites for example antibiotics, enzymes, etc. The fungi showed a great attraction for metal ions as compared to other microbes. These can gather metals by means of biological and physiochemical mechanisms from their external environment **(Cabuk et al., 2004; Preetha & Viruthagiri, 2005).** All the filamentous fungi contain small quantity of protein, large amounts of polymer of N-acetyl, chitin and chitosan, and deacetylated glucosamine on their cell wall. Therefore, large number of potential binding sites are showed by free hydroxyl, amine and carboxyl groups. The amine group containing nitrogen atom and the hydroxyl group containing oxygen atom have the ability to bind a proton or a metal ion.

The knowledge of present investigation will provide information about heavy metal biosorption by filamentous fungus. This high absorption capacity of fungi made them well suited for removal of heavy metals present in very low or diluted concentration from polluted water, bioleaching, bioremediation of polluted sites and effluent treatments. The information of present study will be helpful for further assessment and management of natural biosorbent (fungus) which could serve as an economically viable resource while treating industrial effluents containing toxic heavy metal ions. In this study these five strains were been proved as efficient metal detoxification agent as they have achieved multi lethal tolerance capacity and thus they can well grow in multi polluted environment. This study will provide ample opportunity for field application after pilot trail in near future.

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Isolation and characterization of Arsenic tolerant fungal strains from contaminated sites around urban environment of Kolkata

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Abstract: *Today arsenic is causing an alarming global problem due to its toxicity and day by day this problem will amplify if necessary actions are not taken. In this paper we have isolated two arsenic resistant fungal strains (Aspergillus flavus and A. niger) from some polluted sites of Kolkata. These two strains are capable of removing 50%-76 % of arsenic from different arsenic enriched medium, simultaneously also tolerant to different other heavy metals (Cd, Pb, Hg, Zn and Cr). In near future these tolerant fungal species could be used in remediation of Arsenic and other metal pollution to some extent.*

Key words: *Arsenic, Aspergillus flavus & A.niger, Bioremediation*

I. Introduction

Development in terms of globalization and industrial expansion, heavy metal/metalloid pollution is attaining an alarming problem. Arsenic(As) pollution (a semi metallic element; metalloid) is one of the most concerned topic in recent years, as Arsenic is known to be a dangerous toxin that can lead to death when exposed to a large amounts. Arsenic , an ubiquitous metalloid found in groundwater due to its association with rocks, sediments and soils as well as industrial discharges and pesticide use which enters into the food chain through drinking water, crops and vegetables and causes different serious health problems.

Conventional physical and chemical methods for treating this harmful arsenic are inefficient, costly and not eco-friendly. So a global thrust was felt in searching of an alternative. Hence bioremediation is attaining an important procedure for abatement of metal pollution due to its low cost and high efficacy. Earlier Samal et al.[1] reported that among different algal strain blue green algal species *Oscillatoria- Lyngbya* mixed culture showed maximum efficiency in removing 64% Arsenic (V) and 60% Arsenic(III) after 21 days incubation from 0.1 mg/l arsenic (III) enriched medium. Similarly bacterial cultures also efficient in arsenic removal as reported by a group of researchers, they investigated As(V) reduction characteristics of two different bacteria named *Pseudomonas stuzeri* and *Bacillus cereus* and found 500ppb As(V) was completely reduced to As(III) by *Bacillus cereus* and *Pseudomonas stutzeri* in 114h and 120h respectively[2]. Among different microorganisms used in bioremediation fungi are considered as most effective species for metal removal from metal contaminated sites due to their survibility in higher concentration of metals. Evidence for internal absorption and the mechanism used by *A. niger* to detoxify environmental copper and zinc has been observed by earlier researchers [3**].** Potential of filamentous fungi in bioremediation of heavy metals containing industrial effluents and waste waters has been increasingly reported from different parts of the world [4,5,6,7].

As far Kolkata is significantly less arsenic contaminated area, so we isolated fungal strains from some pollution stressed sites around peri urban areas of Kolkata. Our attempt was to screen out some Arsenic tolerant fungal strains which could be used in remediation of arsenic pollution to some extent in near future.

II. Materials and Methods:

2.1 Isolation of fungal strains:

Fungal strains were isolated from soils of five different sites of Kolkata; namely

(vi) Waste dumping site of Dhapa areas of Kolkata;
(vii) Tannery waste dump sites along Eastern metropolitan bypass areas of Kolkata;
(viii) Contaminated site within the area of Central Bus terminus, Esplanade area, Kolkata ;
(ix) Waste dump sites within the premises of Calcutta medical college hospital, Kolkata.

The fungal strains were isolated through standard dilution plating methods [8] in Czapec-Dox(CD) agar media. Identically Czapecdox medium was prepared (glucose -2gm, $NaNO_3$ – 2.5gm, KCl -0.5gm, $MgSO_4,7H_2O$ - 0.5gm, $FeSO_4$ – 0.5gm, $ZnSO_4$, $7H_2O$ – 0.5gm, KH_2PO_4 – 1gm and agar powder 15gms per liter). (APHA, 1992)

The species were purified by streaking repeatedly on the same medium and were identified on basis of macroscopic (colony character, morphology, colour, texture, shape, diameter and appearance of colony) and microscopic (septation in mycelium, presence of specific reproductive structures, shape and structure of conidia and conidiospore etc) characteristic using fungal identification manuals [9,10].

2.2 Growth optimization

Isolated strains were grown in sterilized CD broth medium for establishment of the optimum temperature, pH that supports the exuberant growth of those species. The pH of the medium was adjusted using dilute HCL or NaOH. The temperature ranges were varied from 20°C- 40°C, and pH was from 3 to 9.

2.3 Antibiotic sensitivity Assessment

Antibiotic (antifungal) sensitivity of each metal tolerant fungal strains were measured using cup assay method [11]. The antibiotics used for this assay were Nystatin, Griseofulvin and Kanamycin.

2.4 Arsenic and another heavy metal tolerance assessment:

To explore the tolerance of the isolates to the As, each freshly prepared growth medium were amended with selected metal concentrations varies from $50mgL^{-1}$ to $5000mgL^{-1}$. After 5 days of incubation, the extent of colony growth (in diameter) were compared with the control one (i.e. growth of fungi in medium without metal). All the experiments were carried out in triplicate.

For other heavy metals (Cd, Pb, Hg, Cr and Zn) resistance of the fungal isolates were determined by conventional cup assay method[11]. Selected metal tolerant fungal strains were grown separately in CD medium supplemented with diverse concentration (sub-lethal to lethal) of each heavy metal separately. Then cups were poured with various other metallic solutions (Cd, Pb, Hg, Cr and Zn). For each test triplicates were made and incubated for 5days. Zone of inhibition of fungal growth around the cup was examined and measured in each case separately

2.5 Arsenic Removal potential of isolated strains

To study the arsenic removal capacity the selected fungal strains were grown in Czapec Dox broth (25ml) with Sodium arsenite ($NaAsO_3$) concentration 20, 100 and 1000 ppm for 3, 6 and 9 days with continuous shaking incubation at 30°C and pH 7. Fungal biomass was harvested by filtration through 0.22µ Millipore filter and the residual arsenic concentration of the broth was measured by FI-HG-AAS (Perkin Elmer Analyst-400) following standard procedure. All results are represented as the mean of triplicates.

III. Results and Discussion:

Almost twenty different fungal strains were isolated from soil samples of four different sites of Kolkata. Two arsenic tolerant strains ASC1 and ASB3 (isolated from Dhapa soil) were more arsenic resistant than other eighteen species , so we have carried out all our experiments with these two strains. The strains were identified as *Aspergillus flavus* (ASC1) and *A.niger* (ASB3) considering their colony color, colony shape, conidiospore color etc.

With increase in pH from 3 to 7 the biomass yield was found to enhance in both cases (ASC1 and ASB3) being maximum at pH 7, further increase in pH caused decrease in biomass. Similarly maximum mycelial weight were obtained at 30°C temperature in both cases (Table 1 & Table-2).Temperature 30°C and pH 7 ,both these physical condition showed maximum growth in terms of mycelial weight so all further experiments were carried out maintaining these growth conditions.

Table-1 Optimisation of pH for growth of two isolated fungi (in terms of mycelia weight) after 5days of incubation

Strain Name	Mycelial weight(g) (Mean ±SD)			
	pH 3	pH 5	pH 7	pH 9
ASC1 (*Aspergillus flavus)*	1.3±0.2	1.57±0.3	1.89±0.52	1.26±0.63
ASB3 *(A.niger)*	1.5±0.78	1.87±0.54	2.1±0.21	1.7±0.45

Table-2 Optimisation of temperature for growth of two isolated fungi(in terms of mycelia weight) after 5days of incubation

Strain Name	Mycelial weight(g) (Mean ±SD)		
	Temp.20°C	Temp.30°C	Temp.40°C
ASC1 (*Aspergillus flavus)*	1.15±0.25	1.95±0.45	1.01±0.32
ASB3 *(A.niger)*	1.3±0.20	2.01±0.35	1.69±0.56

Three different antibiotics (Nystatin, Griseofulvin and Kanamycin) were used to evaluate the antibiotic sensitivity of those two strains. ASB3 is found to be more resistant towards Griseofulvin and Kanamycin than ASC1, while against Nystatin both the strains showed similar range of tolerance (Table 3).

Table 3 Antibiotic sensitivity assessment of both isolated strains

Strains	Antibiotics					
	Nystatin		Griseofulvin		Kanamycin	
	Maximum growing concentration (ppm)	Minimum inhibitory concentration (MIC) (ppm)	Maximum growing concentration (ppm)	Minimum inhibitory concentration (MIC) (ppm)	Maximum growing concentration (ppm)	Minimum inhibitory concentration (MIC) (ppm)
AsC1	60	80	60	80	60	80
AsB3	60	80	80	100	80	100

Our results depict that ASC1 is more arsenic tolerant than ASB3, as Minimum Inhibitory Concentration (MIC) of arsenic for ASC1 was estimated to be around 2000 ppm, where as for ASB3 it is 1200ppm. Similarly ASC1 is found to be resistant against Cd, Pb and Cr, while ASB3 is resistant for Hg, and Zn (Table 4)

Table-4 Metal tolerance assessment of isolated strains

Strain Name	Minimum Inhibitory Concentration (MIC) in ppm					
	As	**Cd**	**Pb**	**Hg**	**Zn**	**Cr**
ASC1 (*Aspergillus flavus)*	2000	500	700	55	650	1000
ASB3 *(A. niger)*	1200	450	625	60	750	900

Similar trend of results were obtained by Gautam et al.[12] where they reported maximum growth of fungi at neutral range of pH. It is reported that temperature is considered as the most important growth factor for fungi [13]. Our results are in tune with the work of Santos & Linardi [14] who have reported earlier that common incubation temperature for the growth of fungi such as *A. niger* , *Fusarium* sp., *Penicillium* sp. and *Graphium* sp. was taken as 30°C. Actually this range of temperatures that encourage growth makes this isolate suitable for use in bioremediation in tropical climates.

Our results depicted that ASC1 is capable of removing more arsenic than ASB3 (Fig 1 & Fig2). In case of both the strains maximum removal efficacy were obtained at 6th day of incubation, with further increase in incubation time (i.e from 6th day to 9th day) a saturation was observed in removal efficacy for both of the cases(i.e. for both strains). Both the strains showed their maximum removal (70%-76%) efficiency when grown in 100ppm concentration of Arsenic. Further increase in metal concentration i.e from 100ppm to 1000ppm a significant decrease in removal percentage was noticed.

Bhar et al.[4] worked with *Aspergillus, Rhizopus and Penicillium* to show the rate of removal of As(III) and As(V) by above mentioned fungal strains and they found *Penicillium* with maximum removal efficiency and removal increases with increase of time interval which coincides with our results that with increasing incubation time (3rd day to 6th day) arsenic removal efficacies also enhanced upto a saturation time(9thday).
Ridvan et al.[15] also worked with *Penicillium purpurogenum* to show bioadsorbtion capacity of heavy metal Pb, Cd, As and Hg and found metal accumulation Pb(II)>Cd(II)>Hg(II)>As(III)

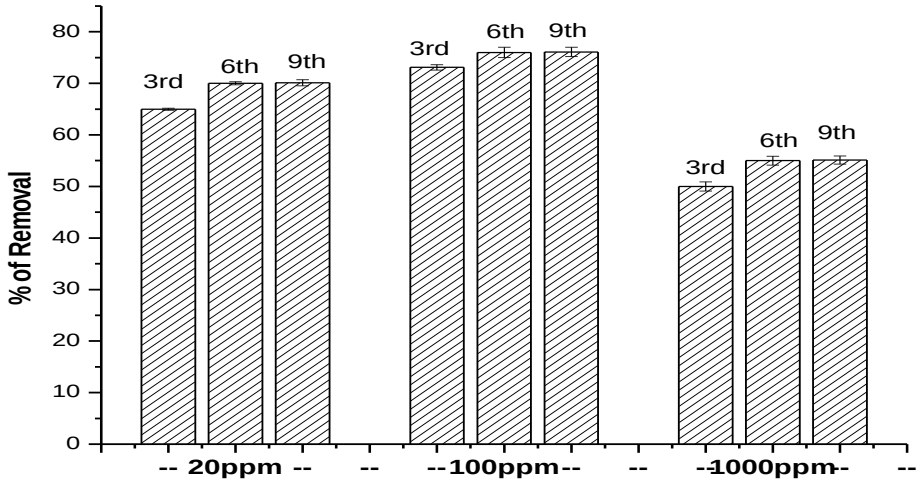

Fig-1 Arsenic removal (%) of ASC1(*A. flavus*) from 20ppm,100ppm and 1000ppm Arsenic enriched media after 3rd day, 6thday and 9th day of incubation period

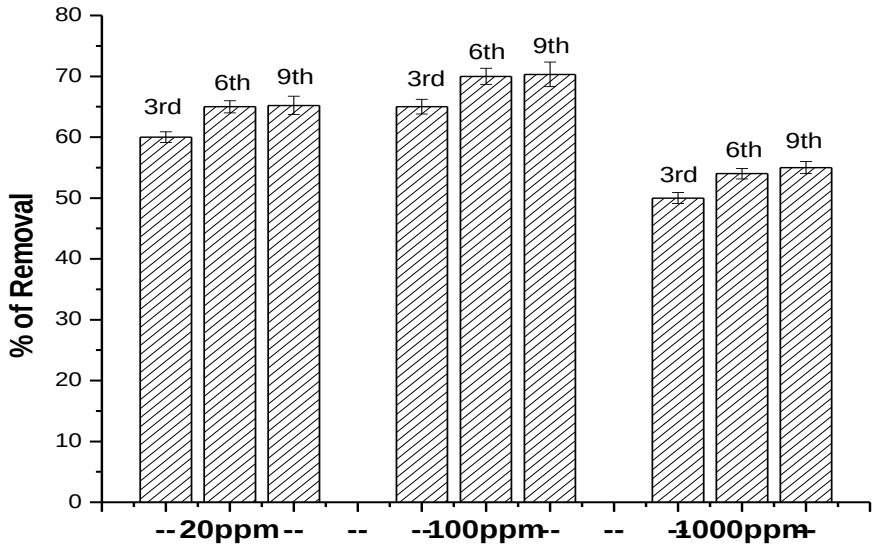

Fig-2 Arsenic removal (%) of ASB3(*A.niger*) from 20ppm, 100ppm and 1000ppm Arsenic enriched media after 3rd day, 6thday and 9th day of incubation period

Our results are in tune with the findings of a group of researchers, who reported arsenic removal potential of *Aspergillus candidus* and *A. flavus* respectively[16,17]. Similarly Srivastava et al.[18] detailed arsenic removal potential of *Trichoderma* and *Aspergillus* sp, isolated from arsenic contaminated sites of West Bengal. Almost ten species they identified which are tolerant upto 5000mg/L of arsenic.

IV. Conclusion:

These two isolated arsenic tolerant fungal strains (ASC1 and ASB3) could remove a significant amount of arsenic from different arsenic enriched media in laboratory condition. In near future these two fungal strains will be effective in arsenic removal planning from arsenic contaminated sites. The study revealed that a cost effective model of bioremediation of arsenic can be developed in a pilot scale.

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