METAL SORPTION BY HALOTOLERANT *PENICILLIUM* SPECIES - MECHANISMS AND APPLICATIONS

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IN

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OCTOBER 2006

DECLARATION

I declare that the present thesis entitled **"Metal biosorption by halotolerant *Penicillium* species - Mechanisms and Applications"** is a consolidation of an original work which has been carried out by me under the supervision and guidance of Dr S. Nazareth at the Department of Microbiology, Goa University, and that the same has not been submitted to any other University or Institution for the award of any other degree, diploma or other such title. The literature review related to the research problem investigated, the facilities availed of and financial support received as 'Research studentship' provided by Goa University have been duly acknowledged.

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CERTIFICATE

This is to certify that Ms Tabitha Marbaniang has worked on the thesis entitled "Metal biosorption by halotolerant *Penicillium* species - Mechanisms and Applications" under my supervision and guidance. This thesis being submitted to Goa University, Taleigao Plateau, Goa, for award of the degree of Doctor of Philosophy in Microbiology is a record of an original work carried out by the candidate herself and has not been submitted for the award of any other Degree, Diploma, Associateship or Fellowship of this or any other University.

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To Jesus

n

To the one I loved and lost

"MOTHER"

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GENERAL INTRODUCTION

1.1 Halotolerant fungi

1.1.1 Fungi

Fungi are present everywhere, and they often form a major and dominant component of the microbiota in soils and mineral substrates. They also play important roles as decomposer organisms; symbionts and pathogens, of animal and plant, spoilage organisms of natural and synthetic materials, and also function in biogeochemical cycles of elements (Gadd, 1993). The flexible mycelial growth strategies of fungi and the ability to produce and exude organic acids, protons and other metabolites make fungi important biological weathering agents of natural rock and minerals (Fomina et al., 2005).

Continuous changes occur in the environment, and with increasing industrialization; heavy metal stress due to contamination is one of the factors the fungi have to cope with. Fungi have developed different response mechanisms to adjust to the environmental changes, the extent of the change determining whether the organism is killed, ceases growth or has increased in the lag time with reduced growth rate. These fungal cultures which are exposed to metals and able to grow under extreme environment eventually develop resistance or tolerance, and these naturally selected organisms represent an important strategy to obtain agents for bioremediation process (Wood and Wang, 1983) and can be used at very low costs, to remove metals from industrial effluents (Vieira and Volesky, 2000).

Algae, bacteria, yeast and fungi have proved to be potential metal biosorbents, and fungi are known to be more tolerant to metals than others; with *Penicillium* spp being quite prominent (Gadd, 1993; Paknikar et al., 1993; Fourest et al., 1994; Niu et al., 1999; Natarajan et al, 1999; Skowronski et al., 2001; Tan and Cheng, 2003; Cabuk et al., 2005).

Fungi are more favoured than bacteria in waste treatment and in pollution control or bioremediation process because of the relative ease of removal of the larger fungal biomass as against removing unicellular organisms from an aqueous solution after treatment. It also yields a greater biomass and thus has a higher potential (Balakrishna et al., 1994). The well-documented ability of fungi biomass to accumulate metals, coupled with their higher tolerance to metals, tolerance to low pH and metal binding to cell walls makes them good candidates for bioremediation of metals, which can also be genetically manipulated (Gadd, 1993; Paknikar et al., 1993).

1.1.2 Ecology and biodiversity of halotolerant fungi

Depending on their occurrence, fungi have shown different adaptations to that particular environment. Some fungi have adapted to environments of low water activity (a_w), and these are referred to as osmophiles or osmotolerant fungi which can be classified into two different groups: xerophilic / xerotolerant or halophilic / halotolerant. The former can grow at low water activity imposed by organic ions such as sucrose and glycerol, while the latter includes the halophilic or halotolerant organisms that can grow in environments dominated by inorganic ions such as Na^+ and Mg^{2+}.

Environments of highly saline conditions exert a strong selective pressure on the biota, favouring the development of halotolerant and halophilic forms (Kis-Papo, 2003). The number of marine fungi is low in comparison to the number of terrestrial fungi, which is more than 60 times as high. As the concentration of salt increases, the biodiversity of fungal life drastically decreases (Kis-Papo, 2003). Halophilic and halotolerant fungi are excellent eukaryotic models for study of salt resistance (Prista et al., 1997; Petrovic et al., 2002).

Fungi have been described in moderately saline environments, such as salt marshes, saline soils and seawater, but were considered unable to grow in highly saline waters. However, a rich diversity of fungi has been isolated even from hypersaline waters (Gunde-Cimerman, 2000). An osmophilic yeast was reported by Kritzman in 1973 (Butinar et al., 2005). Buchalo *et al.* (1998) isolated 25 species of filamentous fungi, *Penicillium westlingii*, *Ulocladium chlamydosporum* and *Gymnascella marismortui*, the last being a new endemic species, as well as other species thought to be indigenous: *Aspergillus terreus*, *Eurotium herbariorum*, *Penicillium westlingii*, and *Cladosporium cladosporoides* were amongst them. Halotolerant black yeasts *H. werneckii* and *P. triangularis* were also recovered from saltpans in Spain (Zalar, 1998). The species *Hortaea werneckii, Phaeotheca triangularis, Trimmatostroma salinum, Aureobasidium pullulans* and *Cladosporium spp* were detected with the highest frequency just before the peak of halite (NaCl) concentration. Since *H. werneckii, P. triangularis and T. salinum* are not known outside saline environments, these results suggest that hypersaline water is their natural ecological niche (Gunde-Cimerman et al, 2000).

More recently, a variety of filamentous fungi were isolated from the Dead Sea (Kis-Papo, 2003). Another species isolated is a *Beauveria alba* strain from salterns near Alicante, Spain, that grows well in 20 % NaCl (Kis-Papo, 2003). The study of microbial ecology in extreme environments reveals the relatively low diversity of the respective microbial communities (Rensing, 2005). The diversity of hypersaline environments and the physiology of representative organisms are only beginning to be understood (Kis-Papo et al., 2003; Rensing 2005). Though it was believed that microbial communities at high salinities are dominated exclusively by archaea and bacteria and one eukaryotic species, the alga *Dunaliella salina*, from studies on the microbial diversity and composition in hypersaline environments such as the Great Salt Lake, the Dead Sea, salterns in California and Spain and ancient salt deposits, melanized fungi, described only in the crystallization pond of Adriatic salterns within the season of salt production, can be considered as a new group of eukaryotic halophiles. They were represented by black, yeast-like hyphomycetes: *Hortaea werneckii*, *Phaeotheca triangularis*, *Trimmatostroma salinum*, *Aureobasidium pullulans*, together with phylogenetically closely related *Cladosporium* species (Butinar et al., 2005).

1.1.3 Importance of halotolerant microorganisms

Yeasts and fungi are well known for their ability to adapt to environments of high osmolarity. Fungal cells synthesize polyols even without osmotic stress and respond to osmotic stress by accumulation of polyols. These compatible solutes may protect enzymes and other cellular components from high salt concentrations (Park and Gander, 1998). Compatible solutes are defined as solutes, which at high concentrations allow enzymes to function efficiently. They are typically low-molecular-weight compounds,

soluble at high concentrations in water, and either uncharged or zwitterionic at the physiological pH. Compatible solutes detected in halophilic and halotolerant microorganisms include polyols such as glycerol and arabitol, sugars and sugar derivatives (sucrose, trehalose, glucosylglycerol), amino acids and derivatives, and quaternary amines such as glycine betaine (Oren, 1999).

The diversity of microorganisms in hypersaline environments is of growing interest. Halotolerant or halophilic microorganisms have found applications in various fields of biotechnology, such as enzymes (new isomerases and hydrolases, hydrolytic enzymes such as DNAases, lipases, amylases, gelatinases and proteases) that are active and stable at high salt contents, compatible solutes which are useful as stabilizers of biomolecules and whole cells, salt antagonists, or stress-protective agents, and biopolymers (biosurfactants and exopolysaccharides) are of interest for microbially enhanced oil recovery. They also play an essential role in food biotechnology for the production of fermented food and food supplements (Margesin and Schinner, 2001; DasSarma, 2004). According to Kogej et al., (2006), "the knowledge of the metabolites of extremophilic fungi is important because they could provide signature molecules in the environment, and they can also contribute nutrients to the otherwise oligotrophic polar conditions". The best-studied fungal extremophiles of which are the halophilic and halotolerant fungi (Kogej et al., 2006).

The degradation or transformation of a range of organic pollutants and the production of alternative energy are other fields of applications of these groups of extremophiles (Margesin and Schinner, 2001). In addition to the technological applications in water

treatment, fungal biosorption may be of natural geochemical importance in the concentration of metals and formation of minerals (Galun et al, 1987).

The use of these organisms to effluent treatment and bioremediation of saline environments is of immense importance. As a result of natural and man-made global changes, hypersaline environments are on the increase. Many natural geological formations, such as petroleum reserves, are associated with hypersaline brines. Industrial processes also use salts and frequently release brine effluent into the environment (DasSarma, 2004). Organisms which are able to grow under extreme environment, offers good potential as indicators of pollution and as biosorbents, and can be used for abatement of pollution in hypersaline conditions or in waters of fluctuating salinity, as well as in non-saline environments.

1.2 Heavy metals

1.2.1 Definition

The term 'heavy metal' has been defined differently, depending on the context of usage of the term 'heavy metal'. Weast (1984) defined heavy metals as those elements with a density higher than 5 g cm^{-3}, and 53 of the 90 naturally occurring elements are heavy metals, but not all of them are of biological importance.

Over the past two decades, this term was associated with chemical hazards and the safe use of chemicals, and often with contamination and potential toxicity. However, the legal regulations which list heavy metals, differ from one set of regulations to the other, or the

term is used without specifying which heavy metals are covered. This eventually led to imprecise definition of the term in scientific literature. The term even included semimetals (metalloids) such as arsenic, presumably because of the assumption that ''heaviness'' and ''toxicity'' are related (Duffus, 2002).

Similarly, definitions have been formulated in terms of atomic weight or mass, on atomic number and on chemical properties. The term "heavy metal" has never been defined by any authoritative body such as IUPAC. Some usage of the term implies that the pure metal and all its compounds have the same physicochemical, biological, and toxicological properties. Thus, sodium metal and sodium chloride are assumed by this usage to be equivalent. However, sodium metal cannot be swallowed without suffering serious, life-threatening damage, while sodium chloride is required in our diet. Epidemiological studies also show that chromium and its alloys can be used safely in medical and dental prostheses even though chromate is identified as a carcinogen. Then again, in medical terms, ''heavy metal poisoning'' can include excessive amounts of iron, manganese, aluminium, or beryllium (the second-lightest metal) as well as the true heavy metals (Duffus, 2002).

One definition states that the heavy metals are a group of elements having atomic weights between 63.55 that of copper and 207.2 that of lead on the periodic table, and specific gravities greater than 4.0, while a stricter definition restricts the term to those metals heavier than the rare earth metals, at the bottom of the periodic table (Duffus, 2002).

1.2.2 Heavy metal toxicity

Living organisms require trace amounts of metals and some heavy metals, the essential metals of which include potassium, sodium, magnesium, calcium, cobalt, copper, manganese, molybdenum, vanadium, strontium, iron, nickel and zinc (Gadd, 1992), but excessive levels can be detrimental to the organism. Other heavy metals such as mercury, lead, silver, gold and cadmium have no known vital or beneficial effect on organisms, and their accumulation over time in the bodies of mammals can cause serious illness. None of these are essential elements in biological systems. Thorium and uranium are sometimes included as well, but they are more often called simply ''radioactive metals''. The concentration of metals in the environment varies greatly, and virtually all metals whether essential or non-essential can exhibit toxicity above a certain threshold level, which varies from metal to metal, and for a highly toxic metal like mercury may be extremely low (Forstner, 1980).

All these elements interact with microbial cells through physicochemical mechanisms and these interactions are responsible for events like biogeochemical cycles and deposition of certain metals in the sea (Gadd, 1990; Konetzka, 1977). These interactions are also responsible for metal removal / recovery from industrial effluents or wastes.

The toxicity of heavy metals has attracted considerable research due to their microbicidal effect (Vieira and Volesky, 2000) and the severe damage to aquatic life (Hussein et al 2003). Metal contamination of the aquatic environment affects organisms at the biochemical, cellular, community and population level.

The toxic effects of heavy metals include the blocking of functional groups of important molecules such as enzymes, polynucleotides, transport systems for essential nutrients and ions, displacement of essential ions from cellular sites, denaturation and inactivation of enzymes and disruption of cell and organeller membrane integrity (Gadd, 1992). Another important mechanism of heavy metal toxicity is their ability to bind strongly to oxygen, nitrogen and sulphur atoms. Because of these features, heavy metals can inactivate enzymes by binding to cysteine residues. Direct effects of cadmium on the sulphydryl homeostasis of cells and inhibition of enzymes have been reported for mammalian and animal cells (Schützendübel and Polle, 2002).

Heavy metal pollution also adversely affects and inhibits growth and the formation and germination of fungal spores. In addition, morphological abnormalities were also induced in sensitive microorganisms along with delayed pigmentation (Babich and Stotzky, 1982). In *Aspergillus niger*, spore germination was inhibited in presence of cadmium. The mechanism of inhibition suggested is that cadmium entering the spores, gets associated with the particulate and soluble cytoplasmic components, then reacts with the cytoplasmic receptor sites, and this reaction internally inhibits spore germination (Babich and Stotzky, 1978).

Growth medium pH is another important factor directly affecting the toxicity of metals. Acidity or alkalinity of the medium can moderate the toxicity of heavy metals, whereby when the pH is less than the value at which an element undergoes hydrolysis, that element will be present as the free ion and is relatively soluble, hence may increase the bioavailability of metal ions resulting in increased toxicity (Modak and Natarajan, 1995).

1.2.3 Heavy metal pollution: An increasing concern

The presence of metallic species in the environment is constantly on the increase due to increasing industrialization (Akthar and Maruthi Mohan, 1995). Heavy metal contamination of the biosphere has increased sharply since 1900 (Nriagu, 1979), and led to increasing economic, public health and environmental concern with increasing industrialization, which in India gained a momentum with initiation of five-year developmental plan in the early 1950's (Ahalya et al, 2003). The discharge of heavy metals into aquatic ecosystems became a matter of concern over the last few decades due to the problems of unwanted and toxic metals such as Pb^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+} which cannot be degraded by biological and chemical processes nor can they be decomposed by *in situ* biological means (Laws, 1993; Scragg, 1999), thus posing a severe threat to living organisms as these metallic species tend to persist indefinitely in the environment, accumulating throughout the food chain (Akthar and Maruthi Mohan, 1995).

The toxic effects of some metal pollutants are given in Table 1.1 The pollutants of concern include lead, chromium, mercury, uranium, selenium, zinc, arsenic, cadmium, gold, silver, nickel and copper, which have found their way into the environment through mining operations, refining ores, metal plating, or the manufacture of electrical equipment, paints, alloys, batteries, pesticides or preservatives. Major lead pollution is through automobiles and battery manufacturers (Ahalya et al, 2003). The World Health Organization (1984) has listed the metals of immediate concern as cadmium, chromium, cobalt, copper, lead, nickel, mercury, and zinc

Table 1.1: Toxic effects of some metals

Metal	Toxic effects / significance	Source
Lead	Toxic, nervous disorder, damage to DNA and RNA, inhibits formation of hemoglobin.	Industrial wastes, paint industry, Auto exhaust emission, plumbing, mining and gasoline.
Copper	Essential trace element, toxic to microorganisms, excess intake causes stomach irritation and nausea, reduced growth, liver damage.	Mining, metal plating, mineral leaching, industrial and domestic wastes, corrosion of copper containing alloys
Cadmium	Extremely toxic to humans, replaces zinc biochemically, causes high blood pressure, kidney damage, destruction to testicular tissues and red blood cells.	Industrial discharge, metal plating, corrosion of water pipes, battery manufacture
Iron	Essential for many biological activities, causes hemochromatosis, liver damage, tension in gastrointestinal tract.	Mining industry, industrial effluents, pesticides, chemical wastes
Manganese	Relatively non-toxic to animals, toxic to microbes at high concentration. Serious and irreversible damage to CNS and brain.	Mining, industrial wastes, acid mine drainage, alloys, and dry cell battery.

1.3 Metal tolerant microorganisms

1.3.1 Metal tolerance-metal resistance

Metal tolerance and metal resistance have come to be used almost synonymously. While tolerance signifies "the ability of an organism to survive metal toxicity by means of intrinsic properties and/or environmental modification of toxicity", resistance is "the ability of an organism to survive metal toxicity by means of a mechanism produced in direct response to the metal species encountered, for example, synthesis of metallothioneins" (Gadd, 1993).

Microorganisms yield to the stress conditions and make suitable provisions for survival or attempting to resist the stress (Beales, 2004). They develop resistance mechanisms to avert the toxicity of metals. Resistance may be expressed in terms of phenotypic or genotypic changes. For most organisms, this tolerance can be pushed to maximum limits if the cell is provided with sufficient opportunity to sense and adapt to the extreme environment. Entire groups of microorganisms such as psychrophiles, acidophiles and halophiles have adapted their lifestyles to prefer these extreme environments. Psychrophiles can grow at 0-20°C and have an optimum growth temperature of 15°C or less, acidophiles grow optimum at pH 1.0-5.5, and halophiles require high levels of sodium chloride for growth such as 2.8 up to 6.2 molal for extreme halophiles (Prescott et al., 1990). Changes in environmental conditions away from the optimal value can cause the induction of many elaborate stress responses. These strategies are generally directed at survival rather than growth.

Tolerance to metal can be a physiological adaptation whereby the culture loses its tolerance when grown in a metal free medium. This can be due to –

- Efflux, that is, pumping of toxic ions from the cells by systems involved in transport of nutrient cations by organisms
- Detoxification mechanisms, such as, valence changes, biomethylation and bioaccumulation.

1.3.2Emergence of metal resistant microorganisms

Heavy metal salts, which are released into the environment from industrial wastes form aqueous solutions and consequently cannot be easily separated by ordinary physical

means of separation (Hussein et al., 2003). As a result, the environment becomes rich in heavy metals selecting only the microorganisms which are able to survive in these conditions. The exposure of organisms to metals usually leads to the establishment of a resistant or tolerant microbial population. Thus, the naturally selected organisms represent an important strategy to obtain agents for bioremediation process (Wood and Wang, 1983).

1.3.3 Diversity of metal-tolerant microorganisms

Studies showed that bacteria, yeasts, algae and fungi exhibited particularly interesting metal binding capacities (Volesky, 1986; Dave and Patwari, 1993; Morley and Gadd, 1995; Naseem et al., 1996; Guptal and Kergan, 1998; Gadd et al., 1999; Gadd and Fomina, 2002; Ahalya et al, 2003; Goyal, 2003); amongst these, the use of *Penicillium spp* has been well documented (Gadd 1993; Paknikar et al., 1993; Niu et al., 1993; Fourest et al., 1994; Natarajan et al., 1999; Skowronski et al., 2001; Tan and Cheng, 2003; Cabuk et al., 2005).

Many types of biomass in living form have been studied for their heavy metal uptake capacities and suitability to be used as bases for biosorbent development. These include bacteria, fungi, yeast, algae and others (Table 1.2). The toxic effect of heavy metal varies significantly for different types of biomass as shown in Table 1.3.

Table 1.2: Some microorganisms used for metal removal

Microorganism	Examples	Reference
Bacteria	*Bacillus subtilis*	Beveridge and Murray, 1980
	Pseudomonas aeruginosa	Strandberg et al., 1981
	Pseudomonas fluorescens	Modak and Natarajan, 1995
Fungi and yeast	*A. niger*	Akthar and Mohan, 1995
	C. paspali	Luef et al., 1991;
	Cladosporium cladosporioides	Pethkar and Paknikar, 1998
	Fusarium solani	Kowshik and Nazareth, 1999
	Ganoderma lucidum	Muraleedharan et al., 1991;
	Mucor miehei	Fourest et al., 1994
	Neurospora crassa	Maruthi Mohan et al., 1984
	Penicillium chrysogenum	Niu et al., 1993; Paknikar et al., 1993; Fourest et al., 1994; Skowronski et al., 2001; Tan and Cheng, 2003.
	Penicillium italicum	Gadd and white, 1998
	Penicillium ochro-chloron	Gadd et al., 1984; Ashby et al., 1997
	Penicillium verrucosum	
	R..arrhizus	Fourest and Roux, 1992; Fourest et al., 1994; Niyogi et al., 1998.
	R..delemar	Tsekova and Petrov, 2002
	R..javanicus	Treen-Sears et al., 1984
	R. oligosporus	Ari et al., 1999
	Saccharomyces cerevisiae	Volesky et al.,1993; Gadd et al., 1984; Volesky, 1998; Vlatka, 2001
	Strepmyces noursei	Mattuschka and Straube, 1993
	Yeast spp	Emilia et al., 2002; Goksungur et al., 2003
Algae	*Sargassum fluitans*	Kratochvil et al., 1998; Yang and Volesky, 1999.
	Sargassum spp	David et al., 2002
	Sargassum seaweed	Davis et al., 2002
	Chlorella vulgaris	Harris and Ramelow, 1990
	Ulva reticulata	Vijayaraghavan, 2004
	Scendesmus quadricauda	Harris and Ramelow, 1990

Table 1.3: Lethal concentrations of metals to some microorganisms

Metal	Microorganism	Toxic level (mg L^{-1})
Lead		
	Chaetomium sp	110
	Pestalotiopsis sp	110
	Pleurophomella sp	275
	Gnomonia platani	275
	Chlamydomonas eugametos	0.1
	Haematococcus capensis	0.1
Copper	*Selenastrum capricornutum*	0.3
Cadmium	*Chlamydomonas reinhardtii*	1.0
	Selenastrum capricornutum	0.65
	Botrytis cinerea	100
	Penicillium vermiculatum	100
	Fomes annosus	100
	Aspergillus niger	1000
	Scopulariopsis brevicaulis	1000
	Phycomyces blakesleeanus	1000
	Scenedesmus quadracauda	6.1
Zinc	*Selenastrum capricornutum*	0.7

1.4 Bioremediation

1.4.1 Microorganisms for abatement of metal pollution

Bioremediation in the area of metal pollution is the use of biological materials to reduce or eliminate the environmental hazards resulting from the accumulation of toxic chemicals and other hazardous wastes as well as recovery of metals. The natural affinity of biological compounds for metallic elements contributes to economically purifying the metal-laden wastewater (Vieira and Volesky, 2000).

A very extensive literature exists in the area of biosorption; the use of microbiological methods for environmental remediation is not an entirely new concept. Microbial

interactions with metals have long been recognised and substantial information on this property of the biomass appeared in literature as early as 1945 (Tsezos, 1990), and since 1960's work in this field have been exploited by biohydrometallurgists and biogeochemists (Vance, 2002). Most of the biosorption studies reported pertain to the use of microbes either grown in the laboratory or obtained as a by-product from industrial fermentation or biological waste treatment process. Over the last few decades, several methods have been devised for the treatment and removal of heavy metals. By the 1990s, a new scientific area developed in the area of biosorption that could help recover heavy metals, and also serve as part of the solution to pollution by toxic heavy metal contamination resulting from human technological activities (Vieira and Volesky, 2000).

The removal of heavy metals from wastewaters was a major ecological problem (Hussein et al., 2003). The conventional methods for metal removal from aqueous solutions are chemical precipitation, sludge separation, chemical oxidation or reduction, ion exchange, reverse osmosis, electrochemical treatment and evaporation (Paknikar et al., 1993; Volesky, 1994). The use of biological methods such as biosorption / bioaccumulation provided an attractive alternative to the physico-chemical method (Paknikar, et al., 1993; Hussein et al., 2003). The noxious effects caused by the release of toxic metals into the environment and the emergence of more severe environmental protection laws, have also encouraged studies about removal/recovery of heavy metals from aqueous solutions using biosorption (Cossich et al., 2002). Compared to the conventional methods of toxic metal removal from industrial effluents, removal of metals using microorganisms has gained importance in recent years because of the good performance and low cost of this complexing material for efficient removal of toxic metals from industrial effluents, the

major advantages of which are its effectiveness in removal of metals present in trace amounts or when very low concentrations of heavy metals are present (Volesky, 1990; Fourest and Roux, 1992; Vinita and Radhanath, 1992; Vieira and Volesky, 2000; Rao et al., 2002; Volesky and Naja, 2005). Other advantages are dead biomass easily available in large quantities as industrial by-products which could not only aid metal removal, but also solves the problem of biomass disposal. Moreover, they are not affected by toxic chemicals and can be regenerated and re-used many times (Paknikar et al., 1993).

The limitations of the technology include that large-scale production of effective biosorbent materials has not been established and that the technology has been tested for limited practical applications (Volesky et al, 2001; Volesky and Naja, 2005). Xu et al., (2002) investigated the peptide-based biosorbents and demonstrated the feasibility of a protein-based technology for heavy metal removal.

The impact of biological process on metal contamination and the potential for bioremediation are also dependent on the nature of the site and the chemical environment. In soils or sediments the potential ecological or public health risk posed by toxic metals depends upon the form in which metals occur, that is, mineral components may contain considerable quantities of metal, but which are unavailable to organisms; soluble metal species however, have greater mobility and bioavailability (Sayer et al., 1997).

1.4.2 Sequestration

The removal and/or recovery of metals from aqueous solutions or wastes by microbial cells or biomass through metabolically mediated or physico-chemical pathways of uptake

is called biosorption (Fourest and Roux, 1992). Either plants (Ensley, 2000; Siva Kumar et al, 2002) or microbial biomass can be utilised for this purpose, the latter being more commonly used (Gadd 1988; Volesky 1990; Gazso, 2001; Lloyd 2002). The biomass for metal sorption experiments can come as a by-product from industrial wastes, easy availability in large amounts in nature and those which grow fast, especially cultivated or propagated for biosorption purposes. The adsorbent materials or biosorbents derived from suitable biomass for the effective removal of heavy metal ions from wastewater streams has been studied (Akthar and Maruthi Mohan, 1995; Vieira and Volesky, 2000). It can also be used for the recovery of precious metals from aqueous solutions in low concentration (Pethkar and Paknikar, 1998) and in their ore or wastes (Balakrishnan et al., 1994).

Pioneering research on biosorption of heavy metal has led to the identification of a number of microbial biomass types, which are extremely effective in concentrating metals. Some examples are *Bacillus subtilis* and *Rhizopus* obtained as a waste by-product of large-scale industrial fermentation or even the brown algae from oceans (Volesky, 1999). These biomass types, serving as a basis for metal biosorption process can accumulate heavy metals – lead, cadmium, uranium, copper, zinc, even chromium and others in excess of 25 % of their dry weight.

The importance of any functional group for biosorption of a certain metal by a particular biomass depends on factors such as the number of sites for biosorption, the accessibility of these sites, and their chemical, and the affinity between the site and the metal, that is, the binding strength. The choice of metal for biosorption process can also be based on

four major categories: (i) toxic heavy metals (ii) strategic metals (iii) precious metals and (iv) radionuclides; toxic heavy metals and radionuclides are of interest in terms of environmental threats, and hence for removal from the environment. It could also be based on the behaviour of metals for application with respect to biosorption. The precious metals though not environmentally threatening are important from their recovery point of view (Pethkar and Paknikar, 1998; Ahalya, et al., 2003).

1.4.3 Recovery of metals

Prioritizations for recovery of metals is mainly ranked into three categories, that is, environmental risk (ER), reserve depletion rate (RDR) and a combination of ER and RDR factors. ER is based on the number of factors which could also be weighed while RDR is an indication of probable future increase in market price of the metal. When coupled with ER, Pb, Hg and Zn are listed as high priority, while Cu is of medium priority (Table 1.4).

Table 1.4: Ranking of Risks Associated with Various Metals

Relative Priority	ER	RDR	Combined factors (RDR+ER)
High	Cd	Cd	Cd
	Pb	Pb	Pb
	Hg	Hg	Hg
	-	Zn	Zn
Medium	Cr	-	-
	Co	Co	Co
	Cu	Cu	Cu
	Ni	-	-
	Zn	-	-
Low	Al	-	Al
	-	Cr	Cr
	Fe	Fe	Fe

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1.4.4 Immobilised biomass

The use of immobilized or pelleted biomass is important from an industrial point of view. It yields mechanically strong rigid particles with a high density, as compared to the native biomass of low density and mechanical strength. Therefore, the native biomass limits the choice of suitable reactors, rendering the effluent separation process difficult (Gadd, 1990, Gadd 2000). The advantages of immobilized biomass are easier separation of cells, better capability of biomass re-use, high flow rates, minimal clogging, controlled particle size and high biomass loading (Gadd, 1990; Modak and Natarajan, 1995). Filamentous fungi can be grown in pelleted form, therefore has some advantages in common with immobilised particles; however, they are prone to disintegration and the similarities in densities with the liquid medium made continuous operation difficult (Gadd, 1990; Pumpel and Schinner, 1993)

1.4.5 Regeneration of biomass

One of the main aims of biosorption is the recovery of metals and regeneration of the biomass. For desorption of metals, an eluent is used which should have a higher binding affinity for the metal than the biomass. Dilute (0.1M) mineral acids can be used for this purpose, of which the best is dilute HCl (Modak and Natarajan, 1995). Reports indicated that the fungal biomass was effectively regenerated by desorbing with 0.1 N HCl (Akthar and Maruthi Mohan, 1995; Akthar et al., 1996; Fernandes and Nazareth, 1999). According to Tsezos (1984), desorption with 1 M HCl or higher concentration, damages the cell wall, and reduces the biosorption efficiency in subsequent cycles. Other

desorbing agents are organic acids such as acetic and lactic acid, and complexing agents such as EDTA (Modak and Natarajan, 1995).

1.5 Factors affecting sorption

The identification of physical and chemical factors regulating the metal sorption is necessary for the development of industrial biosorption technologies. Some of the critical biosorption parameters include factors such as pH, temperature, initial metal concentration, biomass loading, presence of calcium ions and the pretreatment of biomass influence the metal uptake by the biomass (Fourest et al., 1994; Modak and Natarajan, 1995; Sag and Kutsal, 1995). Simultaneously, one or more processes may be operative individually or in combination to sequester metals. Some of the main factors which affects the sorption of metals are discussed.

1.5.1 Growth medium

The microbial growth conditions such as the growth medium significantly influenced the composition of polysaccharides, affecting the metal removal (Muraleedharan et al., 1991). Once polymers are produced, metal removal by this mechanism is probably a passive phenomenon, not requiring the participation of live organisms. However, some reports indicate that the active synthesis of these polymers is induced in the presence of toxic metals (Muraleedharan et al., 1991).

The type of nitrogen source in the cultivation medium, such as $NaNO_3$, and peptone from casein strongly influenced the cell wall composition. The contents of chitosan and phosphorous in the cell wall were observed to be significantly higher with $NaNO_3$ in comparison to peptone. The biomass extracted cell walls with higher contents of chitosan

and phosphorus derived from the cultivation with $NaNO_3$ showed an increased biosorption capacity for chromium (III). The chitosan and phosphorus containing functional groups are the most probable binding sites for trivalent chromium in the cell wall, whereas protein does not play a role in the bisorption of Cr (III) by *Mucor hiemalis* (Ebner, et al., 2002). Other reports also indicate that biosorption could be influenced by the composition of the media used for growth of the cultures (Eccles and Hunt, 1986; Tsezos, 1990; Karna et al., 1996; Zucconi et al., 2003).

1.5.2 Growth phase

Another significant parameter that influences biosorption is the age at which the biomass is harvested (Tsezos, 1990). The maximum metal uptake takes place during the lag periods or the early stages of growth and declines as the culture reach the stationary phase. This was observed in both living and killed biomasses of *Paecilomyes lilacinus* when exposed to media supplemented with known concentrations of the soluble lead (Zucconi, et al., 2003). Mattuschka et al (1993) also reported that the higher accumulation of approximately 80 % of copper occurs in the younger cells in yeast and fungi than that from the stationery growth phase, while Ã‡eribasi and Yetis (2001) indicated that the maximum biosorption capacities of Pb(II) and Ni(II) by *P. chrysosporium* were attained after 41 h of incubation.

1.5.3 Biomass concentration

The amount of biomass used, affects the removal of metals from solutions (Dave, 1994; Sag and kutsal, 1995). At a given equilibrium concentration, the biomass sorbs more metal ions at lesser amount of biomass than at high biomass concentration. This could be

due to enhanced metal/biosorbent ratio which increased the metal uptake per unit of biosorbent, the metal adsorption being larger when the distance between cells is greater. When there is excess biomass concentration, metals are not easily accesible to the binding site (Luef et al., 1991; Fourest and Roux, 1992; Modak and Natarajan, 1995).

1.5.4 Metal concentration

The initial metal ion concentration is another important factor that plays a role in metal sorption (Dave, 1994; Sag and Kutsal, 1995). At lower metal concentration, the ratio of sorptive surface to the metal ion is higher, hence larger amount of metal ions can be bound and removed. While the initial rate of metal uptake increases with increase in initial metal concentration, maximum percentage of metal removal can be obtained from dilute solutions, the increase in metal ion concentration resulting in reduced percent metal uptake (Balakrishnan et al., 1994; Modak and Natarajan, 1995; Niyogi et al., 1998).

1.5.5 Metals singly and in mixture

Most biosorption studies are conducted with single metal ion species in aqueous solutions, however industrial effluents consist of a mixture of metal ions. The presence of metals in mixture significantly affects the removal of the desired metal, whereby other cations compete for the binding sites on the biosorbent due to non-specific functional groups present on the cell wall. The metal uptake from mixed aqueous solutions is therefore lower than that from the single species solution (Modak and Natarajan, 1995; Pethkar and Paknikar, 1998).

Balakrishnan et al., (1994) indicated that the different fungi did have preference in sorption of a particular metal, the uptake of zinc by the species studied being higher than

Cu, Fe and Pb, the relative preference of biomass for a particular metal depending on factors such as, ionic charge, cation radius, initial metal concentration, solution chemistry and toxicity of metal (Gadd, 1990).

Alternatively, metals in mixture may enhance biosorption by forming co-ordination compounds or complexes for the adsorption of a particular metal (Pethkar and Paknikar, 1998). An increase in copper uptake in presence of mercury was observed which may be due to increased permeability of the cell wall (Modak and Natarajan, 1995). However, metal binding is not solely electrostatic as observed by Tsezos (1983) where iron reduced uranium uptake more than copper and zinc although iron is expected to show the least binding (Modak and Natarajan, 1995). Microbial biomass may also show selectivity for a particular metal. This was reported by Pethkar and Paknikar (1998) where the fungus *C. cladosporioides* showed preferential uptake of gold and silver in presence of other metals which may be due to the intrinsic ability of the culture to sorb these metal preferentially.

1.5.6 Pretreatment of mycelia

Fungal pretreatment is another factor that has an effect on the biosorption of heavy metals (Sag and Kutsal, 1995; Pethkar and Paknikar, 1998; Yang and Volesky, 1999; Yan and Viraraghavan, 2000; Ilhan et al., 2004). These are factors such as –

1.5.6.1 Homogenisation

The homogenisation of mycelium is reported to increase the sorption of lead. The effect of homogenisation results in the increase in surface area and consequently the availability of binding sites and thereby enhancing the metal binding capacity (Fernandes and Nazareth, 1999).

1.5.6.2 Chemical treatment

Chemical treatments such as, sodium hydroxide, sodium carbonate, ammonium sulphate, sulphuric acid, ethanol (89 %), dimethly sulfoxide (100 %) and triton-X 100 (1 %) increased or maintained the biosorption efficiency (Paknikar et al., 1993). Mycelial pretreatment by dimethly sulfoxide and boiling enhanced the uptake of metal by 37 % for copper and zinc, as observed with *Penicillium* biomass (Galun et al, 1983; Paknikar et al., 1993).

Disruption of cell components enhances cell permeability and increases the exposure of potential binding sites (Brierly et al., 1987; Luef et al., 1991; Fourest and Roux, 1992). The increased uptake of metal by the biomass subjected to NaOH treatment wherein most of the carboxyl and phosphate groups are removed (Luef et al., 1991), indicates the involvement of other groups in metal binding, other than electrostatic attraction. In *Fusarium solani*, KOH treatment of biomass exhibits only marginal increase in the metal binding capacity (Fernandes and Nazareth, 1999). This marginal increase was also reported by Akthar et al., (1996). Acid pretreatment resulted in a significant reduction in the bioadsorption capacity, while pretreatment with $CaCl_2$ and NaCl slightly reduced the bioadsorption capacity (Yan et al., 2000).

Pretreatment with calcium and magnesium ions restores the sorptive capacity of the biomass with near total regain of the biosorptive capacity of the biomass; the sorption of metals by the biosorb being an exchange of Ca^{2+} and Mg^{2+} ions (Modak and Natarajan, 1995). Hence, a Ca^{2+}/Mg^{2+} based regeneration procedure by desorbing with 0.1 N HCl, and then treating with $CaCl_2$ and $MgSO_4$ (0.1M) solution restored the biosorptive capacity

of the fungal biomass to 85-100 % for the five regeneration cycles tested (Akthar and Maruthi Mohan, 1995; Akthar et al., 1996; Fernandes and Nazareth, 1999). Metal binding due to exchange of Ca^{2+} and Mg^{2+} ions of the biosorb involves the release of equimolar concentrations of both ions into the medium. Biosorbents prepared from *Neurospora, Fusarium* and *Penicillium* exhibited such mechanisms for metal ion binding although they had a lower metal binding capacity compared to *A. niger* (Akthar et al., 1996).

1.5.6.3 Heat treatment

The use of heat-dried microorganisms for sorption from aqueous solutions was investigated (Akthar et al., 1996). It appears attractive in that the toxicity of metals in solution cannot affect the adsorptive function of the biomass, biomass growth conditions need not be satisfied and maintenance of purity of the culture is not a concern (Tsezos, 1990). It also has a longer shelf-life and limits the introduction of viable microbial contamination (Kowshik and Nazareth, 1999). Moreover, it can simultaneously remove several different toxic metals from solution regardless of their concentration (Akthar and Maruthi Mohan, 1995). Fernandes and Nazareth, (1999) reported that dessication by air-drying of the mycelia achieved comparable metal sorption as the undessicated mycelia, with the advantage that the dry mycelial powder can be stored for longer periods. However, dry heat treatment causes loss of almost all capacity of the biomass to sorb metal, an indication of the involvement of certain heat labile groups in metal biosorption.

1.5.7 pH of the solution

The pH of the solution greatly affects the uptake of ions by fungi. An increase in pH values results in increase in cation uptake which was found with fungi biomass (Kuyucak

and Volesky, 1989; Aksu and Kutsal, 1991; Holan et al, 1993; Holan and Volesky, 1994; Kratochvil et al 1998; Luef et al 1991; Muraleedharan et al., 1991; Paknikar, et al 1993; Mattuschka et al., 1993; Fourest, et al., 1994; Sag and Kutsal, 1995; Modak and Natarajan, 1995). A general trend, which was observed, is that the metal uptake is negligible at very low pH values of pH 1 to 2 due to competition between positive ions for binding sites of the biosorbent; the metal uptake increases with the increase in pH from pH 3 to 5, and an optimum of pH 5 to 7 was reached when the uptake is maximum, beyond which a reduction in the uptake was observed, attributed to reduced solubility and precipitation of base metals. However, the uptake of precious metals and radionuclides are reported at alkaline pH of 8 to 10 (Modak and Natarajan, 1995). According to Mahadevan and Tatum, (1995), the increase in biosorption when raising the pH would indicate the involvement of negatively charged groups.

No refs cited

Biosorption being a physical / chemical reaction between positively charged metal ions and anionic groups of all surfaces, the metal uptake is strongly influenced by the pH which affects the speciation of metal and reactive groups. At highly acidic conditions, H^+ and H_3O^+ ions may compete with metal cations for binding sites on the biosorbent. As the pH levels increase, more ligands with negative charge would be exposed, thereby increasing the attraction of positively charged metal ions, while at neutral to alkaline pH, most metals precipitate, thus making them unavailable for biosorption (Paknikar et al., 1993; Modak and Natarajan, 1995). The receptive sorbent groups can also be metal - specific (Muraleedharan et al., 1991) for example, amongst all the tested metals, such as copper, cobalt, chromium, cadmium, nickel, zinc, gold and silver, the fungus *C.cladosporioides* preferentially adsorbed gold and silver (Pethkar and Paknikar, 1998).

A positive influence on sorption at pH controlled conditions by *Rhizopus arrhizus*, *Pencillium chrysogenum* and *Mucor miehei* was observed (Fourest, et al., 1994). The biosorption of cadmium and zinc using *P.chrysogenum* was maximum between pH 4 and 6.0, while in case of copper, chromium and lead was pH 2 (Paknikar et al., 1993), the maximal sorption at low pH is a possible indication that not all binding sites are electrostatic in nature (Modak and Natarajan, 1995), and could be attributed to coordinate bond formation which is not pH dependent (Paknikar et al., 1993) .

1.5.8 Temperature

Temperature affects the metabolism of growing cells. However, the removal of metals by non-living biomass being metabolism-independent, it should have no significant effect on biosorption. According to Modak and Natarajan's reviews (1995), the uptake of Zn, Cu and U was not significantly affected when the temperature was between 4 and 45°C. However, an increase in uptake was found between 4 and 23°C, with only a marginal increase between 23 and 40°C, and a reduced uptake beyond 40°C. The adsorption and ion-exchange processes being exothermic in nature, the rate of these processes increases with increase in temperature, along with increase in metal uptake. However at high temperatures, the cell walls may be permanently damaged, reducing the metal uptake.

1.5 Mechanisms of biosorption

Research on biosorption reveals that adsorption and desorption studies yields information on the mechanism of metal biosorption, how the metal is bound within the biosorbent. A number of different metal binding mechanisms has been postulated to be active in biosorption. The complex structure of microorganisms implies that there are many ways

for metals to be taken up by the microbial cell, which is implicated in fungal survival in presence of toxic metals. They may be classified according to various criteria, and may involve ion exchange, chelation, adsorption by physical forces, biosorption to cell walls polysaccharide, extracellular precipitation, complexation and crystallization, intracellular accumulation, microbial transformation of metal species by oxidation, reduction, methylation and dealkylation, pigments, decreased transport or permeability, efflux, intracellular compartmentation and precipitation and / or sequestration (Brierly, 1990; Gadd, 1993; Ahalya, et al., 2003). These processes may be operative individually or in combination to sequester metals.

Several mechanisms and numerous chemical groups such as hydroxly, carboxly, carbonly, sulfhydryl, thioether, sulfonate, amino, amido, amine, imine, amide, imidazole, phosphate and phosphodiester groups have been suggested to contribute to metal binding by either whole organisms such as algae and bacteria or by molecules such as biopolymers. Microbial polymers consist mainly of neutral polysaccharides and compound such as uronic acid, hexoseamines and organically bound phosphates which complex soluble metals. The hydroxyls in polysaccharides are believed to attract metal ions (Hunt, 1986; Volesky and Holan, 1995). Studies by Sorret, et al (1998) showed that Zn and Pb bind to the predominant phosphoryl (≈ 95%) and minor carboxyl groups (≈ 5%) with a reversed affinity.

The mechanisms responsible for biosorption appear to be multifactorial: the potential ligands within the cell wall comprising of -COOH, $-NH_2$, -SH, -OH and $-PO_4^{3-}$ groups (Tobin et al, 1984). It is not possible to generalize the mechanism of uptake; each factor

has to be defined individually for each biomass and metal-ion pair (Modak and Natarajan, 1995).

Metal sorption mechanisms may involve the release of protons into the solutions and ion exchange plays a role to some extent, as was observed in the biosorption of metals by *P.Chrysosporium*. However, this is not expected to be the principle mechanism (Ã‡eribasi and Yetis., 2001).

Biosorption is considered to be a fast physical or chemical process, the rate of biosorption depending on the type of the process and may be classified according to various criteria based on the cells's metabolism and localisation of metal taken up by the cells. According to Ahalya et al (2003), based on the cell's metabolism, biosorption mechanisms can be divided into:

- Metabolism dependent and
- Metabolism independent

When based on location, that is, where the metal removed from solution is found, biosorption can be classified as:

- Extra cellular accumulation / Cell surface sorption /precipitation and
- Intracellular accumulation.

The transfer of metal ions from aqueous to solid biosorbent phase can be due to passive, facilitated or active transport (Muraleedharan et al, 1991; Balakrishnan et al., 1994). Active biosorption is metabolism-dependent and is a slower process. It is an energy dependent uptake across the cell wall and operate directly on the metal, via precipitation, intracellular accumulation and oxidation / reduction methods. Transport of the metal

across the cell membrane yields intracellular accumulation, which is dependent on the cell's metabolism and therefore may take place only with viable cells. It is often associated with an active defense system of the microorganism, which reacts in the presence of toxic metal (Balakrishnan et al., 1994; Ahalya et al., 2003). Passive sorption is metabolism-independent driven by the concentration gradient across the membrane. It binds to cell walls and other external surfaces, and can take place both in living as well as dead biomass. It acts indirectly by modifying the surrounding medium via complexing with extracellular biological chelates (Balakrishnan et al., 1994). The *Penicillium* ochro-chloron probably uses a detoxification mechanism by forming insoluble copper precipitates within the matrix of fungal mycelia to deal with the potentially lethal concentration of heavy metals such as copper (Ashby et al., 1997).

1.6.1 Cell wall

Metal uptake also takes place by physico-chemical interaction between the metal and the functional groups present on the microbial cell surface based on physical adsorption, ion exchange and chemical sorption, which is not dependent on the cells metabolism. Cell walls of microbial biomass are mainly composed of polysaccharides, proteins and lipids and have abundant metal binding groups such as carboxyl, sulphate, phosphate and amino groups. This type of biosorption, i.e., non-metabolism dependent is relatively rapid and can be reversible (Ahalya et al., 2003).

The following mechanisms are known to occur in the biosorption of metals (Volesky, 1994) -

- Van der Waal's forces wherein uncharged atoms and molecules are loosely bound in the matrix by electrostatic attraction.
- Ionic bonds between a metal cation and an ionic reactive group of the binding matrix.
- Crystallisation of metals at the cell surface.
- Electrostatic attraction, which can result in adsorption of precipitate at the cell envelope

The functional groups on the cell surface of biomass play a role in metal uptake (Fourest and Roux, 1992; Saitoh et al., 2001). Earlier studies on the mechanism of removal of metal ions by microorganism assessed that the cell wall is the primary site of metal ion accumulation (Tobin et al 1984; Zamani et al 1985; Kuyucak and Volesky, 1987). It is not mediated by metabolic processes and can take place in dead as well as living cells. The relative affinity of different metals to bind to fungal cell walls also depends on the chemical composition of the cell wall, and thus, the fungal species involved (Kapoor and ViraRaghavan, 1995). The formation of co-ordination complexes between the metallic species and chitin nitrogen or oxygen was suggested as the initial step in metal binding.

Fungal cell walls contain up to 10-30 % of the cell wall dry weight and chitin contributes significantly to the mechanical strength of the cell wall. Both chitin and α1, 3-glucan biosynthesis are induced in response to cell wall stress most likely as a response mechanism of the fungal cell to change the composition of the cell wall, making it more resistant to cell wall disturbing compounds (Ram et al., 2004). However, other studies indicated the involvement of other components such as functional groups other than chitin in metal binding. Tobin et al (1990) and Fourest and Roux (1992) stated that the metal biosorption involves physical and chemical attractions between positively charged

dissolved species and negatively charged reactive cellular components, the cell walls of microorganisms consisting mainly of negatively charges groups such as carboxyl, hydroxyl, sulphate, phosphate and amino groups which can bind metal ions (Fourest et al., 1996; Sayer et al., 1997). The precipitation of the metal within the cell wall has also been documented (Kuyucak and Volesky, 1987).

Research work revealed that there are differences in the mechanisms of biosorption even in related species, as observed in bacteria (Chakraborty 1978; Treen-Sears, 1984; Volesky 1999; Vance 2002), where the carboxly groups of glutamic acids of peptidoglycans present in the cell walls of Gram positive *B.Subtilis* were the primary sites of metal complexation, while in *B.licheniformis*, the prime site of metal deposition seems to be the phosphate containing teichoic acids, (Chakrabarty, 1978; Beveridge and Murray, 1980; Beveridge et al., 1997; Volesky, 1999 and Vance, 2002), ~~indicating the differences in mechanisms even in related species~~. Numerous metal binding mechanisms are involved in the biosorption of metals, and these mechanisms qualitatively and quantitatively differ according to the species used (Volesky and Holan, 1995), in that the composition of the cell walls of different fungi vary considerably, hence different species exhibit differences in sorption capacities (Modak and Natarajan, 1995). However a comparative study of the metal binding mechanisms between morphotypes of the same genus in fungi has not been dealt with.

1.6.2 Physiological response

Fungal cell walls not only determine the shape of the cell, but also provide physical and osmotic protection with their rigid structure, and are able to adapt to various changes

(Vankuyk et al., 2004). It is mainly composed of mannoproteins, 1,3-β-glucan and chitin. Depending on the species, additional polymers such as 1,3-α-glucan or 1,6-β-glucan polymers may be present. The cell walls of filamentous fungi contain up to 10-30 % of the cell wall dry weight and chitin contributes significantly to the mechanical strength of the cell wall. Chitin and α1, 3-glucan biosynthesis are induced in response to cell wall stress most likely as a response mechanism of the fungal cell to change the composition of the cell wall, making it more resistant to cell wall disturbing compounds (Ram, et al., 2004). In filamentous fungi the response to sub-lethal levels of cell wall stress can result in morphological abnormalities such as swollen apical tips (Vankuyk, 2004).

Toxic metal in the environment induces stress. One aspect of toxic metal tolerance, which can be observed culturally and/or morphologically, is a change in mycelial growth. In filamentous fungi, the response to sub-lethal levels of cell wall stress results in morphological abnormalities such as swollen apical tips (VanKuyk et al., 2004). In general, toxic metal minerals induce slow dense mycelial aggregation or the so-called phalanx growth strategy resulting in a very dense mycelium compared to the control growth pattern. This is well known for its protective function and has been observed in fungi colonizing toxic metal-contaminated domains with higher tolerance for toxic metal stress, but constant biomass yield (Fomina et al., 2005).

Fusarium solani was found to tolerate a number of heavy metals and others, such as Pb, Cu, Hg, As, Cr, Al, Ni, Fe, Co, Mn, Zn and Li. Certain morphological changes, such as, increase in number of spores, thickened cell wall, bulbous hyphae and changes in the shape and size of the cultures in presence of metals were observed during growth of the

culture in response to metal. Pigment production also played a role in higher tolerance to metals (Kowshik and Nazareth, 2000) and increase in the biosorption efficiency (Pethkar and Paknikar, 1998). Similarly, the changes in the morphology of fungi in presence of toxic concentrations of metals were observed by other workers (Ã‡eribasi and Yetis, 2001; Vankuyk, 2004; Katarzyna, 2004).

1.6.3 Metal-binding proteins and peptides

Another factor involved in the resistance mechanism / metal detoxification are chelation of metal ions in the cytosol with thiol-containing compounds, such as glutathione (GSH), phytochelatins (PCs), or metallothioneins (MTs). Reduced glutathione (γ-glutamyl cysteinyl glycine), phytochelatins (PCs), and metallothioneins (MTs), are essential components of Cd detoxification pathways in various organisms (Courbot, et al., 2004). Reduced glutathione is the most abundant nonprotein thiol component of eukaryotic cells, acts as a free radical scavenger, and reacts with various oxidants to produce oxidized glutathione. The cellular resistance to heavy metal cytotoxicity in fungi is mainly mediated by the binding of metal ions either to a metallothionein or phytochelatin (Kameo et al., 2000).

The thiol peptide, GSH (γ-Glu-Cys-Gly), and in some species its variant homoglutathione (h-GSH, γ-Glu-Cys-β-Ala), is considered to influence the form and toxicity of heavy metals such as As, Cd, Cu, Hg, and Zn, in several ways. These include direct metal binding, promotion of the transfer of metals to other ligands, such as MTs and PCs, provision of reducing equivalents for the generation of metal oxidation states more amenable to binding by MTs and possibly PCs, removal of the active oxygen species

formed as a result of exposure of cells to heavy metals, and/or the formation of transport-active metal complexes (Vatamaniuk, et al., 1999; Hall, 2002; Courbot, et al., 2004).

Fungi are known to accumulate heavy metals (Hall, 2002). An important resistance mechanism may be the production of metallothioneins. MTs or low molecular weight, cysteine rich metal-binding proteins (4 to 8 kDa) which bind metal ions in metal-thiolate clusters have abundant cysteine residues (cys) and often possess a characteristic pattern of sulfur containing amino acids (Hamer, 1986; Mali and Bulow, 2001; Bae et al., 2003; Macaskie and Dean, 1990). Earlier studies by Shindo and Brown (1965) revealed that the proteins are capable of complexing with metal ions cystein, and has three possible co-ordination sites, namely sulfhydryl, amino and carboxylate groups.

MTs are synthesized *de novo* upon exposure to elevated concentrations of some metals, and are commonly found in association with essential metal ions such as zinc and copper but have also been shown to bind toxic metals like cadmium, mercury, and lead (Hamer, 1986; Oberdorster et al. 1994; Mali and Bulow, 2001; Macaskie and Dean, 1990). Their metal binding properties are mediated via the abundant cysteine residues and their characteristic organization into -Cys-Cys-, -Cys-X-Cys-, or -Cys-X-X-Cys- sequences (x corresponds to any other amino acid in the protein sequence). MTs can be characterized into three specific classes: class I MTs, which contains most animal MTs, class II MTs and class III MTs or PCs. Upon exposure to metal, fungi synthesizes class II type of MT and PC, a class III type of MT (Hamer, 1986; Macaskie and Dean, 1990; Mali and Bulow, 2001; Hall, 2002). The only fungus known to use both metallothioneins and phytochelatins for metal detoxification is *Candida glabrata*, which produces MTs when

exposed to toxic concentrations of Cu but produces mainly phytochelatins in response to Cd stress (Gardea-Torresdey, et al., 1998; Hall, 2002; Courbot, et al., 2004).

The copper resistance mechanisms can include copper complexing by cell wall components, changes in membrane copper transport, synthesis of intracellular copper binding metallothioneins and phytochelatins and productions of extracellular copper complexing or precipitating metabolites (Fourest et al, 1994). Metal absorption and resistance to toxicity are related phenomenon. This point was made in 1982, when it was shown that multiple copies of copper metallothionein in *S. cerevisiae* enhanced its resistance to copper (Fogel and Welch, 1982).

PCs are a family of small cysteine-rich metal-complexing peptides capable of binding heavy metal ions via their SH group. Unlike MTs, the PCs poly-(γ-Glu-Cys)$_n$-Xaa polymers, are enzymatically synthesized posttranslationally from glutathione and related thiols by the action of γ-glutamylcysteine dipeptidyl transpeptidases (phytochelatin synthases). Phytochelatin synthase catalyzes the addition of the γ-glutamylcysteine (γ-GluCys) moiety of glutathione onto another glutathione molecule to produce PC_2, or onto a pre existing PC_n molecule to produce the corresponding PC_{n+1} molocule. This enzyme is activated by a broad range of heavy metals including Cd, Ag, Pb, and Cu. These peptides consist of repetitive gamma-glutamylcysteine units with a carboxyl-terminal glycine, ser or β-Ala residue [poly-(γ-Glu-Cys)$_n$-Xaa] and range from 5 to 17 amino acids in length. The general structure of this set of peptides is [γ-GluCys]$_n$-gly (n = 2 to 11), and are rapidly induced in plants by heavy metal treatments (Grill, et al., 1987; Vatamaniuk, et al., 1999; Hall, 2002; Courbot, et al., 2004).

PCs bind heavy metals, such as Cd^{2+}, with high affinity, and they play a pivotal role in heavy metal, primarily Cd^{2+} tolerance in plants and fungi by chelating these substances and decreasing their free concentrations. PCs are found in some fungi, algae, and all plant species examined so far, and serve functions analogous to those of metallothioneins in animals and some fungi. Despite the importance of PCs for heavy-metal tolerance, the molecular identity of the enzyme(s) responsible for the elaboration of these peptides has eluded definition. Several investigators have described the partial purification of heavy metal primarily Cd^{2+} activated enzymes, but none have been able to identify or isolate the moiety or moieties concerned at either the protein or gene level (Oberdorster et al. 1994; Grill, et al., 1987; Vatamaniuk, et al., 1999; Hall, 2002; Courbot, et al., 2004).

PC derivatives are not primary gene products, but are glutathione-related peptides. Cellular resistance to heavy metal cytotoxicity in fungi is mainly mediated by the binding of metal ions either to a MT or PC. In contrast to vertebrate MTs, which bind different metal ions, fungal MTs were reported to exclusively contain copper ions. It was reported that incorporated copper in fungi was bound to low molecular weight ligands in the mycelia. An ascomycete *Neurospora crassa* accumulated copper with a concommitant synthesis of copper-binding protein, a class II MT, consisting of a single polypeptide chain of 25 amino-acid residues with 7 cysteine residues and lacking aromatic amino acid, binding only copper. It was also reported that exposure of *Candida glabrata* to copper salts stimulates formation of MTs, whereas PC derivatives were synthesized in response to exposure to cadmium salts. *Beauveria bassiana*, resistant to 10 mM Cu had additional resistance towards zinc, cadmium and lead (Kameo et al., 2000).

Cysteine has a tendency to combine well with metals; the presence of L-cyteine increased the gold-cyanide biosorption by protonated *B. subtilis, P. chrysogenum* and *S. fluitans* biomass at pH 2 (Niu and Volesky, 1999; Niu and Volesky, 2000). Xu et al., (2002) investigated the peptide based biosorbents and demonstrated the feasibility of a protein-based technology for heavy metal removal.

1.6.4 Plasmids

Plasmids are very commonly found in filamentous fungi where they generally have mitochondrial localization. Some plasmids represent defective forms of mitochondrial DNA (mtDNA), while others lack any homology to the mtDNA. True plasmids are DNAs (or RNAs) that have little or no homology to mtDNA and replicate separately from the mitochondrial genome. The majority of the plasmids so far identified belong to the true plasmids, are linear but their functions are still unclear with a few exceptions (Gobbi et al., 1997)

Circular plasmids have been reported to occur in fungi and are not associated with a particular phenotypic trait with the exception of a plasmid of *A.glauca*, which encodes for a surface protein present only in mating type positive strains harbouring the plasmid. Plasmid-containing virulent strains of *C.parasitica* showed morphological alterations during prolonged vegetative propagation seen as abnormalities of the colony development. The phenomenon also includes cessation of growth of the colony with limitations of the morphological development and possibly consequences on the dynamics of the development of the pathogen in the environment (Gobbi et al., 1997)

Genetically, heavy metal resistance in fungi and bacteria can be either chromosomal or plasmid encoded. The changes at the genomic level result in extra cellular or intracellular accumulation of metals (Cervantes and Gutierrez-Corona,1994). Different species may have evolved different mechanisms to tolerate excess metals and that even within same species more than one mechanism could be in operation to withstand excess metals. Adaptive metal tolerance was shown to be governed by a small number of major genes with perhaps contributions from some more minor modifier genes.

Plasmid mediated transport mechanisms have been suggested for metal uptake (Pazirandeh, 1996). Essential metals (like Ca^{2+}, Na^{+}, K^{+}) was studied extensively, however the specific mechanisms by which the non-essential metals are taken up by cells are not well defined. It has been implicated that their uptake may be genetically controlled by genes. Strains of *Klebsiella pneumoniae* that were able to grow in the presence of silver (Ag^{+}), cobalt (Co^{2+}), cadmium (Cd^{2+}), nickel (Ni^{2+}), lead (Pb^{2+}), copper (Cu^{2+}), zinc (Zn^{2+}) at concentrations up to 10 mM showed similar plasmid profiles, ranging in sizes from 1.8 to 120 kb. Resistance to lead, cobalt, nickel, and copper was encoded by a 3.5-kb plasmid of *K. pneumoniae* (Choudhury and Kumar, 1998). Plasmid mediated resistance was also observed in organisms like *Alcaligens eutrophus*. Resistance to copper is known to be determined by large plasmids. The amplification of genes to detoxify metal toxicity was also observed in copper resistant strains of *S.cereviseae*. There is a correlation between the levels of copper resistance and the extent of DNA amplification (Cervantes and Gutierrez-Corona, 1994).

Little work has been done on the morphological changes and protein expression in response to metal stress and plasmid mediated resistance in fungi and particularly more so with regard to *Penicillium* isolates.

1.7 Fungal treatment of industrial effluents

1.7.1 Mining industry

Mines are excavations made in the earth to extract minerals, while mining is the activity, occupation and industry concerned with the extraction of minerals. The minerals commonly mined can be subdivided into:

Metallic ores: These are ferrous metals such as iron, manganese, molybdenum and tungsten, base metals such as copper, lead, zinc and tin, the precious metals which include gold, silver and platinum group metals and the radioactive minerals which include uranium, thorium and radium.

Nonmetallic minerals (also known as industrial minerals): These are the non-fuel mineral ores that are not associated with the production of metals such as phosphate, potash, halite, sand, gravel, limestone, sulfur and many others.

Fossil fuels (also known as mineral fuels): the organic mineral substances that can be utilized as fuels, such as coal, petroleum, natural gas, methane and tar sands.

The factors contributing to the concentrations of heavy metals in soils are the natural sources such as volcanoes and continental dusts, and the anthropogenic activities like mining, combustion of fossil fuels, metal-working industries, phosphate fertilizers, etc., all lead to the emission of heavy metals and the accumulation of these compounds in ecosystems (Schützendübel and Polle, 2002). The major phases in mine development are

(a) exploration (b) mine development (c) extraction (underground and open pit) and mine operation (d) ore beneficiation (e) storage and transport of ore and (f) mine closure and reclamation (World Bank Group, 1998).

In Goa, mining has played a major role for decades and accounts for about 10 % of the total exports of iron and manganese of the country. The larger mines are situated in the Zuari basin, while the rest are in the Mandovi basin (Fig 1.1). The overview of the mining site is shown in Fig 1.2. This industry has placed Goa in a prominent place in the mineral map of India and is an important source of foreign exchange (Deshpande, 1990; Kowshik and Nazareth, 2001).

The iron ore is non-saleable in its raw form; therefore after excavation of the ore, it is upgraded in beneficiation plants where it is washed to yield lumps and fines (Sreeramachandra Rao et al., 1996). The wash waters are known as mine tailings with an iron ore content of approximately 48 %, which is lost out as waste. This residual slurry contains at least 50 % water, but also large amount of chemical reagents and metals, which are usually discharged into storage facilities and retained by dams or embankments. These tailings are processed through modified hydrocyclone circuits, and then further through magnetic separators for the recovery of iron ultra fines. Subsequently, the tailings are treated with limes in tailing ponds and allowed to undergo sedimentation; the clarified waters are then recycled to economize freshwater intake. The final waste wash waters ater treatment and clarification are released into neighbouring rivers (D'souza, 1995)

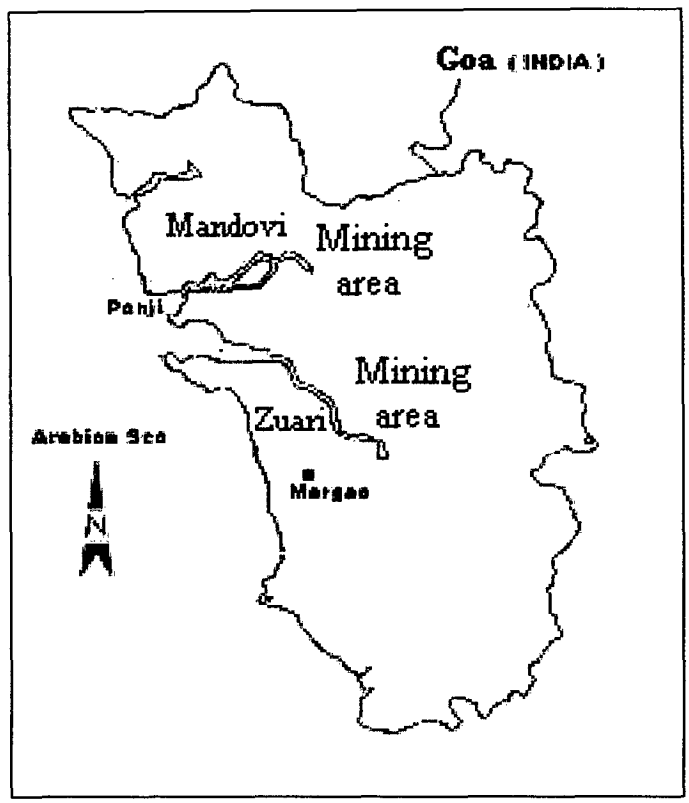

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Fig 1.1: Map of Goa showing mining sites

Fig 1.2: Overview of the mining sites

1.7.2 Environmental effects of mining

The chemicals used in flotation and other metal concentration processes could create toxicity problems when released in effluents. Explosives such as ammonium nitrate may be present in surface runoff. Surface runoffs may also pose significant environmental problems through erosion and carryover of tailings and other mining residues. Transport of mined material and machinery maintenance and repair can lead to contamination of surface water (World Bank Group, 1998).

The deterioration of water quality is another environmental effect of mining which is caused due to discharges of waste water from beneficiation plants, wash offs from wastes tailings, and acid mine drainage (Mahajan et al., 1994). The processed tailing waters which are in some cases, insufficiently treated besides containing iron and manganese, gives rise to pollution of neighbouring rivers, which have turned red and highly turbid and / or find their way into nearby agricultural lands and wells thus adversely affecting crops and living organisms and human life (D'souza, 1995). Hence, proper management of tailings must be ensured to optimize human safety and environmental protection.

The major environmental impacts due to mining (Mahajan et al., 1994) include -

- Noise and vibration impacts - due to blasting, heavy machinery deployment and transport operations, crushers, grinders, conveyers, ventilation fans, etc
- Air quality impacts – in the form of increase in suspended particulate matter due to fugitive emissions, blasting operations and increase in CO, SO_2, NO_2 due to transport activities / mining operations

- Water quality impacts – due to disruption of water regime and hydrology, and water quality deterioration due to wash-offs burdens and discharges of tailings.
- Dereliction of land environment – due to excavations, disposal of mine wastes and tailings, landslides, deforestation, aesthetic impairment of landscape, etc
- Impacts on biological environment – due to removal of existing vegetation, migration of fauna due to human interference, change in soil microbial status
- Socio-economic impacts – impacts on human settlement, mining operations stress, deterioration of cultural, historical and scenic importance.

1.7.3 Clean-up of polluted environment

The main pollution concerning the mining industry is the volume of solid waste generated, of tailings from processing of the ore, which poses a major problem in storage and reclamation. When the concentration or other processing of the ore is done on site, the tailings generated from these also have to be managed. Ores with a low metal content, less than 0.4 %, generate significant quantities of tailings (World Bank Group, 1998). The processed tailings form thick slurries of earth, and carry a lot of suspended particles and metal fines, which would take months to sediment, leading to environmental pollution if discharged into domestic waters untreated. Hence, chemicals such as lime and salt are added to bring about sedimentation. Precautionary measures to safeguard pollution should ensure that the resources are used with maximum efficiency, waste generation is minimised, proper and adequate treatment of residuals, recycling and recirculation of waste products to the maximum extent possible (Mahajan et al., 1994).

The use of chemicals for metal precipitation is not only fairly costly, but also introduces another factor, namely, the increased amounts of calcium and carbonate in water, which results in the formation of hard water. Discharge or run-offs into neighbouring fields and water bodies has an adverse effect on aquatic and human life (D'souza, 1995). Biological systems to remove metal ions from polluted waters could emerge as the potential alternative to chemical treatments (Deshpande, 1990; Volesky, 1990). The application of biosorbent technology is cheaper and more competitive than the conventional technology, thus establishing the feasibility of biosorbent applications and their competitiveness in the market place (Atkinson et al., 1998; Volesky and Naja, 2005).

The role of microorganisms in the mining of ores and to some extent the removal/recovery of metals from waste-waters has been exploited (Belin et al., 1993); however, little had been done on biosedimentation of mine tailings. Exopolymers have been used in flocculation and aggregation (Friedman et al., 1969). The use of algae or cyanobacteria (Avnimelech et al., 1982; Avnimelech and Menzel, 1984; Bar-Or and Shilo, 1988) and bacteria (Deshpande, 1990) in settling of particulate/clay suspension or mine tailings has been reported.

The mechanisms involved are-

- Cell surface modifications resulting in increased adherance to particulate matter (Fattom and Shilo, 1984; Bar-Or et al., 1985), which in turn facilitates sedimentation (Avnimelech and Menzel, 1984)
- Production of flocculants which directly cause clarification of turbid waters (Fattom and Shilo, 1984; Shilo and Bar-Or, 1987; Bar-Or and Shilo, 1988)

The use of biological measures in the sedimentation of industrial effluent has been gaining importance, both for its cost effectivity and the absence of adverse side effects (Kowshik and Nazareth, 2001). The biosedimentation of mine tailings by *Fusarium solani* indicated that sedimentation was achieved in just 30 mins (Kowshik and Nazareth, 2001), which was four to six-fold faster than *Bacillus* and *Arthrobacter* (Deshpande, 1990). The biological compounds mostly act by reducing the charge carried within the ionic atmosphere surrounding each solid particle. Due to electrostatic attraction, the particles come together and sediment (Young and Elvis, 1982). *Fusarium solani* was reported to sorb metals such as iron, manganese and lead (Kowshik and Nazareth, 2001); hence the biosorption of metal fines may also be involved in the biosedimentation of mine tailings.

Some limitations of the technology are that large-scale production of effective biosorbent materials has not been established and that the technology has only been tested for limited practical applications.

Aims of the thesis

The interactions of microorganisms with metals have long been recognized, and numerous workers have exploited the area of biosorption for environmental remediation. Isolates of halophilic penicillia have been reported from hypersaline environments; however, little work has been done with these fungi from coastal waters of Goa, India. Further, the genus *Penicillium* has been studied and used for metal tolerance / removal, however it has not been examined with respect to halophilic / halotolerant species possessing metal resistance. A comparative study of the metal binding mechanisms within isolates of the same genus in halotolerant fungi has also not been dealt with.

The main aims of this thesis was to isolate halotolerant *Penicillium* isolates which are metal resistant and to study the variations in the mechanisms of metal sorption between morphotypes of the same genus by representative isolates of *Penicillium* with respect to their tolerance levels to heavy metals, such as lead, copper and cadmium, and some other metal pollutants, such as, iron and manganese, the total metal sorbed and the localisation of metal during uptake, cultural and morphological responses of the cultures to metal stress, changes in the whole cell protein profiles and plasmid in presence of metals, and to examine the application potential of the isolates for treatment of an industrial waste such as mine tailings, for sedimentation, clarification and metal removal.

Significance of the work

Toxic metal contamination of the environment is of increasing economic, public health and environmental concern as unwanted and toxic heavy metals cannot be degraded by biological, chemical processes or by *in situ* biological means tending to accumulate

throughout the food chain. Removal of these metals is a major pre-requisite, and the use of microorganisms for the abatement of pollution provided an attractive alternative to the physico-chemical method due to the low cost of this complexing material and its effectiveness in removal of metals present in trace amounts. The more severe environmental protection laws have encouraged studies about removal/recovery of heavy metals from aqueous solutions using biosorption. The diversity of microorganisms in hypersaline environments is also of growing interest as the hypersaline environments are on the increase due to natural and man-made global changes. These fungal cultures, which are able to grow under extreme environment, offer good potential as indicators of pollution and as biosorbents and can be used for abatement of pollution in hypersaline conditions or in waters of fluctuating salinity, as well as in non-saline environments.

The mechanisms involved in metal resistance by fungi in its various aspects has not received very much focus, particularly with regard to the morphological responses to metal stress, and especially the variations between morphotypes of the same genus.

This work is a first report of the isolation of extremely halotolerant penicillia from the mangroves and salterns of Goa, India, and a first study of the resistance of these fungi to heavy metals such as Pb^{2+}, Cu^{2+} and Cd^{2+}. It is also the first study on an understanding of the variations arising between morphotypes of the same genus of *Penicillium* in response to metal stress.

An understanding of the factors which governs biosorption can lead to the cultivation of a desired type of biomass which will be most effective in biosorption as well as cost

effective. The knowledge of the mechanism of metal-biomass interaction would open up various possibilities of optimizing the process of biosorption and manipulate certain properties of the biomass to simplify the process of biosorption.

The use of the fungal isolates for effective sedimentation of mine tailings also provides an eco-friendly, cost effective approach which is an attractive alternative to the chemical treatment of lime, the isolates achieving a very good sedimentation and clarification of mine tailings.

CHAPTER 2

ISOLATION OF HALOTOLERANT PENICILLIA WITH HEAVY METAL RESISTANCE

CHAPTER 2 : ISOLATION OF HALOTOLERANT FUNGI WITH HEAVY METAL RESISTANCE

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2.1 Introduction

The isolation of fungi from hypersaline environments has been reported (Gunde-Cimerman, 2000). These highly saline conditions favour the development of halotolerant and halophilic forms (Kis-Papo et al., 2001).

However, there has been little work done on this group of fungi in general, and especially from coastal waters of Goa, India. Further, since the water bodies often become sinks for disposal of waste or run-offs from effluent treatment plants or land fills, or such, which particularly during the heavy rain season find their way into the nearby rivers, work was undertaken to screen for halotolerant isolates and their resistance to heavy metals, the cultures which are able to grow under extreme environment from constant exposure to metals, thus offering good potential as indicators of pollution and as biosorbents (DasSarma, 2001), and naturally selected organisms represent an important strategy to obtain agents for bioremediation process (Wood and Wang, 1983).

Fungi are known to be more tolerant to metals than bacteria or actinomycetes (Gadd, 1993), with *Penicillium* spp being quite prominent (Gadd, 1993; Fourest et al., 1994; Natarajan et al, 1999; Skowronski et al., 2001; Tan and Cheng, 2003; Cabuk et al., 2005). Although, the genus *Penicillium* had been studied and used for metal tolerance / removal, it has not been examined with respect to halophilic/ halotolerant spp possessing metal resistance. Further, a comparative study of the metal binding mechanism between morphotypes of the same genus in halotolerant fungi has not been dealt with.

This chapter deals with the isolation of extremely halotolerant penicillia from the mangroves and salterns of Goa with resistance to heavy metals such as Pb^{2+}, Cu^{2+} and Cd^{2+}, which are listed amongst the pollutants of concern (Ahalya et al, 2003). More attention is given to heavy metals because of the fact that they cannot be decomposed by *in situ* biological means (Laws, 1993). In addition, two transition metals, that is, Fe^{2+} and Mn^{2+} were also used for the study because the sites used for sampling lie along the route of heavy road and water traffic, the latter consisting especially of barges carrying iron ore; the wind-blown dust and / or spillage would cause contamination of the waters.

2.2 Materials and methods

2.2.1 Sample collection

The soil / water samples were collected from the mangroves, salterns and well water as shown in Fig 2.1. Soil samples were collected from the mangroves (M), Ribander, and water samples were collected in sterile Roux bottles: 3 x 800 ml from the mangroves (M), Ribander and 2 x 600 ml from the solar salterns (S), Ribander. The sampling sites, M and S, are shown in Fig 2.1a and Fig 2.1b.

2.2.2 Isolation and purification of halotolerant metal resistant cultures

The samples obtained were filtered through a 0.45mμ membrane filter to eliminate bacteria, which was then placed over Czapek Dox Agar (CDA HI-Media) containing 2 % salt (S-CDA) for well water and mangrove samples, while saltern samples were grown on CDA with 5 % salt. Incubation was done at room temperature (RT) of 30° C and observed for growth. Fungal cultures were picked up and purified on the isolation media containing 1mM lead and then maintained on S-CDA with 0.5 mM lead.

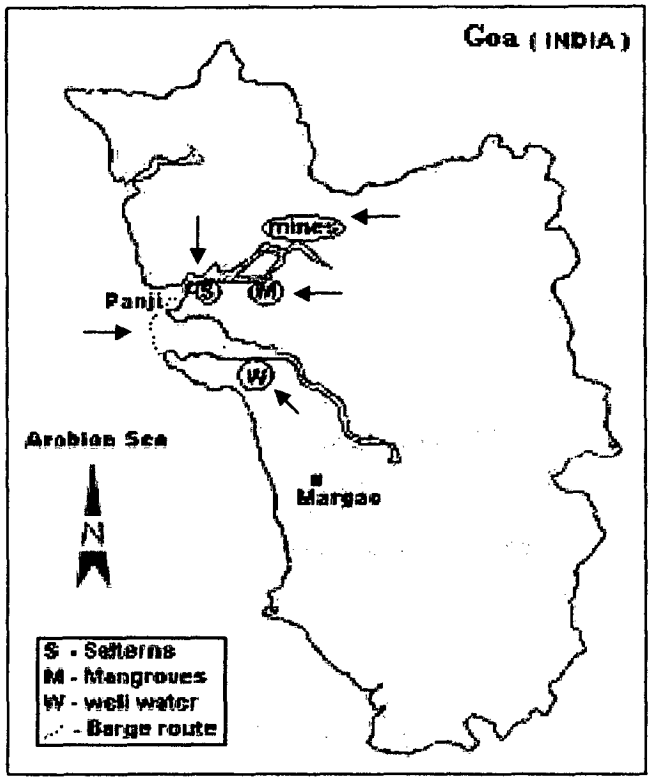

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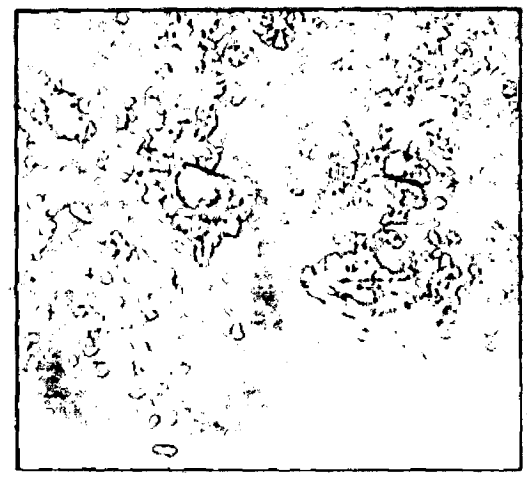
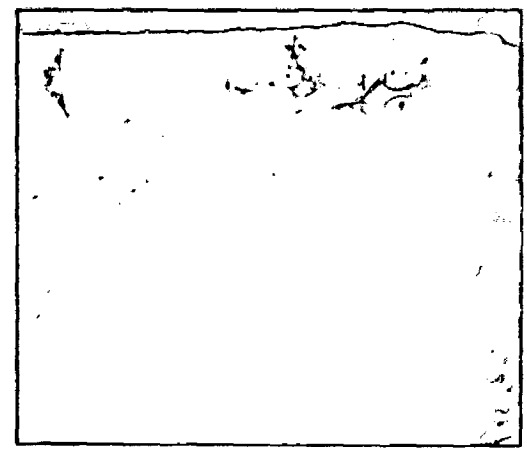
Fig 2.1a: Map of Goa showing location of sampling sites

(a)



(b)

(c)



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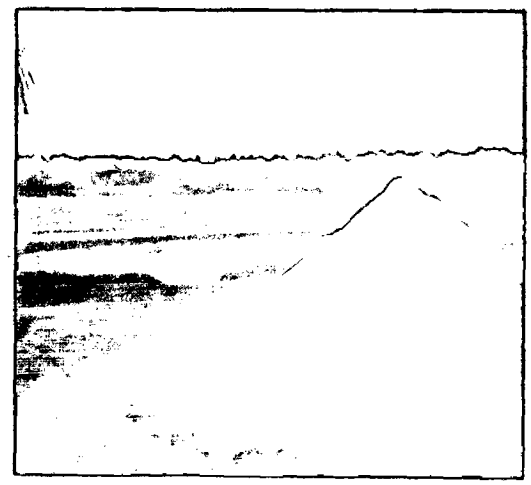

(f)

Fig 2.1b: Sampling sites : Mangroves: (a) soil (b) water samples; Salterns : (c-e); Salt mound (f)

2.2.3 Selection and study of cultural and morphological characteristics of *Penicillium* species

Penicillium species were picked up on basis of cultural and morphological characteristics namely, blue green spores, with mycelium having conidia borne in chains forming a typical brush like spore bearing heads with phialides in clusters. Two isolates previously isolated in the lab from mangroves and from well water (W) close to a copper smelting plant and to the mouth of the river (Fig 2.1a) were also used for the work and studied.

Penicillium cultures were studied for their individual cultural characteristics: nature of growth, colour of spores, pigment production, the colony on the reverse and their morphological characteristics, particularly the branching pattern of the conidiophore (Larone et al., 1995; St-Germain and Summerbell, 1996; Sutton et al., 1998; Christensen et al., 1999; de Hoog et al., 2000), as shown in Fig 2.2.

2.2.4 Sample analysis

The water samples from the mangroves, solar salterns and the well water samples were tested for their pH, salinity and presence of metals such as lead, copper, cadmium, iron and manganese.

2.2.4.1 pH

The pH of the water samples were checked using the pH meter (LABINDIA, PHAN)

2.2.4.2 Salinity

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Standard: Sodium chloride, 1 g was dissolved in 50-75 ml of distilled water and made up to 250 ml. 25 ml of NaCl solution was transferred into a 150 ml titration flask. To this, 1 ml of 5 % potassium chromate indicator solution are added and mixed thoroughly. The silver nitrate (0.1N) was then added from the burette, swirling the liquid constantly until the first sign of a colour change. The titration is continued until a faint reddish colour persists after brisk shaking which is the end point of titration. The reading of the burette is recorded. The titration of the standard was repeated twice. (Grasshoff, et al., 1983; Nadkarny et al., 1975).

Sample: 25 ml of the sample was titrated against silver nitrate in exactly the same way as for the standard.

2.2.4.3 Metals

The concentration of metals, such as, lead, copper, cadmium, iron and manganese were determined by Flame Atomic Absorption Spectrophotometry (AAS; Perkin Elmer Model A Analyst 200 and Model-GBC 932 AA). Samples were aspirated into the AAS, and triplicate readings were obtained for each sample. Standards were prepared using deionized water.

2.2.5 Culture conditions

2.2.5.1 Cultures and maintenance

The *Penicillium* cultures were routinely maintained on S-CDA with 0.5 mM lead, and sub-cultured bi-monthly.

2.2.5.2 Preparation of spore suspensions

A loopful of the culture from S-CDA (HI-Media) slants was inoculated into 60 ml of sterile Czapek's dextrose broth (HI Media) (Appendix A) supplemented with 2 % salt (S-CDB) taken in sterile McCartney bottles and incubated in slanting position, stationary, at RT for 4-6 days till spores covered the whole surface area. The culture broth was filtered through a double layer of muslin cloth under sterile conditions to remove the mycelial mass; the filtrate containing spores was then centrifuged at 1500 g for 15-20 min and the spore pellet washed and resuspended in sterile 0.85 % saline, then dispensed in eppendorf tubes and stored at $4^{o}C$ for use as and when needed.

2.2.5.3 Determination of spore count to determine size of the inoculum in spore suspensions

(a) Viable count (*Spread plate method)*

Ten-fold dilutions of the spore suspension were plated out on S-CDA; and from the number of colony forming units / spore count, the total number of spores in the undiluted spore suspension was determined.

(b) Total count (*Haemocytometer method*)

Appropriate dilutions from (a) above were placed onto a haemocytometer slide with cover slip and covered with a petridish lid to prevent evaporation of the suspension. After 5-7 min, when the cells settled in the square, the slide was viewed under the Microscope

with high power objective. Cells were counted, on five large squares in the following pattern; total number of spores in the original suspension was calculated by the formula:

Calculation:

Total number of large squares	= 25
Each large square has	= 16 smaller squares
Volume of 1 large square	= 0.2 x 0.2 x 0.1 = 0.004 mm^3 = 0.000004 ml
Volume of 5 large squares	= 0.00002 ml = 0.02 µl

$$\text{Cell count / ml} = \frac{\text{Number of cells counted x dilution factor}}{\text{Volume counted}} \times 10^3$$

[10^3 = conversion factor for µl to ml]

(c) Absorbance

The absorbance of a sample of known count of spores was measured at 500 nm and the relation of A_{500} to the spore count was ascertained.

2.2.6 Screening for halotolerance

The *Penicillium* cultures were spot inoculated onto CDA plates containing NaCl concentrations of 0, 2.0, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0 % (w/v). Incubation was done at 30°C for growth and measured in terms of colony diameter up to 7 days. The grown cultures were re-streaked on the same medium for three sub-cultures.

2.2.7 Screening for MTC of metals on growth of isolates

2.2.7.1 MTC of metal on growth on solid medium

Metal stock solutions were prepared as given in Appendix C. The isolates were screened for tolerance to metals by inoculation of 10^3 spores on S-CDA medium containing 0 – 10 mM Pb^{2+} as $Pb(NO_3)_2$, 0 – 5.0 mM Cu^{2+} as $CuSO_4.5H_2O$, 0 – 5.0 mM Cd^{2+} as $3CdSO_4.8H_2O$ and as $Cd(NO_3)_2.4H_2O$, 0 – 50 mM Fe^{2+} as $FeSO_4.7H_2O$ and 0 – 150 mM Mn^{2+} as $MnSO_4.H_2O$ and monitored for growth qualitatively by visual comparative assessment in terms of colony size and intensity of growth.

2.2.7.2 MTC of metal on growth in liquid medium

Penicillium spores (10^7) were inoculated into Czapek's Dox Broth (CDB) medium (100 ml) containing increasing metal concentrations as in 2.2.6 and kept for growth for 3d on a rotary shaker at room temperature (RT) of 30-32°C. The culture broth was centrifuged on Remi C-24 Model 248 at 8000 rpm equivalent of 5438 g for 20 min. The packed cell volume (pcv) and corresponding wet weight was noted, the dry weight obtained by drying at 50-60°C overnight and then kept in a dessicator and checked till constant dry weight was obtained.

2.3 Results

2.3.1 Isolation and purification of halotolerant metal resistant cultures

Altogether 53 ~~54~~ fungal isolates were obtained as shown in Table 2.1a out of which ten isolates of *Penicillium* were obtained. A total of twelve *Penicillium* cultures (Table 2.1b) were used for the screening work, that is, selection for *Penicillium* isolates with high halotolerance and resistance to metals on solid medium, and selection of representative

morphological characteristics such as monoverticillate, biverticillate symmetric, biverticillate asymmetric and triverticillate. The cultures include two previous isolates, that is, from well (W) water; WP1 and from mangrove (M) area; MP2, additional to two

Table 2.1a: Total number of isolated fungi

Sampling site	Fungi	*Penicillium* isolates
Mangroves - soil	11	0
Mangroves - water	08	02
Solar saltern	34	08
Total number	**~~54~~ 53**	**10**

Table 2.1b: *Penicillium* isolates

Sr.No	Source	*Penicillium* isolates	Morphology
1	Well water	WP1	Biverticillate Symmetric
2	Mangroves	MP2	Biverticillate ASymmetric
3	Mangroves	MP3	Biverticillate ASymmetric
4	Mangroves	MP4	Triverticillate
5	Solar salterns	SP5	Biverticillate Symmetric
6	Solar salterns	SP6	Biverticillate Symmetric
7	Solar salterns	SP7	Biverticillate Symmetric
8	Solar salterns	SP8	Biverticillate Symmetric
9	Solar salterns	SP9	Biverticillate ASymmetric
10	Solar salterns	SP10	Monoverticillate
11	Solar salterns	SP11	Monoverticillate
12	Solar salterns	SP12	Biverticillate Symmetric

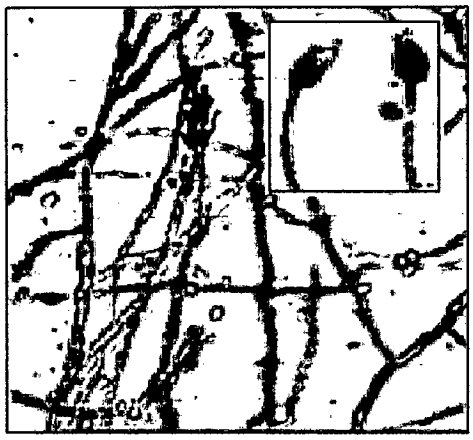
isolates from mangroves water samples (M); MP3 - MP4, and eight isolates from salterns (S); SP5 - SP12.

The isolates showed variations in growth pattern, with SP5 - SP12 having wider colony diameter on CDA than did WP1, MP2 - MP4.

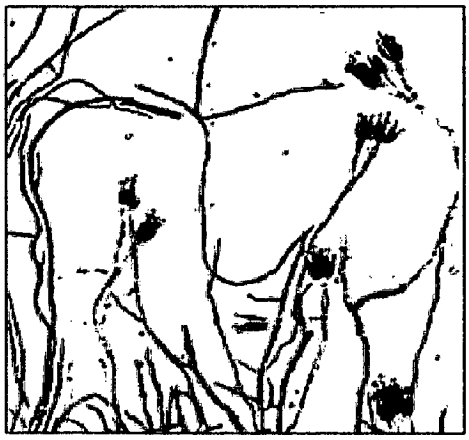
2.3.2 Cultural and morphological characteristics of *Penicillium* isolates

The cultural and morphological characteristics of all 12 *Penicillium* isolates are indicated in Table 2.2. Cultures WP1, MP2 - MP3 showed similar cultural characteristics with rapid growth and the culture on the reverse was off-white producing a diffusible yellowish green pigment with age. Culture MP4 also showed rapid growth, however sporulating till 54h growth and differs in colony characteristics, the culture on the reverse becoming reddish brown with age and greenish yellow at the centre. The cultures from salterns showed similar growth pattern with a wider diameter, however, SP5 - SP7 on the reverse was pale with no pigment production even with age, while SP8 - SP12 showed more rapid spreading growth with a wider diameter than the previous 7 cultures, the cultures on the reverse were off-white, changing to reddish brown with age.

Morphological studies revealed that two isolates SP10 and SP11 were monoverticillate, five isolates MP1, SP5 - SP8 and SP12 were biverticillate symmetric, three isolates MP2, MP3 and SP9 were biverticillate asymmetric and MP4 was triverticillate. Microscopic mounts indicating the different morphological characteristics of the four main forms of *Penicillium* as representative isolates are shown in Fig 2.3.



Monoverticillate



Biverticillate Symmetric

Biverticillate Asymmetric

Triverticillate

Fig 2.3: Morphology of representative isolates:
Monoverticillate (SP10); Biverticillate Symmetric (WP1)
Biverticillate Asymmetric (MP2); Triverticillate (MP4)

Table 2.2: Cultural and Morphological characteristics of WP1 - SP12

Isolates	Cultural	Morphological
WP1	Rapid growth, blue green spores sporulated in 2d, colonies flat, dry, velvety, plate reverse is pale with a tinge of yellowish green pigment.	Mycelium branching and septate, conidiophores erect, septate at the apex with a verticel of erect primary branches borne directly on the conidiophore i.e., one staged branch, phialides in groups of 2-3, conidia borne in chains forming a typical brush like spore bearing heads with phialides gone in clusters. Penicilli is Biverticillate-Symmetric.
MP2	Rapid growth, blue green spores sporulated in 2d, colonies flat, dry, velvety, plate reverse is pale with yellowish green pigment.	As in WP1, however the verticel of erect primary branches bore a verticel of secondary branchlets (metulae) i.e., two staged branch, metulae in terminal whorls of 2-4 members even within the whorl. Penicilli is Biverticillate-Asymmetric.
MP3	Rapid growth, blue green spores, colonies cottony, plate reverse is pale with yellowish green pigment.	As in MP2, metulae in whorls of 3 members even within the whorl, penicilli few in number, Biverticillate-Asymmetric.
MP4	Rapid growth, sporulation in 3d, colonies cottony with raised central areas, blue green at the centre to creamish at the borders, plate reverse is reddish brown with age.	As in WP1, however the verticel of erect primary branches bore a verticel of secondary (metulae) and tertiary branchlets i,e., three staged branch. Penicilli few in number with phialides in groups of three, Penicilli – Triverticillate.

Table 2.2: Cultural and Morphological characteristics of WP1 - SP12 (contd)

Isolates	Cultural	Morphological
SP5	Colonies cottony with raised central areas, blue green spores central with creamish margins, however sporulating in 3d, plate reverse is pale.	As in WP1, Penicilli Biverticillate-Symmetric, however primary branches are uneven, with phialides in groups of three.
SP6	As in SP5	As in SP5, Penicilli Biverticillate-Symmetric.
SP7	As in SP5	As in SP5, Penicilli Biverticillate-Symmetric.
SP8	Rapid spreading growth more than previous 7 cultures, mycelium cottony white, turning pinkish with age, plate reverse is pale changing to reddish brown with age.	As in WP1, penicilli few, Biverticillate-Symmetric, with phialides in groups of 4.
SP9	As in SP8, however colonies are creamish turning pinkish, plate reverse is creamish changing to reddish brown with age.	As in MP2, phialides in groups of 3-4 members, penicilli few in number, Biverticillate-Asymmetric.
SP10	As in SP8	As in WP1, however conidiophore is unbranched with no metulae, Penicilli Monoverticillate, few with phialides in groups of 3-4.
SP11	As in SP8, however plate reverse is pale changing to reddish brown with age.	As in SP10.
SP12	As in SP11	As in MP2, phialides in groups of 3-4 members, penicilli few in number, Biverticillate-Asymmetric

2.3.3 Sample analysis

2.3.3.1 pH

The pH of the soil and water samples from the mangroves region, salterns and well water are shown in Table 2.3.

2.3.3.2 Salinity

Salinity tests of the water samples of mangroves, solar salterns and the well water are indicated in Table 2.3.

2.3.3.3 Metals

Tests for the presence of lead, copper, cadmium, iron and manganese from the water samples collected are shown in Table 2.3.

Table 2.3: Salinity and metal concentration in water samples

Source	pH	Salinity (% NaCl)	Metal (ppm)				
			Pb^{2+}	Cu^{2+}	Cd^{2+}	Fe^{3+}	Mn^{2+}
Mangroves soil	7.5	ND	nd	0.865	0.0	0.019	0.086
Mangroves water	7.5	3.16 %	3.315	0.241	1.034	1.9	0.425
Solar salterns	7.2	15.4 %	1.817	0.182	0.567	0.075	0.378
Well water	7.5	0.04 %	0.12	0.046	nd	nd	0.076
Salt (1%)	ND	ND	0.018	0.022	nd	nd	0.116

nd: not detected; ND: Not done

2.3.4. Corelation of methods for determination of spore count

Table 2.4 shows the spore count as determined by the viable count, which registered an approximate hundred fold higher total count, the corresponding absorbance of which was noted. A viable count of 10^6 was equivalent of an absorbance of 0.1 at 500 nm.

Table 2.4 shows the spore count as determined by the viable count, which registered an approximate hundred fold higher total count, the corresponding absorbance of which was noted. A viable count of 10^6 was equivalent of an absorbance of 0.1 at 500 nm.

Table 2.4: Corelation of absorbance of spore suspension to total count and viable count

Culture	A_{500}	Total count	Viable count
WP1	0.220×10^2	3.33×10^{10} spores ml^{-1}	6×10^8 cfu ml^{-1}
MP2	0.175×10^2	1.19×10^{10} spores ml^{-1}	5×10^8 cfu ml^{-1}
MP4	0.165×10^2	1.14×10^{10} spores ml^{-1}	4.7×10^8 cfu ml^{-1}
SP10	0.150×10^2	0.44×10^{10} spores ml^{-1}	1.8×10^8 cfu ml^{-1}

2.3.5 Screening for halotolerance

The growth of all isolates in presence of increasing concentrations of sodium chloride showed a lag in growth (Fig 2.4) and later as a decrease in sporulation. All the cultures could grow in absence of salt; however, optimal growth of WP1 and SP5 - SP12 was obtained at 0 - 2 % salt, growth of WP1 and SP10 was enhanced in presence of 2 % salt, while that of MP2 - MP4, was optimal at 2 - 5 % salt.

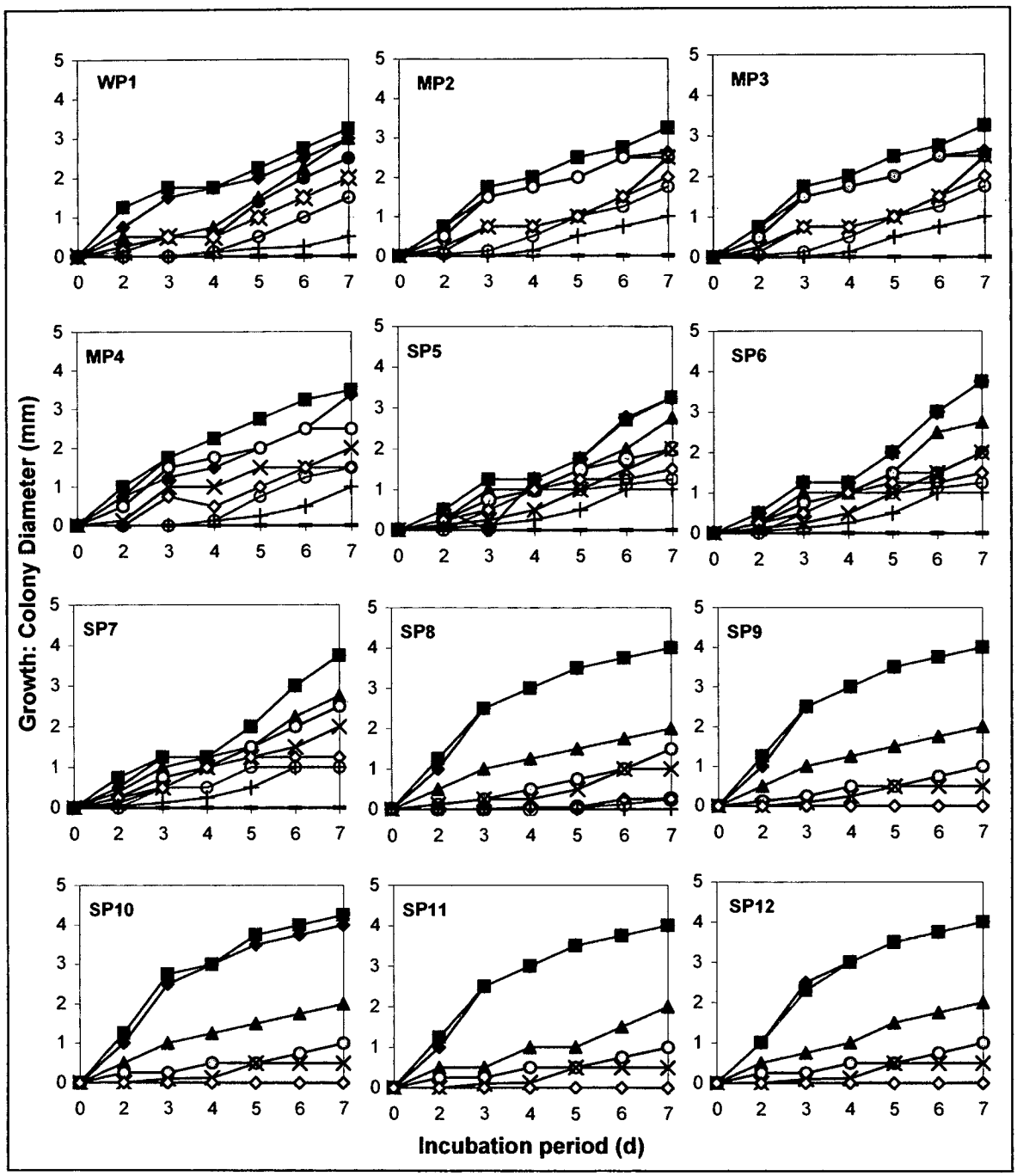


Fig 2.4: Day-wise growth of cultures in presence of increasing concentrations of salt (NaCl) at: ◆ 0%, ■ 2%, ▲ 5%, ⊖ 7.5%, x 10%, ◇ 12.5%, ○ 15%, + 17.5%, - 20%

Maximal tolerance level of WP1, MP2 - MP4, SP5 - SP7 to salt concentration was at 17.5 %, of SP8 was 15 %, while SP9 - SP12 tolerated upto 10 % salt (Fig 2.5). The decrease in growth with respect to increasing concentrations of saline upto 17.5 % NaCl was gradual in WP1, MP2 - MP4, and SP5 - SP7, while a rapid 50 % biomass reduction was observed in the remaining isolates from the salterns (SP8 - SP12) with every 2.5 % increase in salt concentration.

2.3.6 Screening for MTC of metals in solid media

The growth on medium containing increasing concentrations of metals was measured by a qualitative visual comparative assessment in terms of units. The growth of the cultures at increasing concentrations of metals is shown in Fig 2.6 and MTC of heavy metals to the cultures are indicated in Fig 2.7 and Table 2.5.

All cultures were resistant to Pb^{2+} at a concentration of 7.5 mM, while most could also tolerate either Cu^{2+} and/or Cd^{2+} as sulphate or as nitrate salt, with MP3 and MP4 showing resistance to all the heavy metals tested. WP1, MP2 - MP4, and only one from salterns, SP6, tolerated upto 3 mM Cu^{2+}; MP4 could resist upto 5 mM Cu^{2+}. WP1, SP5 and SP11 could resist Cd^{2+} as $CdNO_3$ but not as $CdSO_4$ while MP2, MP4 and SP10 could resist Cd^{2+} only as $CdSO_4$; two isolates, MP3 and SP7 could resist Cd^{2+} both as $CdNO_3$ and $CdSO_4$. SP8, SP9 and SP12 could not tolerate either Cu^{2+} or Cd^{2+} (Fig 2.7).

Further, while 50 % of the isolates could resist upto 30 mM Fe^{3+} (MP4 upto 20 mM only), 50 % showed tolerance level upto the maximum of 50 mM Fe^{2+} tested, beyond which it precipitates. Similarly all cultures showed extreme tolerance, to a minimum of

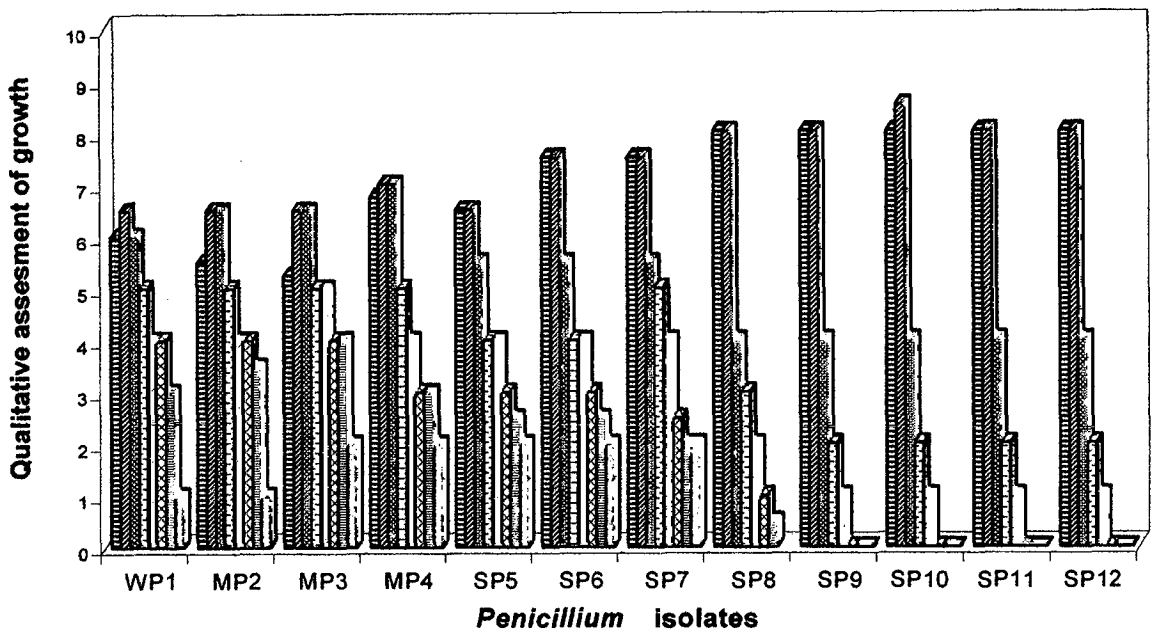


Fig 2.5:Growth of the cultures in presence of NaCl at:
0%, 2%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5%

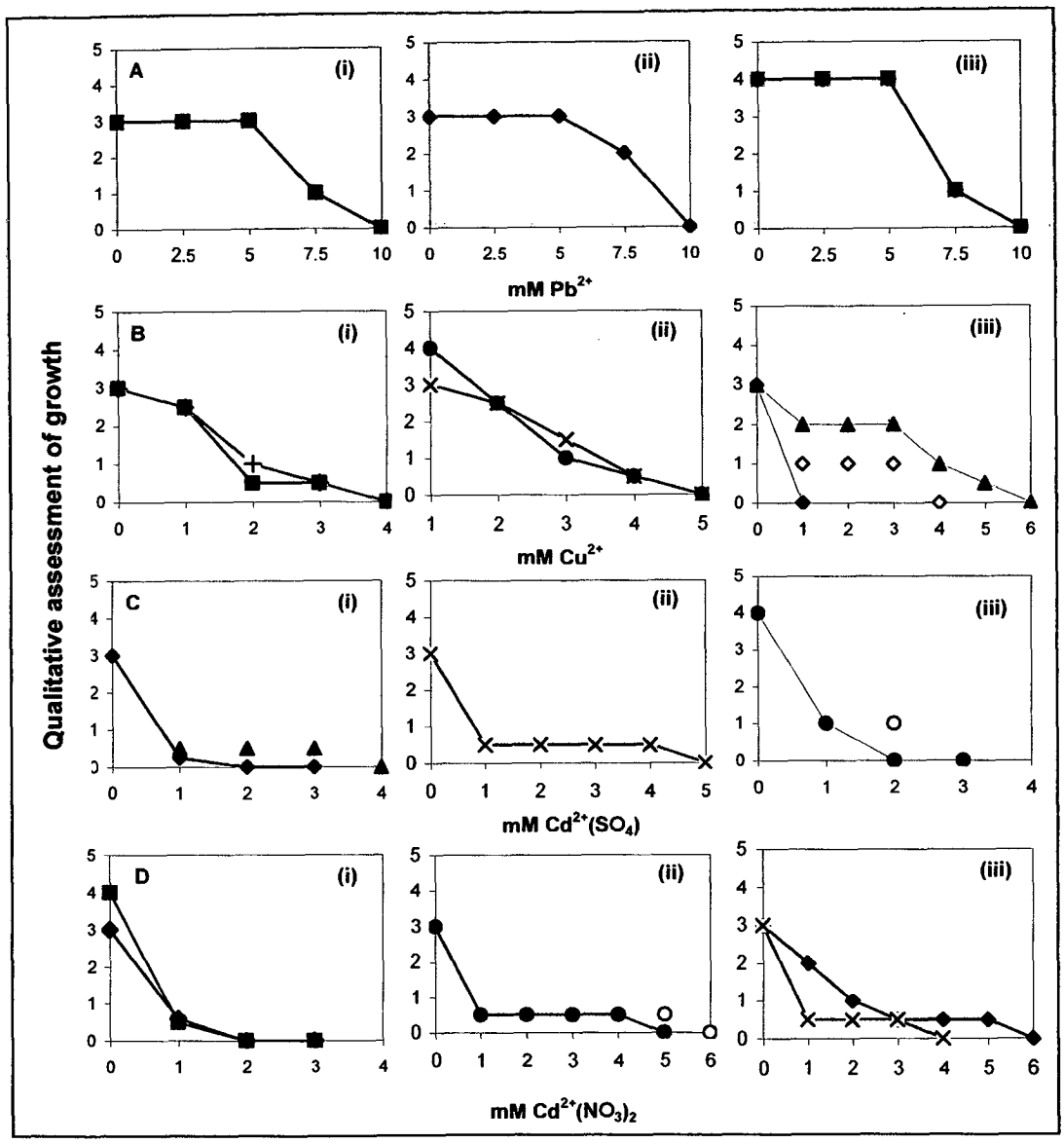


Fig 2.6: Growth of cultures after 3d on CDA medium containing increasing concentrations of metals:

A: Pb^{2+}: of cultures (i): ■ WP1/MP2/MP3/SP5-SP7; (ii):◆ MP4; (iii): ● SP8-SP12

B:Cu^{2+}: of cultures (i): + WP1, ■ SP6; (ii): x MP2, ● SP8; (iii):◆ MP3, ◇ MP3 (7d), ▲ MP4

C: $Cd^{2+}(SO_4)$ of culture: (i):◆MP2/MP3/ SP7;▲ MP3(7d); (ii): x MP4; (iii):●SP10, ○SP10 (7d)

D: $Cd^{2+}(NO_3)_2$ of culture:(i):◆ WP1, ■ SP11; (ii): ● MP3/ SP5, ○ MP3/SP5(7d); (iii):◆ MP4, x SP7

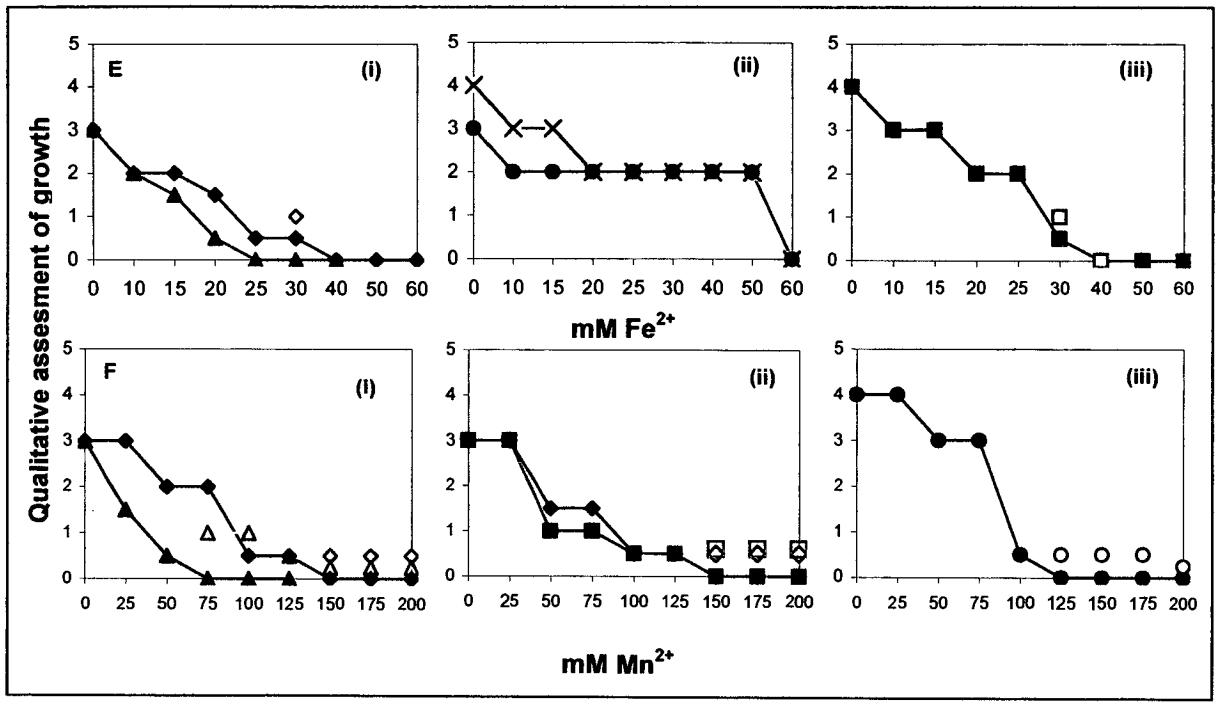


Fig 2.6E: Growth of cultures after 3d on CDA medium containing increasing concentrations of Fe^{2+}:
(i) : ◆WP1/MP2/MP3, ◇WP1/MP2/MP3 (7d), ▲ MP4;
(ii) :● SP5-SP7, x SP8;
(iii) :■ SP9-SP12, □SP9-SP12(7d)

2.6F:Growth of cultures after 3d on CDA medium containing increasing concentrations of Mn^{2+}:
(i) : ◆WP1/MP2/MP3,SP7;◇ WP1(7d) /MP2/MP3/SP7 (10d), ▲MP4;△MP4(10d);
(ii) : ◆SP5,◇ SP5(10d), ■ SP6,□ SP6(10d);
(iii): ●SP8-SP12,○SP8-SP12(10d)

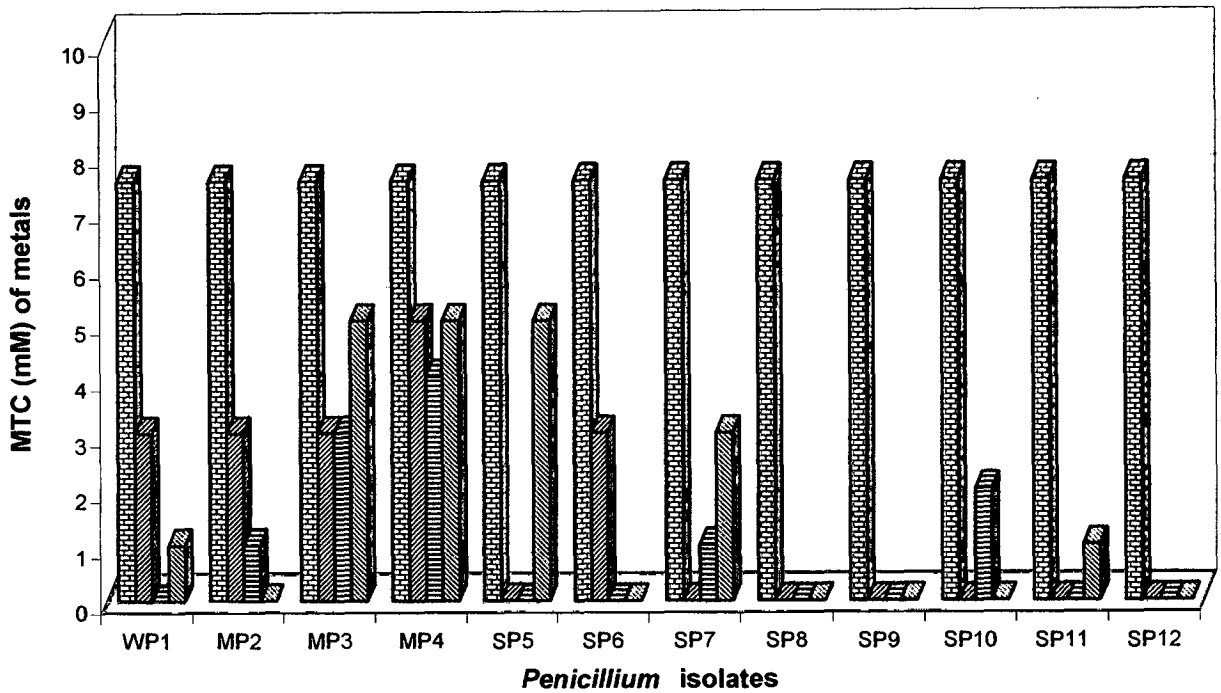


Fig 2.7: MTC of cultures to metals on solid media (7d):
Pb^{2+} $[Pb(NO_3)_2]$, Cu^{2+} $[CuSO_4]$, $Cd^{2+}[CdSO_4]$ and $Cd^{2+}[Cd(NO_3)_2]$

Table 2.5: MTC of metals to WP1- SP12 cultures (7d) on solid media

Metal (mM)	*Penicillium* species											
	WP1	MP2	MP3	MP4	SP5	SP6	SP7	SP8	SP9	SP 10	SP 11	SP 12
	B/S	B/A	B/A	T/A	B/S	B/S	B/S	B/S	B/A	M	M	B/S
Pb^{2+}	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Cu^{2+}	3.0	3.0	3.0	5.0	-	3.0	-	-	-	-	-	-
Cd^{2+} (SO_4)	-	1.0	3.0	4.0	-	-	1.0	-	-	2.0	-	-
Cd^{2+} $(NO_3)_2$	1.0	-	5.0	5.0	5.0	-	3.0	-	-	-	1.0	-
Fe^{2+}	30	30	30	20	50	50	50	50	30	30	30	30
Mn^{2+}	150	150	150	150	150	150	150	150	150	150	150	150

M: Monoverticillate; B/S: Biverticillate Symmetric; B/A: Biverticillate Asymmetric
T: Triverticillate

150 mM Mn^{2+}. However precipitation of metals occurred at 50 mM Fe^{2+} and 150 mM Mn^{2+}, hence the MTC of SP5 - SP8 to Fe^{2+} and all cultures to Mn^{2+} could not be obtained.

2.3.7 Screening for MTC of heavy metals in liquid medium

Four cultures, WP1, MP2, MP4 and SP10 were selected for further study based on their high halotolerance and resistance to metals on solid medium and selection of representative morphological characteristics such as monoverticillate, biverticillate symmetric, biverticillate asymmetric and triverticillate. The day-wise growth of WP1, MP2, MP4 and SP10 on solid medium (CDA) in presence of increasing concentration of metal is shown in Fig 2.8. The lag in growth and decrease in sporulation with respect to increasing metal concentrations was seen. Results revealed that the MTC in liquid medium (Fig 2.9) of Pb^{2+} for WP1/MP2//SP10 is 4 mM, while MP4 is 5 mM. MTC of Cu^{2+} is 2 mM for WP1/MP2, 2.5 mM for MP4 and 0.5 mM for SP10. MTC of Cd^{2+} $Cd(S0_4)$ is 1.0 mM for MP4 and 0.5 mM for MP2/SP10. MTC of Cd^{2+} $Cd(N0_3)_2$ is 0.5 mM for WP1 and 2mM for MP4 (Table 2.6).

Table 2.6: MTC of metals (mM) in liquid medium of reprsentative isolates

Penicillium isolate	WP1	MP2	MP4	SP10
Pb^{2+}	4.0 mM	4.0 mM	5.0 mM	4.0 mM
Cu^{2+}	2.0 mM	2.0 mM	2.5 mM	0.5 mM
$Cd^{2+}(CdS0_4)$	-	0.5 mM	1.0 mM	0.5 mM
$Cd^{2+}(CdN0_3)_2$	0.5 mM	-	2.0 mM	-

Note: Fe^{3+} and Mn^{2+} precipitated at 50 mM and 150 mM respectively and could not be tested higher

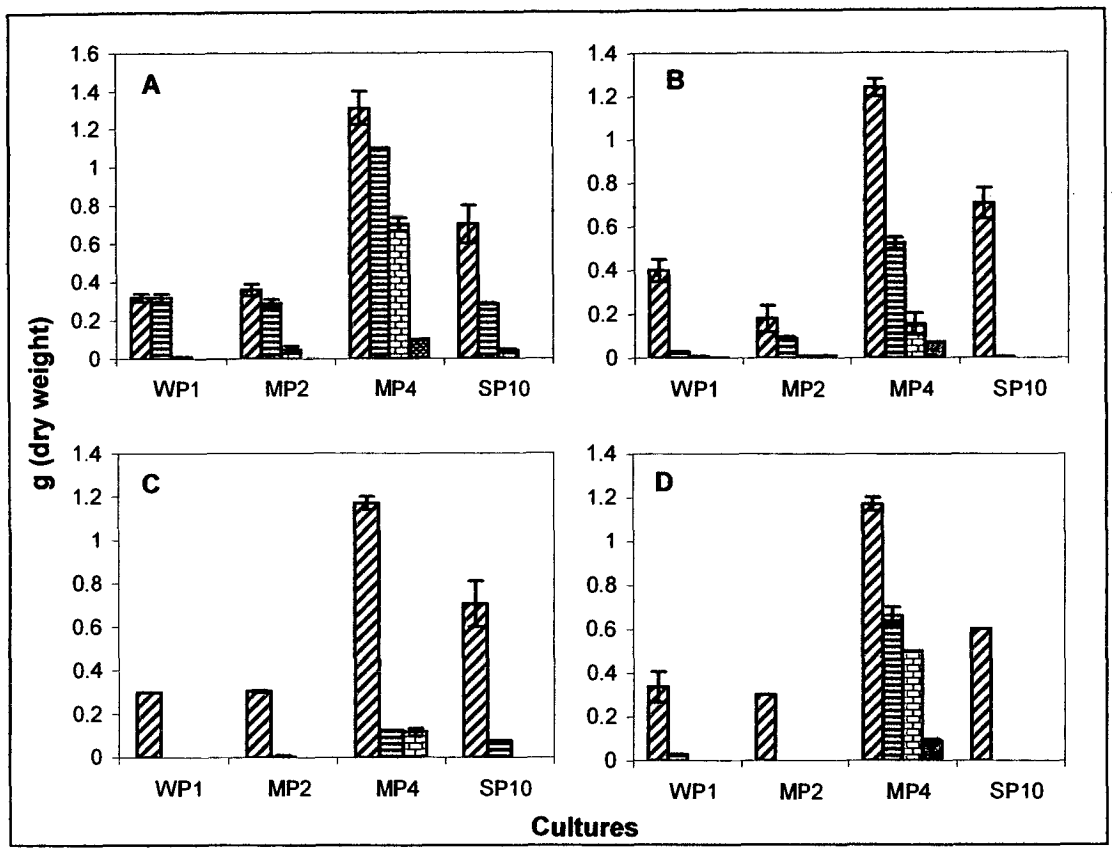


Fig 2.9: MTC of heavy metals: Growth of WP1, MP2, MP4 and SP10 in liquid medium (CDB) in presence of increasing concentrations of:

A: Pb^{2+} at: ▨ 0mM, ▤ 2.0mM and ▦ 4mM ■ 5mM

B: Cu^{2+} at: ▨ 0mM, ▤ 0.5mM, ▦ 1mM and ■ 2mM

C: $Cd^{2+}(SO_4)$ at: ▨ 0mM, ▤ 0.5mM and ▦ 1mM

D: $Cd^{2+}(NO_3)_2$ at: ▨ 0mM, ▤ 0.5mM, ▦ 1mM and ■ 2mM

2.4 Discussion

The cultural characteristics of the *Penicillium* isolates revealed that WP1, MP2 - MP4 have smaller colony diameter, growth diameter of SP5 - SP7 colonies were more and SP8 - SP12 showed rapid spreading growth more than the previous 7 cultures. The rate of radial growth differs for different fungi (Delpech, 2004), and within isolates of the same genus.

All the 12 *Penicillium* isolates obtained from the mangroves and salterns of Goa were extremely halotolerant, able to grow in absence of salt and at a high concentration of 10 - 17.5 % NaCl. All cultures grew well at 0% NaCl. Of these, WP1 the isolate obtained from well water (close to the mangrove region) and MP2 – MP4 from the mangroves tolerated upto 17.5 % NaCl. A striking observation was that only three of the isolates from salterns showed a 17.5 % tolerance level, while the rest showed a lower halotolerance of 10 % and one at 15.0 % NaCl, although the NaCl content was lower than the salterns. Fungi, including yeasts, are well known for their ability to adapt to environments of high osmolarity and respond to osmotic stress by accumulation of predominantly polyols, such as glycerol; these solutes may protect enzymes and other cellular components from high salt concentrations (Park and Gander, 1998).

Cellular adaptation to extreme environmental conditions, such as high-saline environments, is a fundamental biological process needed for survival and growth of the organisms (Gadd et al., 1984; Da Costa et al., 1989; Park and Gander, 1998). Zalar et al

(2005) also reports the isolation of halotolerant black yeast and not halophilic cultures from salterns. A similar tolerance to high salt concentration of 25% NaCl by *Penicillium* spp has been recorded by Jennings et al (1996). With increase in NaCl concentration, a lag period and subsequent decrease in growth was observed for all cultures. Growth of fungi is known to be inhibited by increasing salinity (Mulder et al 1989). According to Park and Gander (1998), it is generally observed that *Penicillium* species survive in environments containing high concentrations of carbohydrates, protein degradation products and salts. Their data records that *P. fellutatum* survives and grows at a reduced rate in an environment containing 3 M NaCl.

Further, WP1, MP2, MP3 - MP4 and SP10 showed some halophilism, growth being better in presence of 2 % salt, with MP2 - MP4 growing better in 2 – 5 % salt; the rest of the cultures grew equally well without or with 2 % salt. Kogej et al (2005) reported a similar observation wherein growth of *Hortaea werneckii* was improved upon addition of 5% NaCl, while even the minimal addition of NaCl to the growth medium slowed down the growth rate of *Aureobasidium pullulans*, confirming their halophilic and halotolerant nature respectively. Although salts are required for all life forms, halophiles are distinguished by their requirement of hypersaline conditions for growth (DasSarma, 2001). The optimal growth of some isolates at salt concentrations of 2 % and more could indicate these isolates to be of marine origin.

The isolates were seen to have a good resistance to the heavy metals tested, with resistance to lead being common to all and at a high level of 7.5 mM, while most could

also tolerate either Cu^{2+} and/or Cd^{2+} as sulphate or as nitrate salt. Fungi isolated from coal mining environments of Santa Catarina were also reported to be resistant to one metal but not to the other, that is, resistance to Cd^{2+} was observed, and none of the isolates presented resistance to Cu^{2+}, resistance being associated with the capacity to accumulate these elements (Castro-Silva et al 2003). The genus *Penicillium* is known to tolerate metals. Studies by Levinskaite (2001), showed that 32 isolates belonging to 27 different species of the genus *Penicillium* examined for their tolerance towards heavy metals, such as, cadmium, chromium (Cr^{6+}), cobalt, copper, nickel, and zinc indicated that the range of fungal response to the metals was quite wide, *P. funiculosum, P. italicum* and *P. viridicatum* being tolerant to most of the tested metals.

It was also noted that in general, the mangrove isolates showed higher resistance than isolates from salterns, to copper and cadmium as cadmium sulphate, while resistance to cadmium as its nitrate salt varied amongst the isolates. The metal toxicity is affected also by the form in which they exist in the medium, the amount of cells in the medium and the stage of growth of the cell culture (Zlatarov and Yakimov, 2001). Different salts of the same metal do not have equal toxicity, the sulphates of heavy metals being much less toxic than the nitrates or chlorides, determined by the product of the normality and conductance ratio (Jones, 1935), however, it was noted that some isolates were resistant to the nitrate salt but not to the sulphate, where possibly the nitrate helped to support growth. The MTC of Cd as nitrate or sulphate salt, by WP1, MP2 and SP10 is 0.5mM (154μg ml^{-1}) and for MP4 is 1-2 mM (308-616μg ml^{-1}). The detected resistance levels to Cd were similar to those reported by Ahmad et al (2006). Their results showed that metal tolerance by *Aspergillus* and *Penicillium* in term of minimum inhibitory concentration

(MIC) is 125-550 μg ml^{-1} for Cd (~~Ahmad et al.~~, ~~2006~~). MTC of the *Penicillium isolates* to lead is 7.0 mM and copper is 3.0 mM. Kowshik and Nazareth reported MTC of *Fusarium solani* to lead as 10 mM, and to copper is 3.0 mM. It was also noted that the resistance level in solid media was higher than the liquid media, the distribution of metal in liquid media being uniform by constant agitation throughout, may have resulted in lower tolerance to the metal. Yilmaz (2003) also reported that inhibitory concentrations in solid media were higher than those in liquid media.

The tolerance to metals by these *Penicillium* isolates indicates pollution of the sites used for sampling. Heavy metal salts released into the environment from industrial wastes form aqueous solutions (Hussein et al., 2003) causing the environment to become rich in heavy metals selecting only the microorganisms which are able to survive under these conditions. The exposure of organisms to metals leads to the establishment of a resistant or tolerant microbial population. ~~Thus, the naturally selected organisms represent an important strategy to obtain agents for bioremediation process (Wood and Wang, 1983).~~ All the sites used for sampling lie along the route of heavy road and water traffic, the latter consisting especially of barges carrying iron ore as indicated in Fig 2.1a. Iron ores are known to contain manganese and lead (varying with the ore, as detailed in Chapter 4); wind-blown dust and / or spillage would cause contamination of the waters. In addition, lead has been commonly used in automobile fuels, and in fungicides in agriculture, paint and water pipes, which would find its way into these waters. Copper contamination could arise from industrial or agricultural pollution, and from corrosion of water pipes, while cadmium is found naturally in small quantities in air, water and soil which are released into the air when household or industrial waste, coal or oil are burned, and from cigarette

smoke and car exhaust. The air-borne cadmium spreads with the wind and settles onto the soil or surface water as dust, tending to sink in water.

The tolerance to metals also differs amongst the *Penicillium* isolates studied indicating a difference in the resistance level between morphotypes of the same genus. It was interesting to note that the triverticillate isolate (MP4) was not only resistant to all the heavy metals tested, but that it also showed the highest tolerance levels amongst all the isolates, especially to the more resistant metals, copper and cadmium.

The diversity of microorganisms in hypersaline environments is of growing interest. Fungal cultures which are able to grow under extreme environment offer good potential as indicators of pollution and as biosorbents (DasSarma, 2001), and in application for bioremedial measures, naturally selected organisms representing an important strategy to obtain agents for bioremediation process (Wood and Wang, 1983). The results obtained by Ahmad et al (2006) revealed that fungi from metal-contaminated soil have high level of metal tolerance and biosorption properties.

Many natural geological formations, such as petroleum reserves, are associated with hypersaline brines. Industrial processes also use salts and frequently release brine effluent into the environment. These extremely halotolerant *Penicillium* isolates that are able to grow at high concentrations of salt and possessing a high resistance to heavy metals have a very high potential to be used as agents for abatement of pollution in hypersaline conditions or in waters of fluctuating salinity, as well as in non-saline environments.

2.5 Conclusions

Altogether ~~54~~ 53 fungal isolates were obtained out of which ten isolates of *Penicillium* were identified and selected. A total of twelve *Penicillium* cultures were used for the screening work; two previous isolates, one from well water, WP1 and the other from mangroves, MP2, present isolates: from mangroves, MP3 - MP4 and eight isolates from salterns, SP5 - SP12. Two isolates SP10 and SP11 were monoverticillate, five isolates WP1, SP5 - SP8 and SP12 were biverticillate symmetric, three isolates MP2, MP3 and SP9 were biverticillate asymmetric and MP4 was triverticillate. All the cultures showed extreme tolerance upto 10-17.5 % NaCl and could grow in absence of salt as well; hence they are halotolerant cultures. Optimal growth of ~~WP1 and~~ SP5 - SP12 was obtained at 0 - 2 % salt, growth of WP1 and SP10 was enhanced in presence of 2 % salt, while that of MP2 - MP4, was optimal at 2 - 5 % salt indicating slight halophilism. Maximal tolerance level of WP1, MP2 - MP4, SP5 - SP7 to salt concentration was 17.5 %, of SP8 was 15 %, while SP9 - SP12 tolerated up to 10 % salt.

Studies on the metal tolerance of the cultures to Pb^{2+}, Cu^{2+} and Cd^{2+} revealed that all cultures were resistant to Pb^{2+} at a concentration of 7.5 mM, while most could also tolerate either Cu^{2+} and / or Cd^{2+} as sulphate or as nitrate salt, with MP3 and MP4 showing resistance to all the heavy metals tested. The lag in growth and decrease in sporulation with respect to increasing metal concentrations were also seen in presence of metals. Further, 50 % of the isolates could resist upto 30mM Fe^{2+} (MP4 upto 20 mM only) while the remaining 50 % showed tolerance level upto 50 mM Fe^{2+}. Similarly, all

cultures showed extreme tolerance, a minimum of upto 150 mM Mn^{2+}, however precipitation of metals occurred at 50 mM Fe^{2+} and 150 mM Mn^{2+}, hence the tolerance levels beyond these concentrations could not be obtained.

Four representative isolates, that is, WP1, MP2, MP4 and SP10 were selected for the work based on morphological characteristics such as monoverticillate, biverticillate symmetric, biverticillate asymmetric, triverticillate, and their high halotolerance and resistance to metals on solid medium. The MTC of the metals on growth of the selected cultures revealed that the MTC in liquid medium of Pb^{2+}, Cu^{2+}, Cd^{2+} as $CdS0_4$ and as $CdN0_3$ for WP1 / MP2 / MP4 / SP10 ranged from 0.5 mM - 4 mM.

CHAPTER 3: RESPONSE TO HEAVY METAL STRESS

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3.1. Introduction

The natural affinity of biological compounds for metallic elements contributes towards economically purifying metal-laden wastewater. Bacteria, yeasts, algae and fungi exhibited particularly interesting metal binding capacities, and these microbial cells or biomass can be used for removal or recovery of metals from wastes or aqueous solutions (Vieira and Volesky, 2000). The development of industrial biosorption technologies needs the identification of the physical and chemical factors regulating the metal sorption (Fourest et al, 1994), the mechanisms associated with biosorption being affected by several factors such as temperature, pH, cell wall functional groups, growth medium, initial metal ion concentration, biomass concentration, proteins, presence of calcium ions and the pretreatment of biomass (Paknikar, et al 1993; Modak and Natarajan, 1995; Vieira and Volesky, 2000).

According to Modak and Natarajan (1995), it is not possible to generalize the mechanisms of uptake, therefore, each factor has to be defined individually for each biomass and metal ion pair. Differences in the mechanism of biosorption even in related species were observed in bacteria (Vance, 2000; Volesky, 1999). The (sorbed) metals may be cell bound as well as accumulated intracellularly (Balakrishnan et al., 1994; Kowshik and Nazareth, 1999). Cultural and morphological changes (Venkateswerlu et al., 1989; Kowshik and Nazareth, 2000; Ram et al., 2004; VanKuyk et al., 2004; Fomina et al., 2005), stress proteins (Gardea-Torresdey, et al., 1998; Hall, 2002; Courbot, et al., 2004) and plasmids (Pazirandeh, 1996) are also induced in response to metal.

There are few reports on plasmid and plasmid-mediated resistance in fungi, however, a study of plasmid in the *Penicillium* species, and in particular, plasmid mediated resistance to heavy metals by *Penicillium* species has not been reported.

The chapter describes the response to heavy metal stress of lead nitrate, copper sulphate, cadmium nitrate and cadmium sulphate by selected representative isolates. It deals with the factors affecting sorption of metals by the isolates, their sorption isotherms, studies on metal uptake and cellular localisation of the sorbed metals. It also describes the changes in cultural and morphological characteristics, the whole cell protein profiles for induction or repression of proteins, and the plasmid bands in response to metals.

This chapter is divided into four sections-

3A. Optimization for growth of the cultures and metal sorption.

3B. Sorption isotherms and metal sequestration.

3C. Cultural and morphological studies

3D. Whole cell protein profiles and plasmid response

Each section details the methodology followed by the result and discussion.

3A. OPTIMISATION OF CONDITIONS FOR GROWTH OF THE CULTURES AND METAL SORPTION BY THE MYCELIAL BIOMASS

3A.2 Materials and methods

3A.2.1 Growth media

The growth media (Appendix A) used in the study were the following, each with 2 % NaCl added:

(a) Czapek Dox Broth (S - CDB)

(b) Redefined Czapek with glucose instead of sucrose (S - CDB*.G)

(c) Czapek Dox Broth + Glucose (S - CDB+ G)

(d) Mineral salts with glucose medium (S - MG)

The effect of growth media was studied with respect to biomass production and lead sorption. The involvement of the cell surface compound (SC) in metal sorption is also checked, therefore SC production in terms of its constituent carbohydrate, protein and lipid was estimated.

a) *Growth of the culture*

The test culture studied for this purpose was WP1.

Spores (10^6) were inoculated into 60 ml growth medium as given above (a) – (d) and incubated at RT of 30°C under shaker conditions. The mycelial mass obtained was centrifuged to obtain the packed cell volume (pcv) and corresponding wet weight was determined. The dry weight was obtained by drying an aliquote at 50-60° overnight and then kept in a dessicator and checked till constant dry weight was obtained.

b) *SC production*

Extraction of cell surface compound (SC) - The surface compound (SC) was extracted by adding saline to the mycelial mass and 8 - 10 glass beads in a flask that was then shaken at 100 rpm for 15 min. The mycelial mass with the SC removed [M^-] was filtered to obtain the filtrate containing the SC. To the filtrate was added 3 vol of alcohol and kept at 4°C overnight. The precipitated SC was centrifuged at 5000g for 20 min. The supernatant was discarded and the precipitated SC dissolved in 200 microlitres of distilled water. [M^+] was used to denote the intact mycelia with the [SC].

Quantitation of SC: The SC, 50 µl made up to 1ml with distilled water was analysed for its protein, carbohydrate and lipid content.

Proteins - Protein reacts with the Folin-Ciocalteau reagent to give a coloured complex. The colour so formed is due to the reaction of the alkaline copper with the protein and the reduction of phosphomolybdate by lysine and tryptophan present in the protein. The intensity of the colour depends on the amount of these aromatic amino acids present and will thus vary for different proteins.

The protein content of the SC was estimated using Folin-Lowry method (Plummer, 1988). To the sample (1 ml), alkaline $CuS0_4$ solution (5 ml) was added (Appendix B), mixed and allowed to stand at RT for 10 min. Folin-Ciocalteau reagent (0.5 ml) was added (Appendix B), mixed well and incubated at RT in the dark for 30 min to develop colour. The intensity of the blue colour formed was measured spectrophotometrically

against the blank at 660 nm. Bovine serum albumin was used as a standard and the concentration of the sample was determined from the standard curve.

Carbohydrates - In hot acidic medium, glucose is dehydrated to hydroxy methylfurfural. This forms a pinkish colured product with phenol and has an absorption maximum at 490 nm.

Carbohydrate concentrations of the SC were estimated following the phenol-sulphuric acid method of Dubois et al, 1956 (Plummer, 1988). The sample, 1ml, was mixed with 5% aqueous phenol, 1 ml, and sulphuric acid, 5ml (Appendix B). It was shaken and kept on ice for 10 min. The intensity of the yellowish-orange colour formed was measured spectrophotometrically against the blank at 490 nm. Glucose was used as a standard and the concentration of the sample was determined from the standard curve.

Lipid - Lipid estimation was done by stearic acid method (Pande, 1963) with slight modification. The principle involved is the oxidation of lipid with acid dichromate. The oxidation reaction is followed by a decrease in the dichromate colour (micromethod). Therefore, the extinction of the reaction medium has an inverse relationship based on the decrease of dichromate colour.

The precipitate was extracted from the SC (100 μl) with the mixed organic solvent (chloroform: methanol: water = 1: 2: 0.8 v/v). The tube containing the sample was pre-cooled in cold water for at least 1 min; then, 2 ml of chloroform and 2 ml of distilled water were added. This was shaken well in a 50 ml capacity separating funnel and the lower layer (chloroform layer) collected in a 25ml capacity rotary evaporater flask and

evaporated to dryness under vacuum and 0.15 % acid dichromate (2 ml) was added. This was vortexed and kept in a boiling water bath for 15 min, then cooled under running tap water and 4.5 ml of distilled water was added and mixed thoroughly. The absorbance of the lipid free blank against the sample was read at 440 nm. Stearic acid of concentration 3 μg of 0.1 % in 95 % ethanol was used as a standard. The concentration was calculated by the formula -

$$\mu g \text{ lipid} = \frac{E \times F}{V}$$

where, E = Absorbance of sample

$F = \frac{C}{E}$ where, C = concentration of std; E = absorbance of std

V = Total sample

c) *Lead sorption experiments*

5 % (pcv/v) of the saline washed mycelial mass [M^+] and [M^-] were each added to 1mM lead solutions. The flasks were incubated on a shaker at 150 rpm for 30 min and the contents were filtered. Metal controls were maintained. The amount of metal in the filtrate was estimated as follows –

Estimation of Pb^{2+} - The filtrate, 2 ml, was added into 40 ml of deionised water. To the solution was added 1:2 ammonia solution, 25ml (to make the solution ammoniacal) followed by 10% w/v sodium sulphide solution, 0.5 ml. The volume was made up to 100 ml. A blank of deionised water was treated in the same way. The absorbance was measured at 430 nm.

The standard was prepared by dissolving 0.160 g lead nitrate in 100 ml deionised water. 10 ml of this was diluted to 100 ml for a working solution, the latter containing 0.1 mg of Pb ml^{-1} (Vogel, 1978).

3A.2.2. Incubation conditions

The test culture was grown in the selected growth medium and checked for total biomass and [SC] production and maximum metal sorption at the following incubation conditions:

(a) Stationary and shaker conditions (160 rpm) for 48h.

(b) Incubation period of 48h / 72h / 96h each under stationary and shaker conditions (160 rpm).

3A.2.3. Factors affecting sorption

a) Effect of growth period of mycelial biomass on sorption of metal

The effect of growth period of the mycelial biomass was studied with respect to metal sorption. The test culture, WP1 was grown in CDB with 2 % salt (S-CDB) for incubation period of 48h / 72h / 96h each under shaker conditions. 5 % (pcv/v) of the saline washed mycelial mass were each added to 1mM lead solutions. The flasks were incubated on a shaker at 150 rpm for 30 min and the contents were filtered. Metal controls were maintained. The amount of metal in the filtrate was estimated as in 3A.2.1c (Vogel, 1978).

b) Effect of pH on metal removal by *Penicillium* (WP1)

The culture was grown in S-CDB under shaker conditions for 48h. Solutions of pH 3, 5.6, 7.0, 9.0 were prepared in deionised water, the pH adjusted with 0.1N HCl / NaOH. To each pH, metal salts were individually added to give a final concentration of 0.5 mM Pb^{2+}

as $Pb(NO_3)_2$, Cu^{2+} as $CuSO_4.5H_2O$, Cd^{2+} as $3CdSO_4.\ 8H_2O$ and as $Cd(NO_3)_2.4H_2O$. The saline washed mycelium, 1% pcv/v, was added to each and the flasks were incubated for 10 min at RT and 160 rpm. The mycelial mass was filtered off, and the amount of $Pb^{2+}/Cu^{2+}/Cd^{2+}$ in the filtrate was estimated as given below. Controls of mycelia in deionised water, and metal in deionised water were maintained at all pH.

Estimation of lead was done in 3A.2.1c

Estimation of copper - To 25 ml of the filtrate, 10% KI solution, 5 ml, was added, and the liberated iodine was titrated with 0.1N sodium thiosulphate solution till brown colour of iodine turns pale. Starch solution, I ml, was then added, and the titration with 0.1N sodium thiosulphate continued till the blue colour starts fading to colourless which is the end point (Vogel, 1978).

Estimation of cadmium - To 25 ml of the filtrate, ammonia solution was added dropwise till the initial precipitate just redissolves. The indicator (solochrome black T, 0.5% w/w) was added and the sample was then titrated with 0.1M EDTA solution. The end point is achieved when the colour changes from red to blue colour (Barnard and Chayen, 1965).

c) Rate of metal biosorption

Mycelial mass, WP1, 1% (pcv / v) was added to 0.5 mM Pb^{2+} solution in de-ionized water at pH 5.6, incubated at RT and 150 rpm and aliquotes removed at 0', 1', 5', 10', 30' (and 60'). The aliquots were filtered and the amount of Pb^{2+} in the filtrate was estimated. A graph of the metal sorbed against time was then plotted.

3A.3. Results

3A. Optimisation for growth of the cultures

3A.3.1 Growth media

The fungus grown in S-CDB + G yielded the maximum mycelial mass (Fig 3.1a), the amount of mycelial mass being measured in terms of pcv (packed cell volume), which is approximately equal to the wet weight. However, the mycelia grown in CDB and CDB*.G produced marginally higher amount of [SC] in terms of protein, carbohydrate and lipid than when grown in S-CDB + G or S-MG medium as well as achieved maximum lead sorption capacity by both [M+] and [M-] (Fig 3.1b). This also indicated the possible involvement of the [SC] in metal sorption. S-CDB was therefore selected for growth of all cultures, as the mineral salts of MG would influence metal sorption by the cells per se

3A.3.2 Incubation conditions

A comparison of stationary and shaker growth conditions for 48 h revealed that the shaker grown culture yielded greater mycelial biomass (Fig 3.2a); metal sorption ml^{-1} pcv was comparable, but with respect to the total biomass obtained, was higher under shaker conditions (Fig 3.2b). A comparison of the growth period under both stationary and shaker conditions showed that the culture grown under shaker conditions yields greater mycelial mass of 0.558, 3.333 and 2.717 gm % (100 ml^{-1} medium) at 48, 72 and 96 h incubation respectively, as compared to the mycelial yield at stationary growth conditions of only 0.141 gm % at 48h and 0.541 % at 72 and 96 h growth (Fig 3.2a), and therefore can achieve more total metal sorption. The culture grown for 48h under shaker conditions

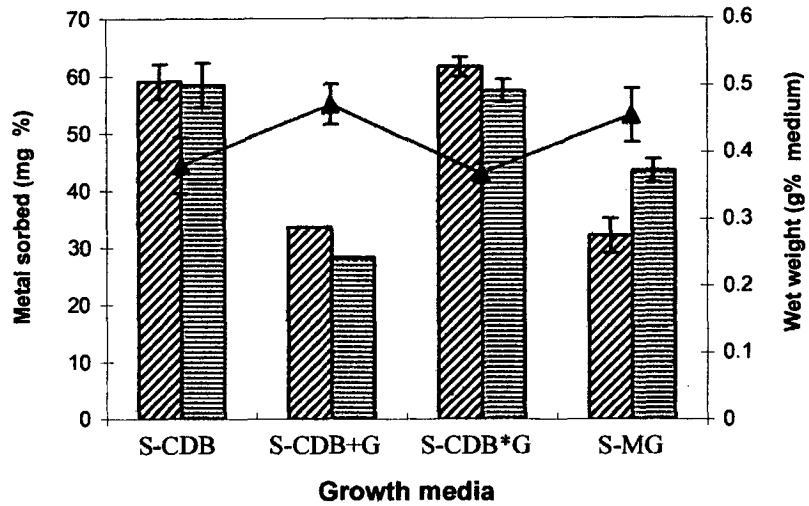


Fig 3.1a: Mycelial mass produced (▲, wet weight) in S-CDB, S-CDB+G, S-CDB*G and S-MG media and metal sorbed by: ▨ M+ and ▤ M-

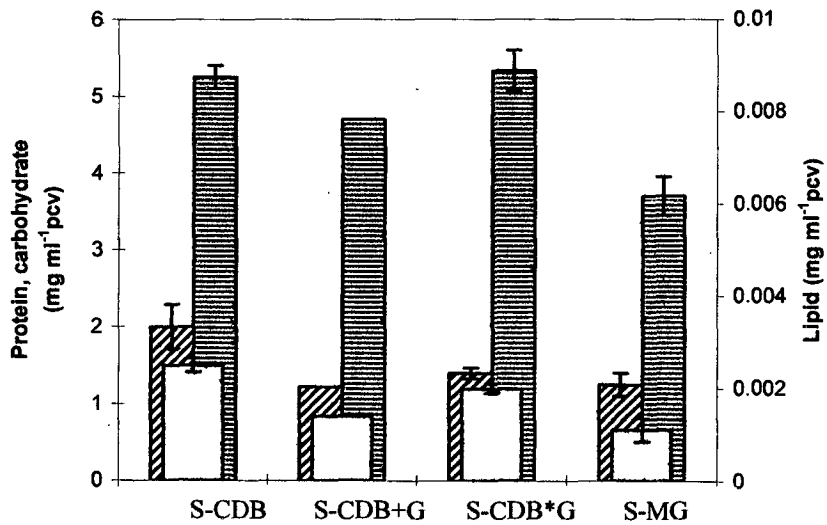


Fig 3.1b: SC production by culture grown in CDB, CDB+G, CDB*G and MG media: ▨ Protein, ▤ carbohydrate and ■ lipid components

yields comparable metal sorption ml^{-1} pcv to the cultures grown for 48h and 72h under stationary conditions (Fig 3.2b). Further growth period decreases the amount of metal sorption by the mycelial biomass with the culture grown under stationary conditions achieving a relatively better sorption of metal than that grown under shaker conditions (Fig 3.2b).

Quantitation of the SC by estimation of carbohydrate, protein and lipid composition revealed that the culture grown under stationary state produced more SC than under shaker conditions, and decreasing thereafter with further incubation during growth with a yield of 3.34 mg protein, 3.965 mg carbohydrate and 5 μg lipid ml^{-1} pcv after 48h under stationary growth conditions, and a decreased yield of 0.98 mg protein, 2.96 mg carbohydrate and 1.4 μg lipid ml^{-1} pcv under shaker conditions of 48h incubation. The 72 h grown cultures produced a lower yield of SC both under stationary as well as shaker conditions with 2.4 mg protein, 2.6 mg carbohydrate and 1.9 μg lipid ml^{-1} pcv under stationary growth conditions, and a lower yield of 0.16 mg protein, 1.265 mg carbohydrate and 0.7 μg lipid ml^{-1} pcv under shaker conditions. When the culture was incubated for an even longer period of 96 h, the SC produced further decreased with only a yield of 1.3 mg protein, 1.0 mg carbohydrate ml^{-1} pcv and almost negligible lipid under stationary growth conditions, and an even further decrease under shaker conditions with only 0.3 mg protein, 0.6 mg carbohydrate ml^{-1} pcv and no lipid. The ratio of carbohydrate is also more than the protein in all cases (Fig 3.2a).

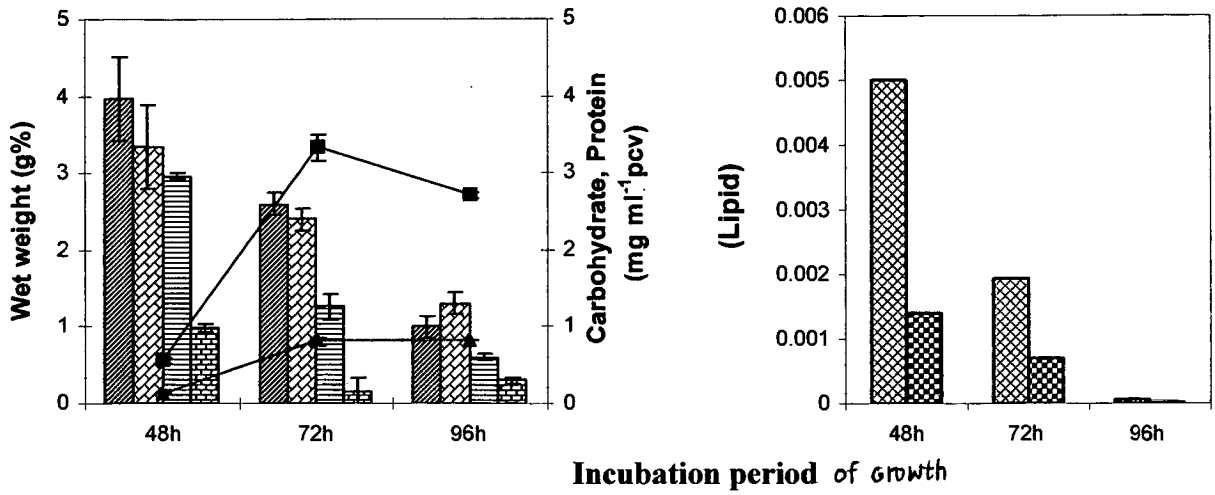


Fig 3.2a: Mycelial mass produced under: ▲stationary, ■shaker conditions and SC production during growth at 48h, 72h and 96h growth:

▨ /▨ : Carbohydrate / Protein under stationary growth conditions

▤ /▤ : Carbohydrate / Protein under shaker growth conditions

▩ /▩ : Lipid under stationary / shaker growth conditions

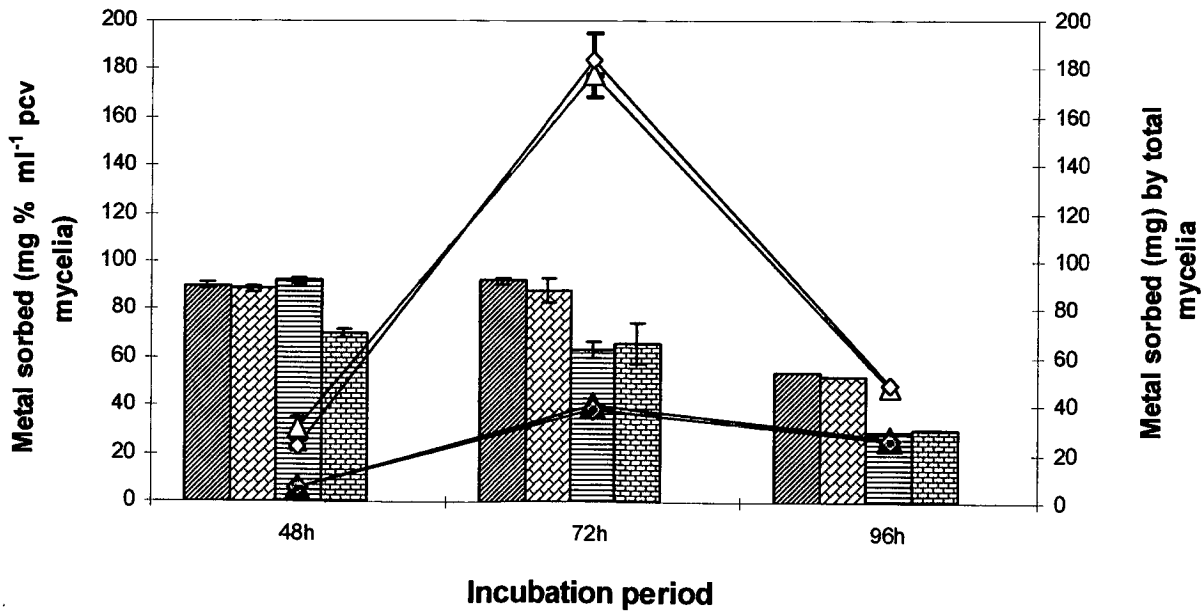


Fig 3.2b: Metal sorption by 48h, 72h and 96h grown culture:

ml^{-1} pcv: ▨M+ (stat), ▨M- (stat), ▤M+(shak), ▤ M-(shak)

by total pcv: ▲ M+ (stat), ◆ M- (stat), △ M+(shak), ◇ M-(shak)

3A.3.3. Factors affecting sorption

a) Effect of growth period of mycelial biomass on sorption of metal

The culture grown for 48h achieved the maximum metal sorption per ml^{-1} pcv achieving 91.95% removal, 72h and 96h grown cultures giving 63.5% and 29.3% removal of metal from solution respectively (Fig 3.3).

b) Effect of pH on metal binding

As seen in the Fig 3.4, Pb^{2+} uptake was maximum between pH 3.0-5.6 (Fig 3.4), and decreases with increasing pH of 7.0 and 9.0 at which point metal precipitation occurs in a 1mM solution. Sorption at pH 9.0 was a little higher than at pH 7.0. The pH selected for experimental purposes was 5.6 as maximum uptake of cadmium was observed at pH 5.6, not much difference between pH 3.0 and pH 5.6 for Pb^{2+} and Cu^{2+} uptake.

c) Rate of metal biosorption

Rate of biosorption increased linearly upto 1min at 11-12 % metal removal by WP1,MP2 and 17.5 % removal by MP4, SP10 and then was steady in case of WP1, MP2, SP10 with maximum metal removal achieved in the first min; MP4 however continued to show a further increase in metal sorption with a decreased rate and a maximum removal of 24.6 % in 30 min. All cultures showed a slight desorption of metal after 30 min (Fig 3.5).

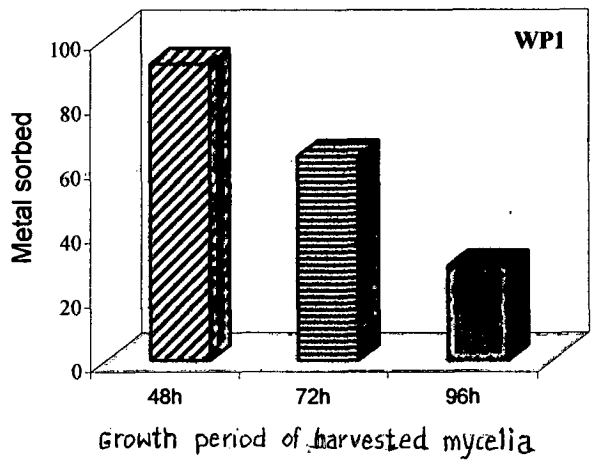


Fig 3.3: Metal sorption by the culture grown for: ▨48h, ▩ 72h and ▦ 96h

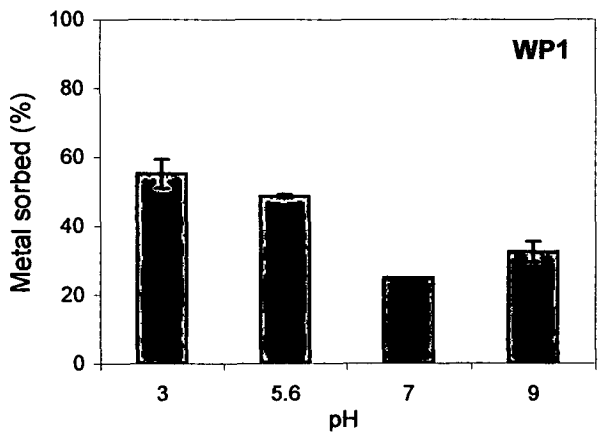


Fig 3.4: Sorption of Pb^{2+} at different pH

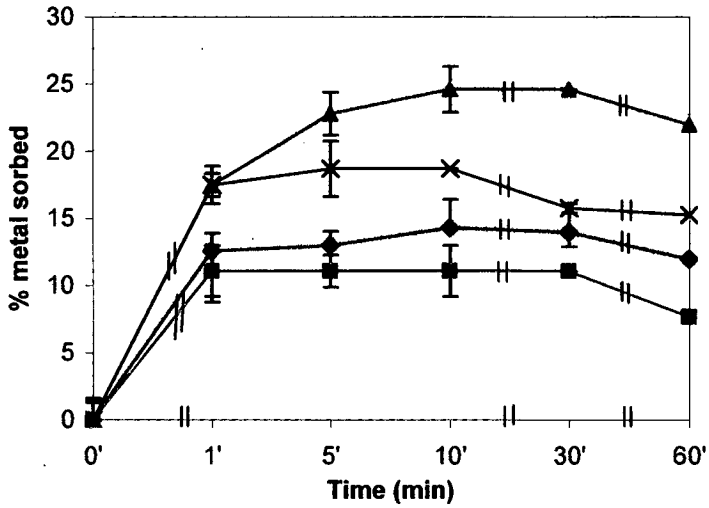


Fig 3.5: Rate of Pb^{2+} removal at pH 5.6 by:
◆ WP1, ■ MP2, ▲ MP4, x SP10

3B. SORPTION AND SORPTION ISOTHERMS

3B.2. Materials and methods

3B.2.1. Sorption isotherms

Equilibrium experiments (Volesky and Holan, 1995) were done to assess the binding capacity of cultures WP1, MP2, MP4 and SP10 to Pb^{2+}, Cu^{2+} and Cd^{2+}. Biomass, 1 % of culture grown for 48h, washed with deionized water and lightly pressed was added to increasing concentrations of heavy metal solutions prepared in de-ionized water as given in (Table 3.1). This was incubated at RT on a rotary shaker at 160 rpm for 24h to allow each metal-biosorbent system to reach equilibrium with the pH maintained at 5.6 throughout. The biosorbent was then filtered off and filtrate was analyzed for residual metal concentration by the formula:

$$q = \frac{V\,(C_i - C_f)\ \text{mg L}^{-1}}{S}$$

where, V = volume of the metal solution (L)
C_i, C_f = initial and equilibrium (residual) metal in solution respectively (mg L^{-1})
S = Biosorbent (g)

3B.2.2. Uptake of metals

a) Metal biosorption

Cultures WP1, MP2, MP4 and SP10 were inoculated in Czapek's Dox broth (CDB) supplemented with 2% salt and grown for 2-3d at RT under shaker conditions at 160 rpm. The grown cultures were filtered and washed with deionized water. 1% mycelial mass of each culture was added separately to 1mM metal solution of Pb^{2+} as $Pb(NO_3)_2$, Cu^{2+} as $CuSO_4$, Cd^{2+} as $Cd(N0_3)_2$ and as $CdS0_4$, Fe^{2+} and Mn^{2+}. Incubation was done for 1h at RT

and at 160 rpm. The mycelial mass was filtered off and the filtrate used for estimation of residual metal to obtain the total metal sorbed.

Table 3.1: Metal concentrations for equilibrium isotherm experiments

Culture	Metal	mM concentration						
WP1	Pb^{2+}	0.0	0.25	0.5	0.75	1.0	1.25	1.5
	Cu^{2+}	0.0	0.5	1.0	1.5	2.0	2.5	3.0
	Cd^{2+} (Nitrate)	0.0	0.25	0.5	0.75	1.0	1.25	1.5
MP2	Pb^{2+}	0.0	0.5	1.0	1.5	2.0	2.5	3.0
	Cu^{2+}	0.0	0.5	1.0	1.5	2.0	2.5	3.0
	Cd^{2+} (Sulphate)	0.0	0.5	1.0	1.5	2.0	2.5	3.0
MP4	Pb^{2+}	0.0	0.25	0.5	0.75	1.0	1.25	1.5
	Cu^{2+}	0.0	0.5	1.0	1.5	2.0	2.5	3.0
	Cd^{2+} (Nitrate)	0.0	0.5	1.0	1.5	2.0	2.5	3.0
	Cd^{2+} (Sulphate)	0.0	0.5	1.0	1.5	2.0	2.5	3.0
SP10	Pb^{2+}	0.0	0.5	1.0	1.5	2.0	2.5	3.0
	Cd^{2+} (Sulphate)	0.0	0.5	1.0	1.5	2.0	2.5	3.0

b) Quantitation of cell-bound and intracellular uptake of metals

The filtered mycelial mass was then treated with 0.1N HCl at 100 rpm for 10-15' to release the cell bound metal. The filtrate was used for estimation of cell bound metal. The filtered, washed mycelial mass was then digested at 100°C with perchloric acid to release the intracellular metal till a clear solution was obtained. Further digestion was done with $HN0_3$ which was heated to dryness. After cooling, the digests were made up to 10 ml in a volumetric flask with de-ionized water. The metal content was determined by Flame atomic absorption spectrophotometry (AAS; Perkin Elmer Model A Analyst 200).

3B.3. Results

3B.3.1. Sorption isotherms

The equilibrium isotherm experiments were done for those metals which could be tolerated by the cultures as shown in chapter 2. As shown in Fig 3.6, the general shapes of the isotherms were similar for all cultures, however the sorptive capacities were significantly different. The uptake capacity of WP1 was highest for Cu^{2+} at 0.6 mmol followed closely at 0.51 mmol by Pb^{2+} then by Cd^{2+} (nitrate) at 0.44 mmol g^{-1} dry weight. For MP2, the uptake capacity was highest for Cu^{2+} at 0.67 mmol, followed by Cd^{2+} (sulphate) at 0.57 mmol, then by Pb^{2+} at 0.5 mmol g^{-1} dry weight. For MP4, the uptake capacity was highest for Pb^{2+} at 0.52 mmol followed closely by Cu^{2+} at 0.5 mmol, then by Cd^{2+} (sulphate) at 0.45 mmol and Cd^{2+} (nitrate) at 0.4 mmol g^{-1} dry weight and for SP10 the uptake capacity was higher for Pb^{2+} at 0.5 mmol than for Cd^{2+} (sulphate) at 0.45 mmol g^{-1} dry weight.

The sorptive capacity for Pb^{2+} is similar, about 0.5 mmol g^{-1} dry weight for all cultures. For Cu^{2+}, the sorptive capacity was highest by MP2 at 0.67 mmol followed by WP1 at 0.6 mmol g^{-1} dry weight. For Cd^{2+} (sulphate) was highest by MP2 at 0.57 mmol followed by MP4 /SP10 both at 0.45 mmol g^{-1} dry weight and for Cd^{2+} (nitrate), the sorptive capacity was highest for WP1 at 0.44 mmol followed by MP4 at 0.4 mmol g^{-1} dry weight.

3B.3.2 Cell-bound and intracellular uptake of metals

All four isolates of *Penicillium* achieved efficient removal of metals such as Pb^{2+}, Cu^{2+}, Cd^{2+} (nitrate) and Cd^{2+} (sulphate), Fe^{3+} and Mn^{2+} (Fig 3.7), the metal sorbed is given in

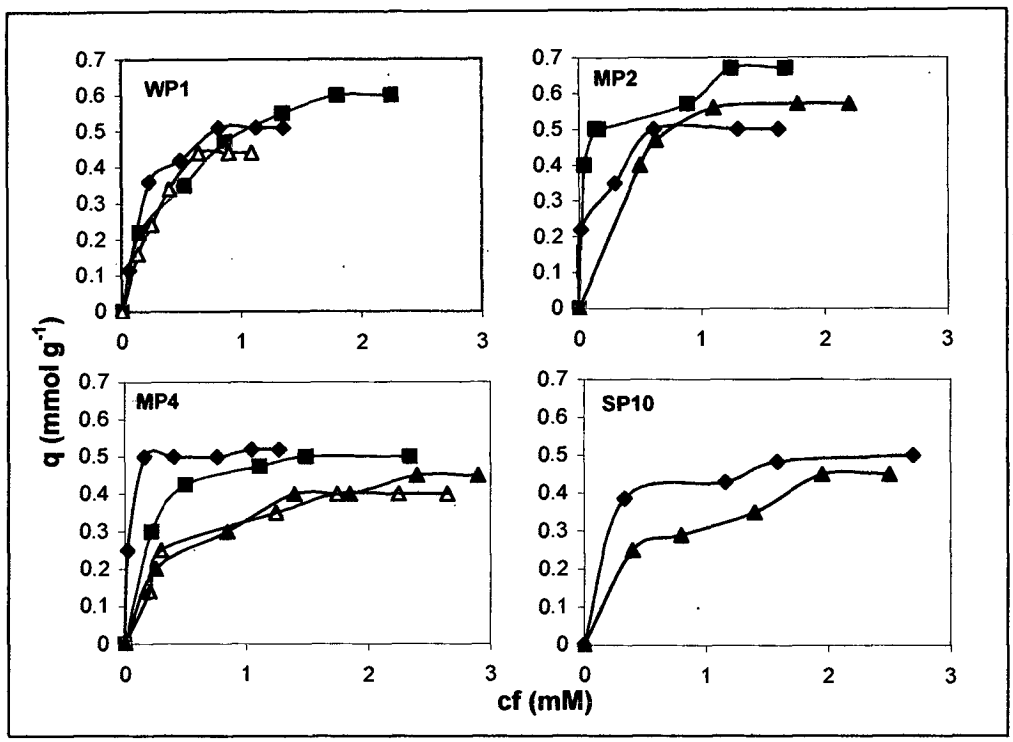


Fig 3.6: Equilirium isotherms of WP1, MP2, MP4, SP10 for: ◆Pb, ■ Cu, ▲ Cd ($CdS0_4$),△ Cd($CdN0_3$)

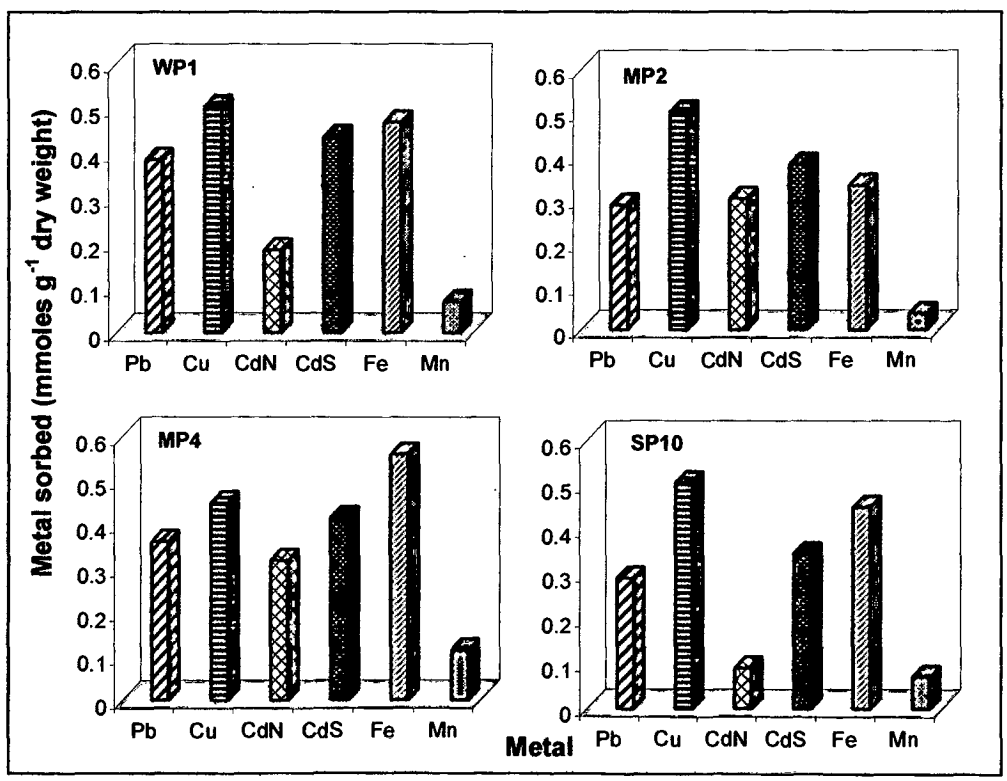


Fig 3.7: Metal sorption by cultures WP1, MP2, MP4 and SP10

Fig 3.7. As seen in the Fig, the heavy metals studied can be arranged in the following order according to their sorption. For WP1 sorption decreases in the following order: Cu > Cd (SO_4) > Pb > Cd $(NO_3)_2$, for MP2 is: Cu > Cd (SO_4) > Cd $(NO_3)_2$ > Pb, for MP4 is: Cu > Cd (SO_4) > Pb > Cd $(NO_3)_2$ and for SP10 is: Cu > Cd (SO_4) > Pb > Cd $(NO_3)_2$; the percentage of heavy metal removal was maximum for copper, about 50.5 % removal by all four *Penicillium* isolates. In case of transition metals, removal of iron was more than manganese (Fig 3.7).

The sorbed metals were observed to be mainly cell bound for all four *Penicillium* isolates, with intracellular accumulation of the heavy metals at only 7-15 % of the total metal removed from solution. The amount of total metal sorbed and its distribution on the cell surface and intracellular accumulation is given in Table 3.2, Fig 3.8.

Table 3.2: Cell-bound and intracellular distribution of metals

Metal sorbed (mmoles g^{-1} dry weight)				
Culture	Metal	Total sorbed	Cell bound	Intracellular
WP1	Pb^{2+}	0.386	0.311	0.036
	Cu^{2+}	0.505	0.437	0.018
	Cd^{2+} (Nitrate)	0.185	0.173	0.0116
	Cd^{2+} (Sulphate)	0.437	0.394	0.027
MP2	Pb^{2+}	0.288	0.238	0.012
	Cu^{2+}	0.503	0.225	0.001
	Cd^{2+} (Nitrate)	0.303	0.317	0.002
	Cd^{2+} (Sulphate)	0.382	0.361	0.004
MP4	Pb^{2+}	0.361	0.326	0.02
	Cu^{2+}	0.450	0.370	0.017
	Cd^{2+} (Nitrate)	0.319	0.297	0.002
	Cd^{2+} (Sulphate)	0.416	0.396	0.013
SP10	Pb^{2+}	0.291	0.265	0.019
	Cu^{2+}	0.505	0.353	0.004
	Cd^{2+} (Nitrate)	0.091	0.061	0.001
	Cd^{2+} (Sulphate)	0.346	0.324	0.013

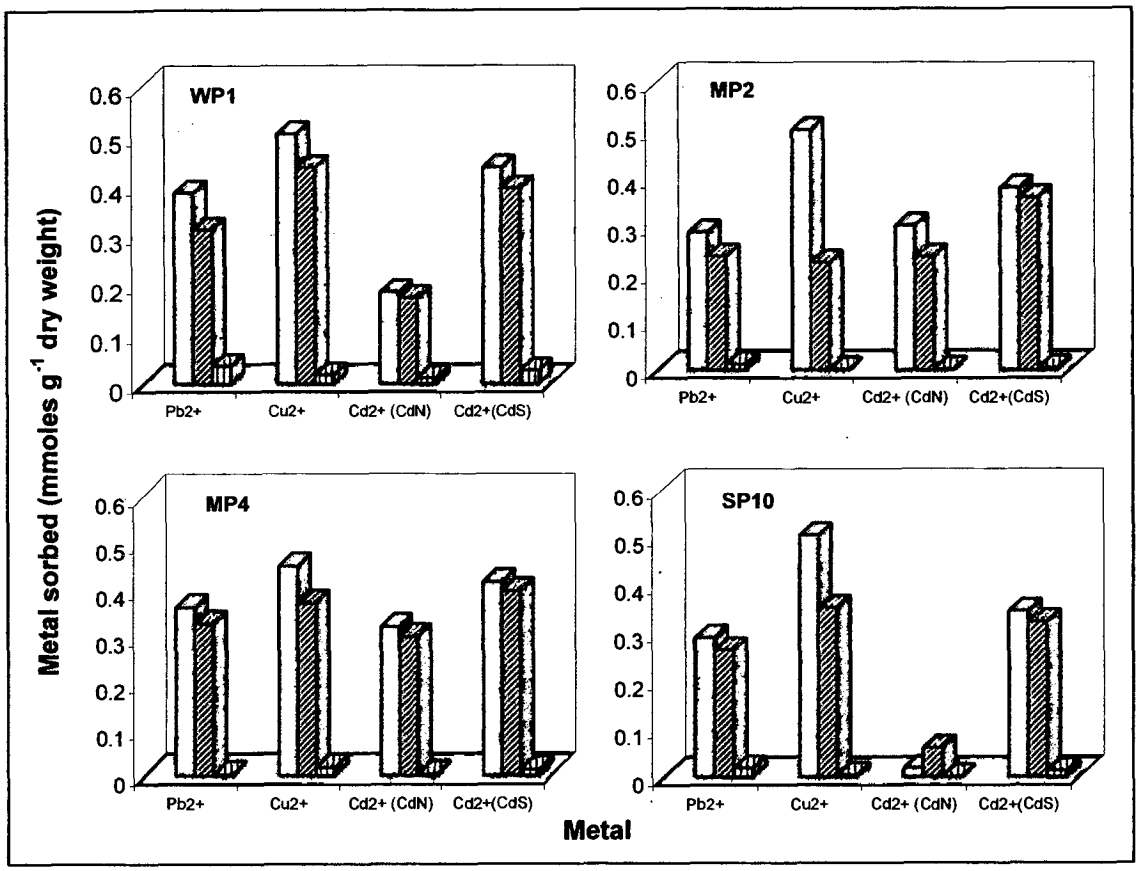


Fig 3.8: Metal localisation in WP1, MP2, MP4 and SP10:
□Total sorbed metal, ▨ Cell bound metal, ▥ Intracellular metal

3C. CULTURAL AND MORPHOLOGICAL RESPONSE TO METALS

3C.2. Materials and methods

3C.2.1 Cultural and morphological changes in response to metal

Cultures WP1, MP2, MP4 and SP10 were grown on Czapek's Dox Agar (CDA) plates containing increasing metal concentrations as shown in Table 3.4.

Table 3.4 Metal concentrations for cultural and morphological studies

Culture	Metal	mM concentration		
WP1	Pb^{2+}	0.0	2.5	5.0
	Cu^{2+}	0.0	1.0	2.0
	Cd^{2+} (Nitrate)	0.0	1.0	-
MP2	Pb^{2+}	0.0	2.5	5.0
	Cu^{2+}	0.0	1.0	2.0
	Cd^{2+} (Sulphate)	0.0	1.0	-
MP4	Pb^{2+}	0.0	2.5	5.0
	Cu^{2+}	0.0	2.0	3.0
	Cd^{2+} (Nitrate)	0.0	1.0	2.0
	Cd^{2+} (Sulphate)	0.0	1.0	2.0
SP10	Pb^{2+}	0.0	2.5	5.0
	Cd^{2+} (Sulphate)	0.0	1.0	-

The cultural characteristics of fungi were defined as described by Dubey and Maheshwari, (2001):

1. Appearance- The colonies are fluffy and cottony in appearance, developing a dry chalky appearance with age.

2. Form- The shape of the colony may be circular, filamentous, rhizoidal, punctiform (dot like), irregular or spindle shape.
3. Elevation – This is used to describe the depth of the colony which may be flat (thin film over the agar surface), raised, convex or with papillate surface.
4. Margins- The margins may be entire, undulate (wavy), crenate, dentate, lobate, rhizoidal or filamentous.
5. Pigment- Some colonies produce pigments which are soluble or insoluble; the soluble pigments diffuse out into the medium.

The cultures were examined visually for changes in cultural characteristics such as growth pattern, pigment production and sporulation and microscopically for changes in the morphological characteristics such as penicilli structure, thickening of cell wall, bulbous hyphae and/or any other changes from the control, using Olympus binocular microscope model-CH201 with microscope image projection system (MIPS) for image capture. A control of cells grown in absence of metals was maintained.

3C.3 Results

3C.3.1 Cultural and morphological changes in response to metals

a) Colony description of control cultures

All control cultures, that is, WP1, MP2, MP4 and SP10 showed rapid growth, sporulating in two days; MP4 showing slightly delayed sporulation till 54 hours growth. WP1 and MP2 showed a tinge of yellowish green pigment, which increased with age. MP4 produced a tinge of yellowish brown pigment, which also increased with age, and no visible pigment was observed in culture SP10. The colony appearance of all four isolates is circular, however colony margins of WP1, MP2 and SP10 are entire, while MP4 is undulate. WP1 and MP2 are flat, MP4 is convex with papillate surface, and SP10 is slightly raised (Fig 3.9). The colony of MP4 with age turned to greenish yellow at the centre.

b) Response to metal

The cultural and morphological changes brought about during growth in presence and absence of metals is summarised in Table 3.5 - 3.6. The cultures grown in presence of metals showed striking cultural (Fig 3.10A - 3.10F) and / or morphological changes (Fig 3.11A - 3.11I) with increasing metal concentration as compared to the control.

Lead (Pb^{2+}): All four isolates could resist Pb^{2+} at a much higher concentration as compared to copper and cadmium. Interestingly, although growth in presence of Pb^{2+} was not affected, distinct cultural changes in terms of growth pattern, sporulation, as well as

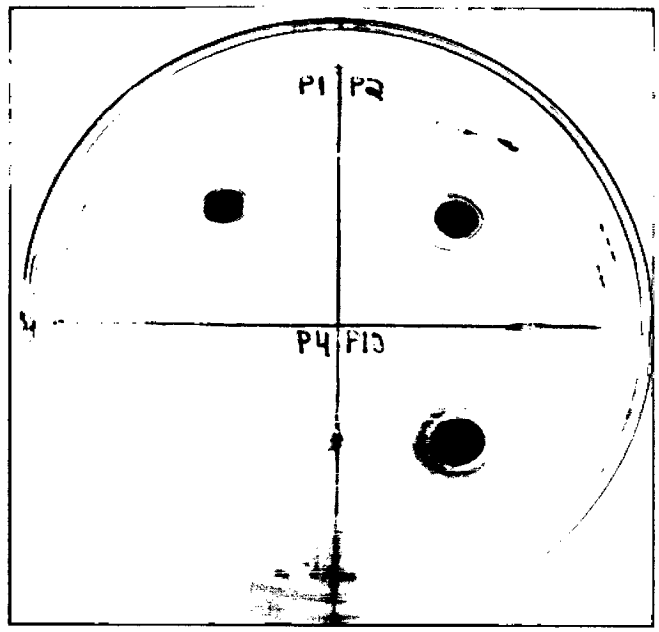


Fig 3.9: Cultures WP1, MP2, MP4 and SP10 on CDA plate at 2d growth

pigment production were observed. When the cultures were grown in the presence of 2.5 mM Pb^{2+}, pigment production was suppressed in cultures WP1 and MP2, while growth and sporulation were not affected as observed upto 7 days. MP4 showed decreased level of sporulation in the outer part of the colony and SP10 showed delayed sporulation and growth, with changes in growth pattern. Delayed pigment production of yellowish orange tinge was observed on the 6th day for MP4 as well as in SP10 which is not produced in the control. When the cultures were grown at a higher concentration of 5.0 mM Pb^{2+}, growth was not affected, however, all the isolates showed distinct changes in the growth pattern and inhibition of sporulation was observed for MP4 and SP10, as well as uneven sparsely distributed spores for all cultures with age. WP1 and MP2 also produced a diffusible yellowish green pigment turning into a brownish red tinge with age. MP4 produced a tinge of yellowish brown pigment; the yellow pigment diffusible into the medium on the 6th day. SP10 also produced a brownish red pigment on the 6th day.

Morphological characteristics were not very much affected when grown in the presence of Pb^{2+} for all four isolates of *Penicillium*. The cultures grown in presence of 2.5 mM Pb^{2+} showed not much morphological changes, while growth at a higher concentration of 5 mM Pb^{2+} showed more prominent changes with thickened hyphal cell walls for WP1, MP2 and SP10, while for MP4, bulbous hyphal cells was observed. Close and distinct septa were also seen.

<u>Copper (Cu^{2+})</u>: WP1, MP2 and MP4 could resist Cu^{2+}. Growth and sporulation of WP1 and MP2 when grown in the presence of 1mM copper were as in the control, however the appearance of the colony changed from entire to undulate form, and at a slightly higher

concentration growth was totally inhibited. Diffusible pigment production was arrested, however a greyish brown pigment, which turned brownish red with age, was observed. In contrast, growth of MP4 was not very much affected, however an increase in a greenish brown pigment which turned yellowish brown with age was observed; the yellowish pigment diffusible into the medium along with significant morphological changes.

When the cultures were grown in the presence of 2mM copper, growth appearance of WP1 and MP2 showed very less filamentous growth with inhibition of sporulation and pigmentation and pronounced morphological changes. MP4 showed fairly good growth at 2 mM copper, producing a greenish brown pigment with rich yellowish green pigment diffusible into the medium.

MP4 could also resist 3 mM copper, however, sporulation was delayed till the 4th day. Decreased growth was noted, which also produced a greenish brown pigment with yellowish green pigment diffusible into the medium, and more so on the 4th day.

Morphologically, as the metal concentration increases, the penicilli heads were not fully formed, and at higher concentration of 2 mM Cu^{2+} revealed the presence of rounded hyphal cells in WP1 and MP2. MP4 showed loss of triverticillate structure.

<u>Cadmium nitrate [Cd^{2+} $(NO_3)_2$]:</u> WP1 could resist cadmium as its nitrate form upto 1 mM, while MP4 could resist upto 2 mM, although the growth was significantly reduced in both, with inhibition of sporulation. WP1 showed very less filamentous growth with total inhibition of sporulation and pigmentation observed upto the 7th day, while MP4

showed slow growth with sparse sporulation on the 6th day, the culture on the reverse showing a yellowish brown turning greenish brown with age both at 1mM and 2 mM, however with a decreased growth at 2 mM.

Morphologically, at a concentration of 1mM Cd^{2+} the presence of rounded hyphal cells was observed in WP1, while MP4 showed thickening of cell wall.

Cadmium sulphate [Cd^{2+} (SO_4)]: MP2, MP4 and SP10 could resist cadmium as its sulphate form, growth being significantly reduced in MP4. MP2 and SP10 showed almost negligible filamentous growth with total inhibition of sporulation and pigmentation observed upto the 7th day at 1 mM concentration, while growth for MP4 at 1 mM and 2 mM cadmium is as cadmium nitrate.

Morphologically, at a concentration of 1mM Cd^{2+} the presence of rounded hyphal cells was observed in MP2, while MP4 showed thickening of cell wall.

Iron (Fe^{2+}): Growth and sporulation in WP1, MP2 and MP4 was not affected at 15 mM Fe^{2+} with production of yellowish green turning yellowish brown pigment, while delayed sporulation was observed in SP10; the colony showing a tinge of pinkish orange with yellowish brown pigment. WP1, MP2 and SP10 could resist upto 30 mM Fe^{2+}, however growth was significantly reduced with delayed sporulation observed on the 4th day with greenish yellow pigment for WP1 and MP2 and yellowish brown tinge for SP10. MP4 resisted lower concentration of Fe^{2+} upto 20mM only.

Table 3.5a: Cultural and morphological characteristics of WP1 grown in absence and presence of heavy metals.

Culture	Metal salt	Conc (mM)	Cultural Characteristics	Morphological Characteristics
WP1	Control	0.0	Rapid growth, blue green spores sporulating in 2d, with a tinge of yellowish green pigment diffusible into the medium with age. Colonies flat, dry and velvety, the reverse of the colony is creamish, margin is entire.	Mycelium branching and septate, conidiophores erect, septate at the apex with a verticel of erect primary branches borne directly on the conidiophore i.e., one stage branch, well formed phialides in groups of 2 - 3, conidia borne in chains forming a typical brush like spore bearing heads with phialides in clusters. Penicilli are biverticillate - symmetric.
	Pb $(N0_3)_2$	2.5	Variation: No yellowish green pigment observed. Decreased levels of conidiation in the outer parts of the colony as compared to the control	No variations from control
	Pb $(N0_3)_2$	5.0	Variation: Decrease in sporulation, yellowish green pigment diffusible into the medium.	Variation: Thickened hyphal cells
	$CuS0_4$	1.0	Variations: delayed sporulation, colony on the reverse is greyish white, colony margin is undulate	Variations: Thickened mycelia, penicilli head not well formed.
	$CuS0_4$	2.0	Variations: Pronounced lag / inhibition in growth, sporulation and pigment production inhibited.	Variation: Thickened mycelia, bulbous hyphal cells, septa very close and distinct and very few penicilli heads.
	Cd $(N0_3)_2$	1.0	Variation: Pronounced lag / inhibition in growth, sporulation and pigment production inhibited.	Variation: Thickened mycelia, bulbous hyphal cells, septa very close and distinct. No penicilli head observed.

Table 3.5b: Cultural and morphological characteristics of MP2 grown in absence and presence of heavy metals.

Culture	Metal salt	Conc (mM)	Cultural Characteristics	Morphological Characteristics
MP2	Control	0.0	Similar to control of WP1	Similar to WP1, with two-stage branching: the verticel of erect primary branches bore a verticel of secondary branchlets (metulae), metulae in terminal whorls of 2-3 members. Penicilli are biverticillate - asymmetric.
	Pb $(N0_3)_2$	2.5	Variation: No yellowish green pigment observed. Decreased levels of conidiation in the outer parts of the colony as compared to the control	Variation: change in morphology, from two-stage to one- stage branching.
	Pb $(N0_3)_2$	5.0	Variations: Decrease in sporulation, increase in pigment production diffusible into medium	As above
	$CuS0_4$	1.0	Variations: Delayed sporulation, plate reverse greyish white. Colony margin crenated.	Variations: Very few penicilli heads observed, two - stage branching changed to one - stage or unbranched conidiophore.
	$CuS0_4$	2.0	Variations: Pronounced lag / inhibition in growth, sporulation and pigment production inhibited.	Variations: No penicilli head observed. Thickened mycelia with slightly bulbous hyphal cells and septa very close and distinct.
	$CdS0_4$	1.0	Variations: Pronounced lag / inhibition in growth, sporulation and pigment production inhibited.	Variations: Thickened mycelia, bulbous hyphal cells, septa very close and distinct. No penicilli heads observed.

Table 3.5c: Cultural and morphological characteristics of MP4 grown in absence and presence of heavy metals.

Culture	Metal salt	Conc (mM)	Cultural Characteristics	Morphological Characteristics
MP4	Control	0.0	Rapid growth, sporulation in 54 h, greenish yellow at the centre with age. Colony on the reverse is creamish changing to reddish brown with age. Colony margin is crenated.	Similar to WP1, however the verticel of erect primary branches bore a verticel of secondary (metulae) and tertiary branchlets i,e., three stage branch. Penicilli few in number with phialides in groups of three, Penicilli - triverticillate
	Pb $(N0_3)_2$	2.5	Variations: Delayed sporulation observed on 3d. Decreased levels of conidiation in the outer parts of the colony as compared to the control. colony on the reverse is brownish yellow. Colony margin is entire.	Variations: Triverticillate structure not well formed.
	Pb $(N0_3)_2$	5.0	Variations: Delayed/ inhibition of sporulation, colony on the reverse is brownish yellow. Colony margin is entire.	Variations: Triverticillate structure not well formed. Thickened mycelia with bulbous hyphal cells, septa very close and distinct.
	$CuS0_4$	2.0	Variations: Lag / delayed growth, sporulation in 3d, and after 3d diffusible pigment produced into medium. Colony on the reverse is greenish yellow, colony margin is entire.	Variations: Three - stage branching changed to two - stage branching
	$CuS0_4$	3.0	Variations: Lag / delayed growth, sporulation in 4d, after 3d diffusible pigment produced into medium. Colony on the reverse is greenish yellow, colony margin is entire	Variations: As above, however penicilli heads were not well formed.

Table 3.5c: Cultural and morphological characteristics of MP4 grown in absence and presence of heavy metals (Contd).

Culture	Metal salt	Conc (mM)	Cultural Characteristics	Morphological Characteristics
MP4	Control	0.0	Rapid growth, sporulation in 54 h, greenish yellow at the centre with age. Colony on the reverse is creamish changing to reddish brown with age. Colony margin is crenated.	Similar to WP1, however the verticel of erect primary branches bore a verticel of secondary (metulae) and tertiary branchlets i,e., three stage branch. Penicilli few in number with phialides in groups of three, Penicilli - triverticillate
	Cd $(N0_3)_2$	1.0	Variations: Lag / inhibition in growth, sporulation inhibited, colony on the reverse is pale greenish yellow. Colony margin is entire, colony surface slightly broken with age.	Variations: Very few penicilli heads not well formed, thickened mycelia.
	Cd $(N0_3)_2$	2.0	Variations: Lag / inhibition in growth, sporulation inhibited, colony on the reverse is greyish white. Colony margin is entire, colony surface broken with age.	Penicilli heads were not observed. Thickened mycelia.
	$CdS0_4$	1.0	Variations: Lag / inhibition in growth, sporulation / pigment inhibited, colony margin is entire.	Variations: Three-stage branching changed to two-stage branching.
	$CdS0_4$	2.0	Variations: Lag / inhibition in growth, sporulation inhibited, colony on the reverse is greyish white. Colony margin is entire, surface slightly broken with age.	Variations: Thickened mycelia, bulbous hyphal cells and septa very close and distinct. No penicilli heads observed

Table 3.5d: Cultural and morphological characteristics of SP10 grown in absence and presence of heavy metals.

Culture	Metal salt	Conc (mM)	Cultural Characteristics	Morphological Characteristics
SP10	Control	0.0	Rapid cottony white growth, bluish green spores, sporulating in 2 d, colony on the reverse is creamish green changing to reddish brown with age. Colony margin is entire.	Similar to WP1, with unbranched conidiophore having no metulae, penicilli heads few in number, with phialides in groups of 2-3. Penicilli - monoverticillate
	Pb $(N0_3)_2$	2.5	Variations: Lag in growth / sporulation, colony on the reverse is greyish white. Colony margin is crenated.	Variations: very few penicilli heads were observed.
	Pb $(N0_3)_2$	5.0	Variations: Lag in growth / sporulation, colony on the reverse is greyish white. Colony margin is crenated.	Variations: As above
	$CdS0_4$	1.0	Variations: Pronounced lag / inhibition in growth, sporulation / pigment production inhibited. Colony on the reverse is greyish white.	Thickened mycelia, bulbous hyphal cells and septa very close and distinct. No penicilli heads were observed.

Table 3.6a: Cultural and morphological characteristics of WP1 grown in presence of iron and manganese.

Culture	Metal	Conc (mM)	Cultural Characteristics	Morphological Characteristics
WP1	Control	0.0	Rapid growth, blue green spores sporulating in 2d, with a tinge of yellowish green pigment diffusible into the medium with age. Colonies flat, dry and velvety, the reverse of the colony is creamish, margin is entire.	Mycelium branching and septate, conidiophores erect, septate at the apex with a verticel of erect primary branches borne directly on the conidiophore i.e., one stage branch, well formed phialides in groups of 2 - 3, conidia borne in chains forming a typical brush like spore bearing heads with phialides in clusters. Penicilli are biverticillate - symmetric.
	Fe^{2+}	15	Variations: Decreased sporulation, with production of yellowish green turning yellowish brown pigment, colony on the reverse is creamish.	Variations: Thickened hyphal walls.
	Fe^{2+}	30	Variations: Pronounced lag in growth, sporulation inhibited, colony on the reverse is greenish yellow.	Variations: Bulbous hyphal cells. No penicilli heads observed.
	Mn^{2+}	50	Variations: Pronounced lag in growth / sporulation, colony on the reverse is greenish yellow.	Variations: Bulbous cells, penicilli not well formed.
	Mn^{2+}	100	Variations: Pronounced lag in growth / sporulation, colony on the reverse is greenish yellow.	Thickened mycelia, bulbous hyphal cells and septa very close and distinct. No penicilli heads were observed.

Table 3.6b: Cultural and morphological characteristics of MP2 grown in presence of iron and manganese.

Culture	Metal	Conc (mM)	Cultural Characteristics	Morphological Characteristics
MP2	Control	0.0	Similar to control of WP1	Similar to WP1, with two-stage branching: the verticel of erect primary branches bore a verticel of secondary branchlets (metulae), metulae in terminal whorls of 2-3 members. Penicilli are biverticillate - asymmetric.
	Fe^{2+}	15	Variations: Decreased sporulation, with production of yellowish green turning yellowish brown pigment, colony on the reverse is creamish.	Variations: Thickened hyphal walls.
	Fe^{2+}	30	Variations: Pronounced lag in growth / sporulation, colony on the reverse is greenish yellow.	Variations: Hyphal cells bulbous. No penicilli heads were observed.
	Mn^{2+}	50	Variations: Lag in growth and sporulation, colony on the reverse is greenish yellow.	Variations: Bulbous cells, penicilli not well formed.
	Mn^{2+}	100	Variations: Lag in growth / sporulation, colony on the reverse is greenish yellow.	Variations: Thickened hyphal walls.. No penicilli head observed.

Table 3.6c: Cultural and morphological characteristics of MP4 grown in presence of iron and manganese.

Culture	Metal	Conc (mM)	Cultural Characteristics	Morphological Characteristics
MP4	Control	0.0	Rapid growth, sporulation in 54 h, greenish yellow at the centre with age. Colony on the reverse is creamish changing to reddish brown with age. Colony margin is crenated.	Similar to WP1, however the verticel of erect primary branches bore a verticel of secondary (metulae) and tertiary branchlets i,e., three stage branch. Penicilli few in number with phialides in groups of three, Penicilli - triverticillate
	Fe^{2+}	15	Variations: Lag in growth / sporulation. Production of yellowish green turning yellowish brown pigment, colony margin is entire.	Variations: Triverticillate structure not well formed.
	Fe^{2+}	20	Variations: Pronounced lag / inhibition in growth / sporulation. MP4 resisted lower concentration of Fe^{2+} upto 20 mM only.	Variations: Bulbous hyphal cells observed.
	Mn^{2+}	50	Variations: Lag in growth, sporulation inhibited. Colony turning pinkish orange.	Variations: Bulbous hyphal cells with thickened walls. Penicilli heads were not observed.
	Mn^{2+}	100	Variations: Pronounced lag / inhibition in growth / sporulation, growth observed on the 10th day.	Variations: Thickened mycelial walls. No penicilli heads were observed.

Table 3.6d: Cultural and Morphological characteristics of SP10 grown in presence of iron and manganese.

Culture	Metal	Conc (mM)	Cultural Characteristics	Morphological Characteristics
SP10	Control	0.0	Rapid cottony white growth, bluish green spores, sporulating in 2 d, colony on the reverse is creamish green changing to reddish brown with age. Colony margin is entire.	Similar to WP1, with unbranched conidiophore having no metulae, penicilli heads few in number, with phialides in groups of 2-3. Penicilli - monoverticillate
	Fe^{2+}	15	Variations: Pronounced lag / inhibition in growth / sporulation, colony showing a tinge of pinkish orange with yellowish brown pigment.	Variations: Thickened mycelia. Penicilli heads not well formed.
	Fe^{2+}	30	Variations: Pronounced lag / inhibition in growth / sporulation, colony turning pinkish brown with age.	Variations: Thickened mycelia. No penicilli heads were observed
	Mn^{2+}	50	Variations: Lag / inhibition in growth / sporulation, colony turning pinkish orange.	No variations from control
	Mn^{2+}	100	Variations: Pronounced lag / inhibition of growth / sporulation, colony showing a tinge of pinkish orange.	Variations: Thickened mycelia. No penicilli head observed.

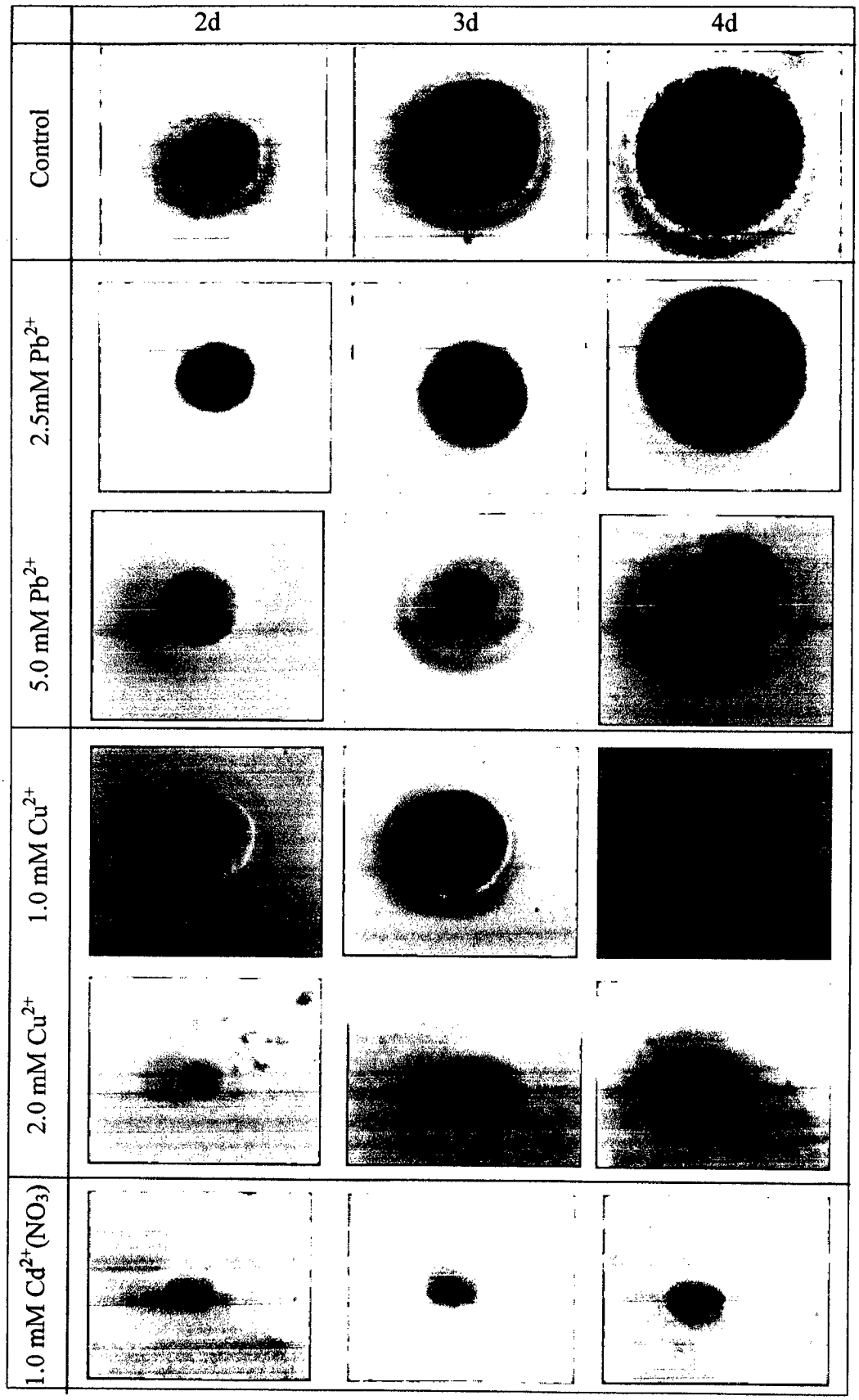


Fig 3.10A: Cultural changes in WP1 in presence of metals

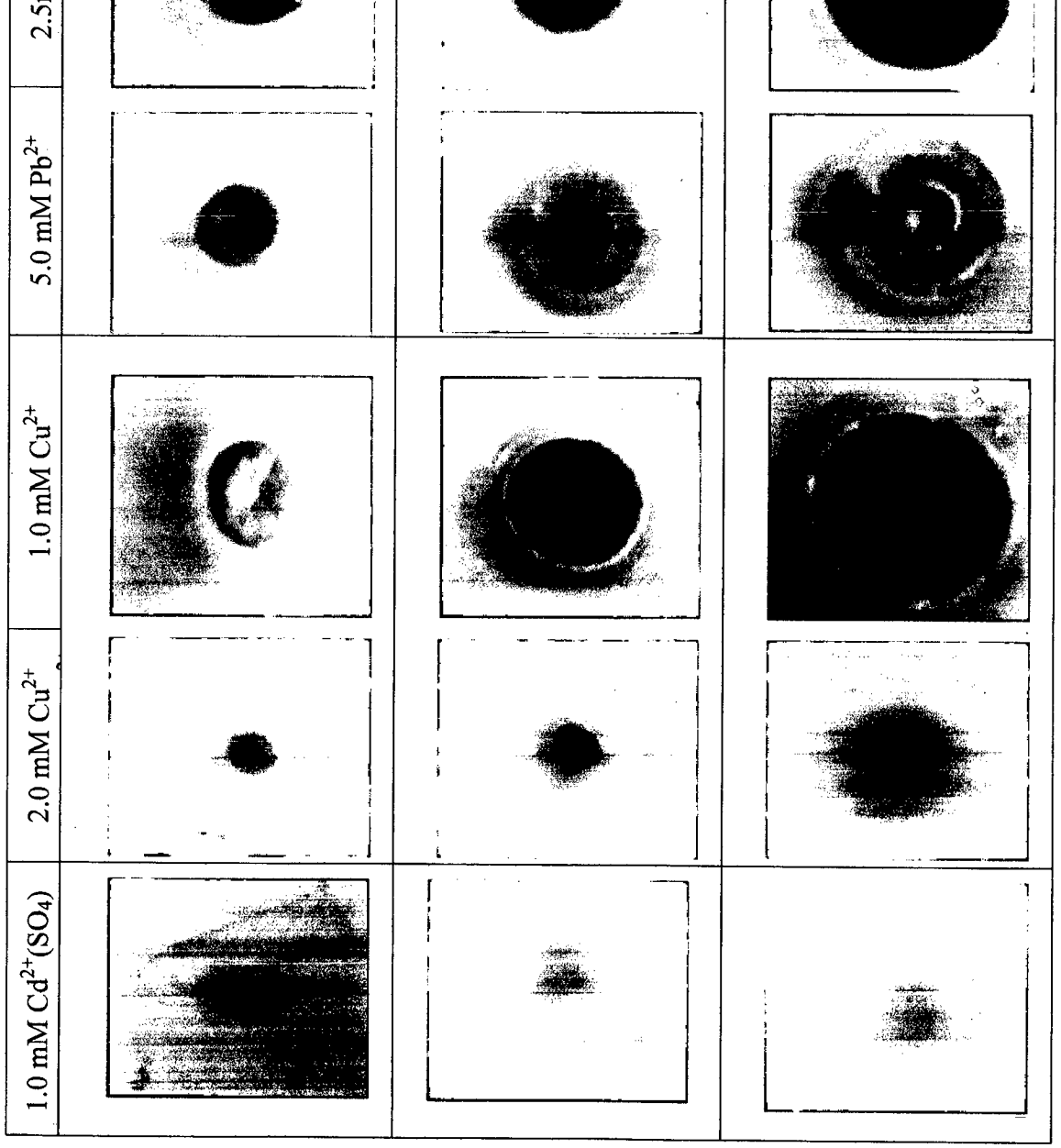


Fig 310 B: Cultural changes in MP2 in presence of metals

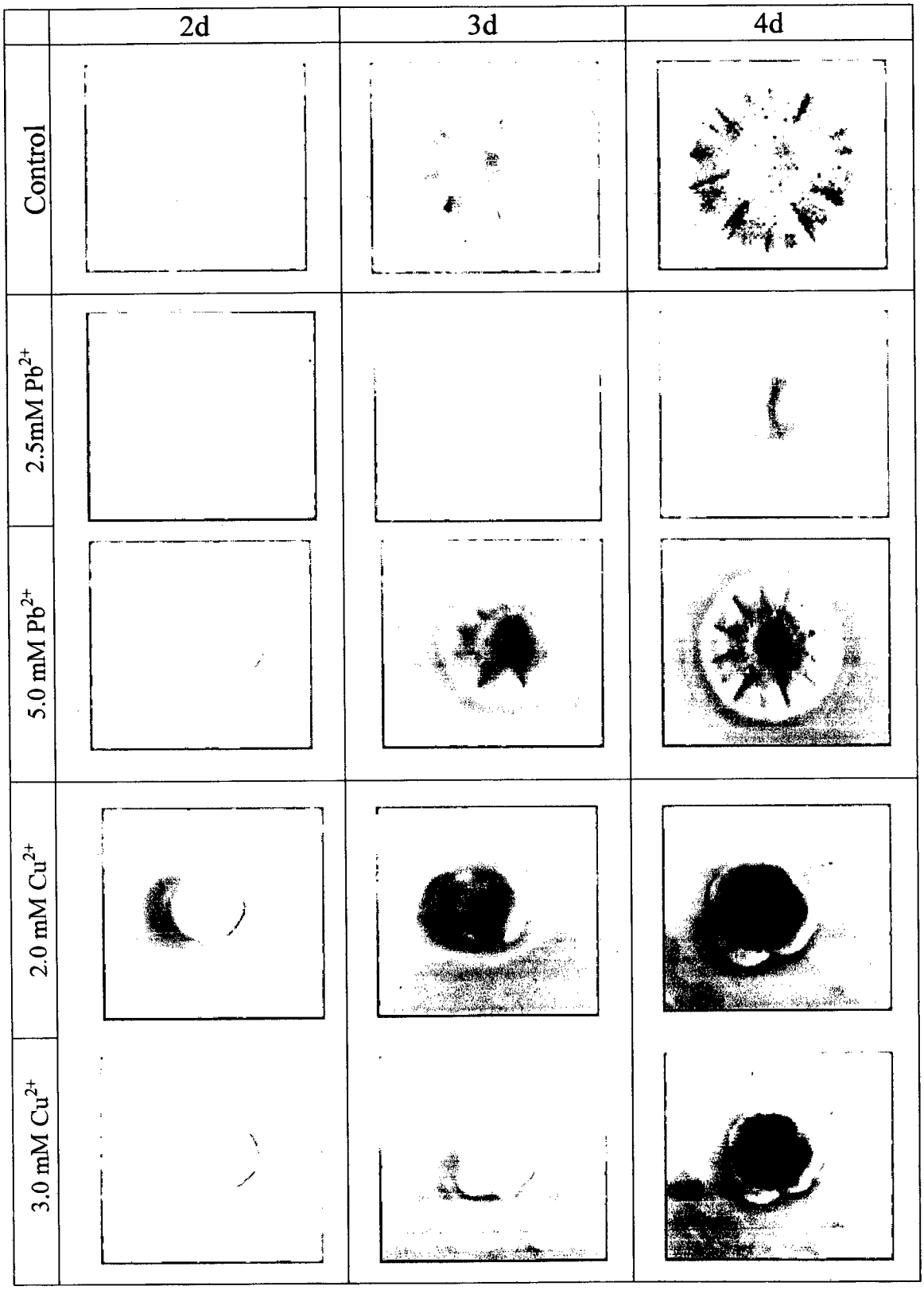


Fig 3.10 C: Cultural changes in MP4 in presence of metals

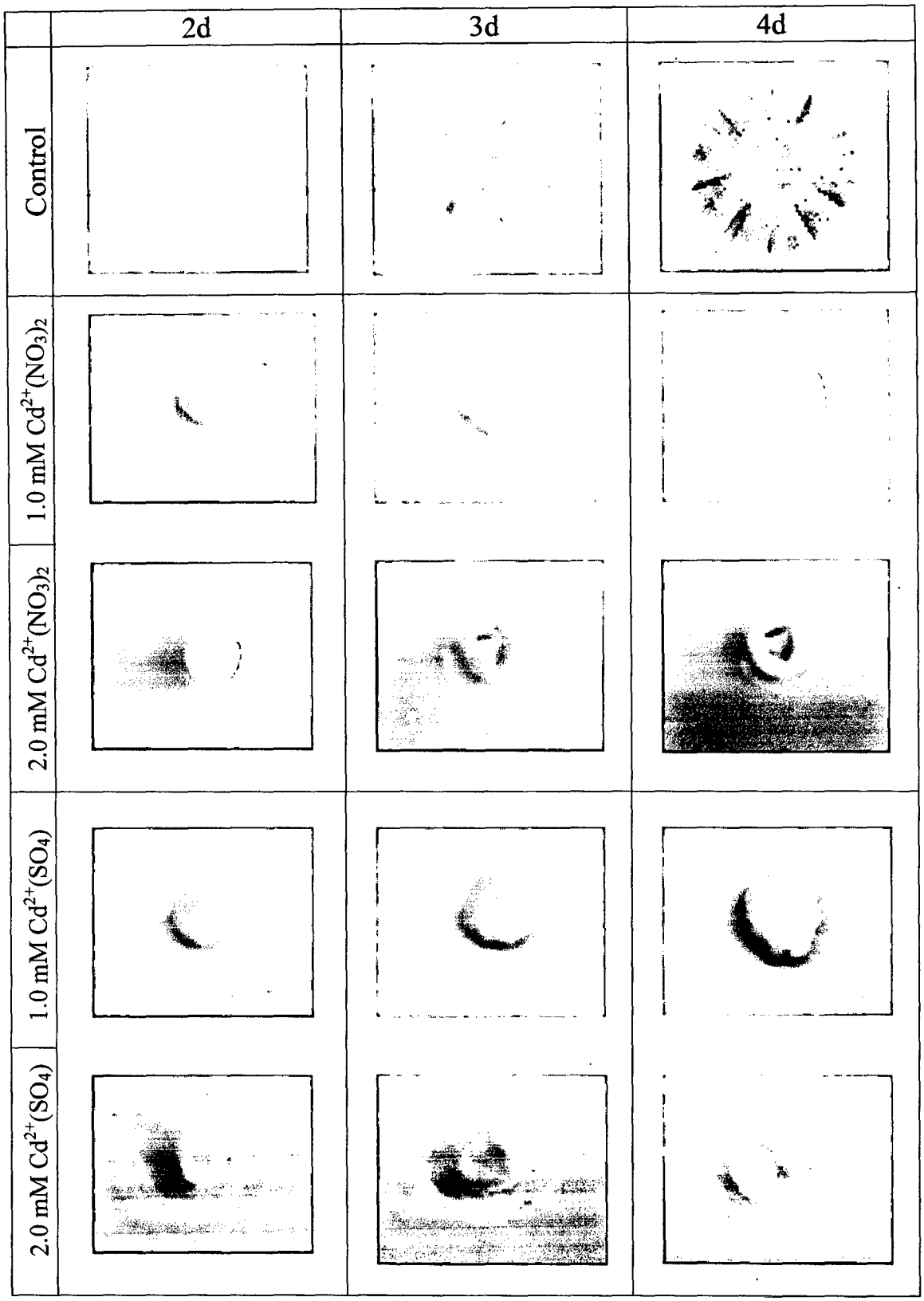


Fig 3.10 D: Cultural changes in MP4 in presence of metals (contd)

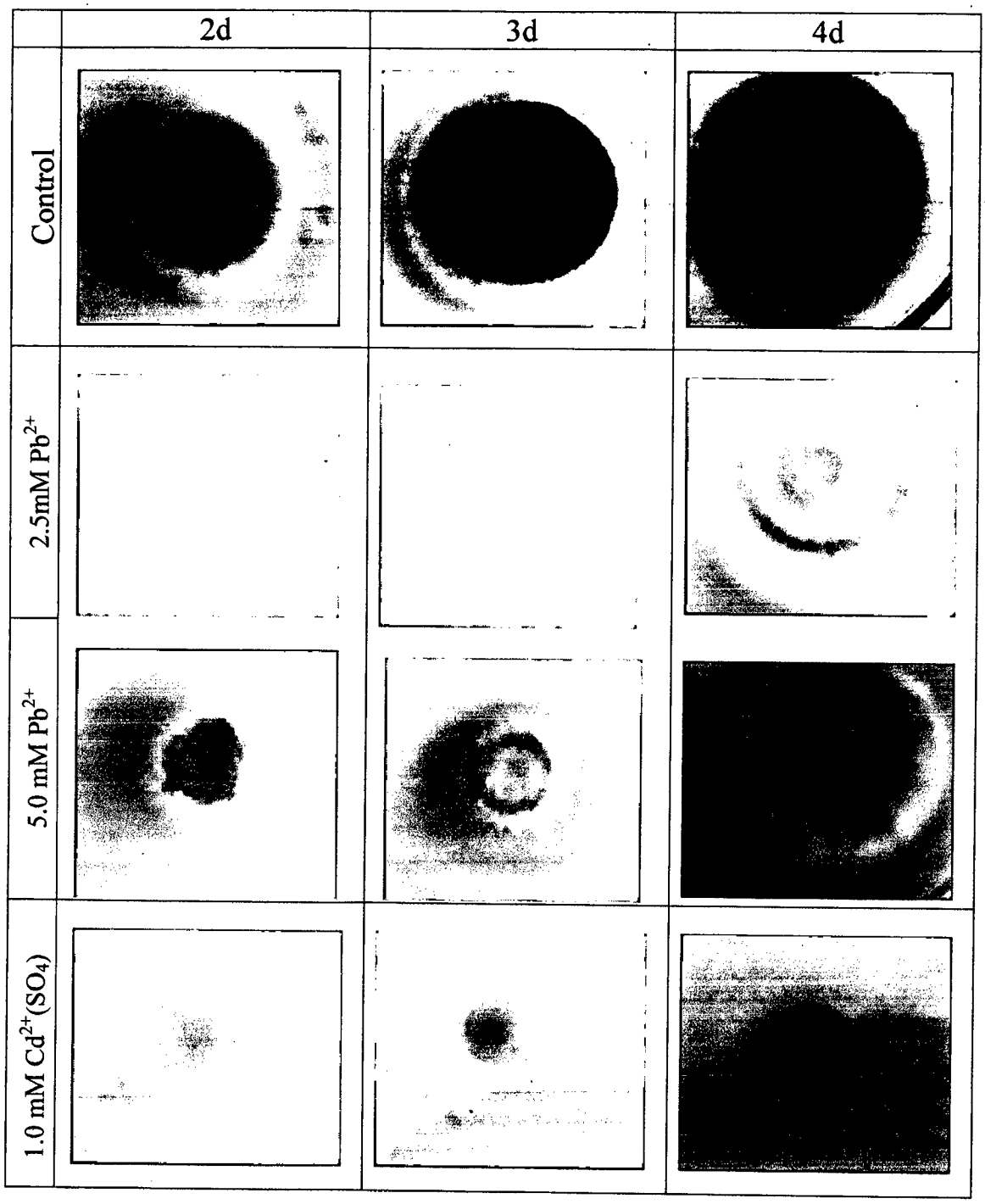


Fig 3.10 E: Cultural changes in SP10 in presence of metals

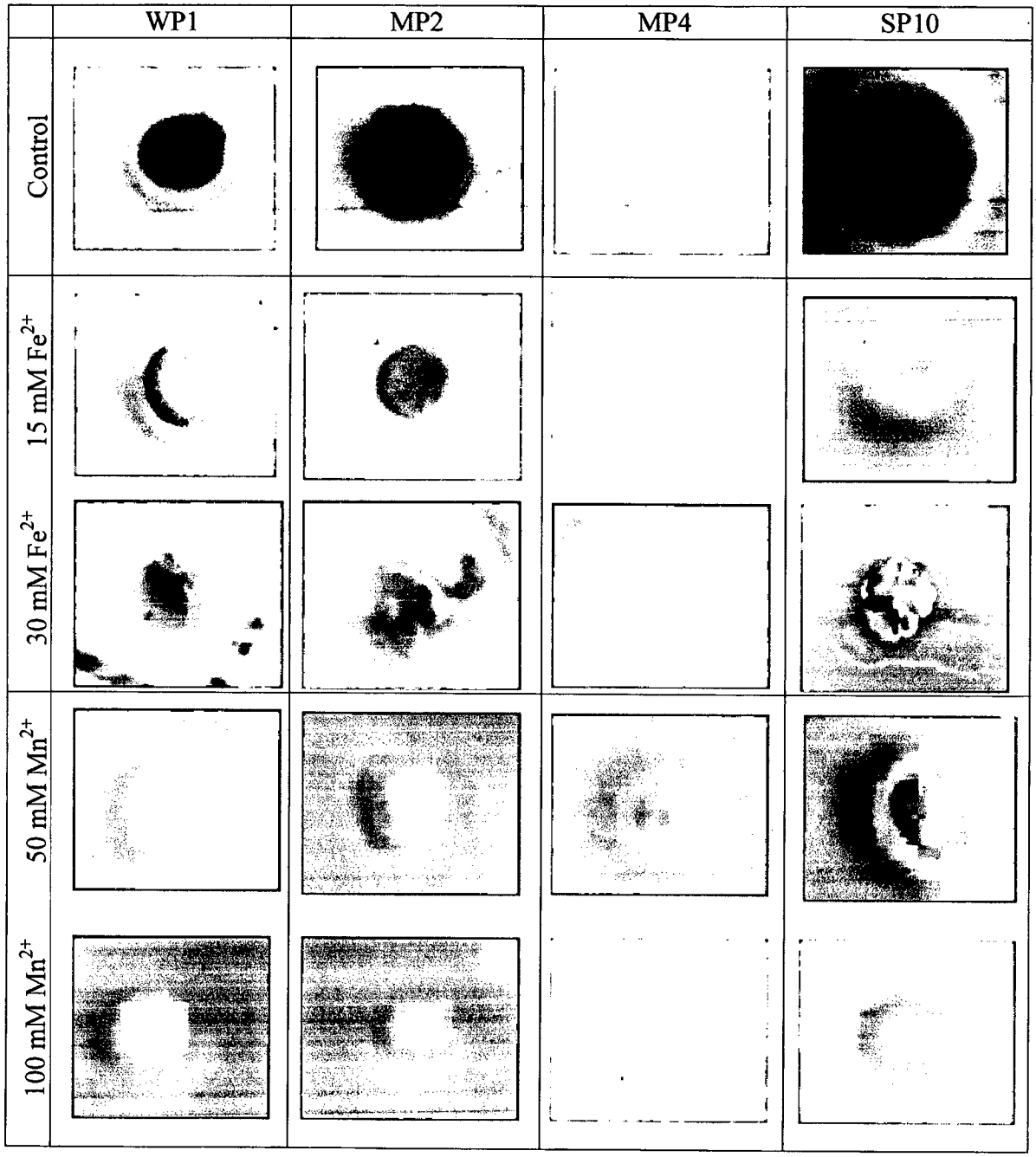


Fig 3.10F: Cultural changes in WP1, MP2, MP4 and SP10 in presence of metals

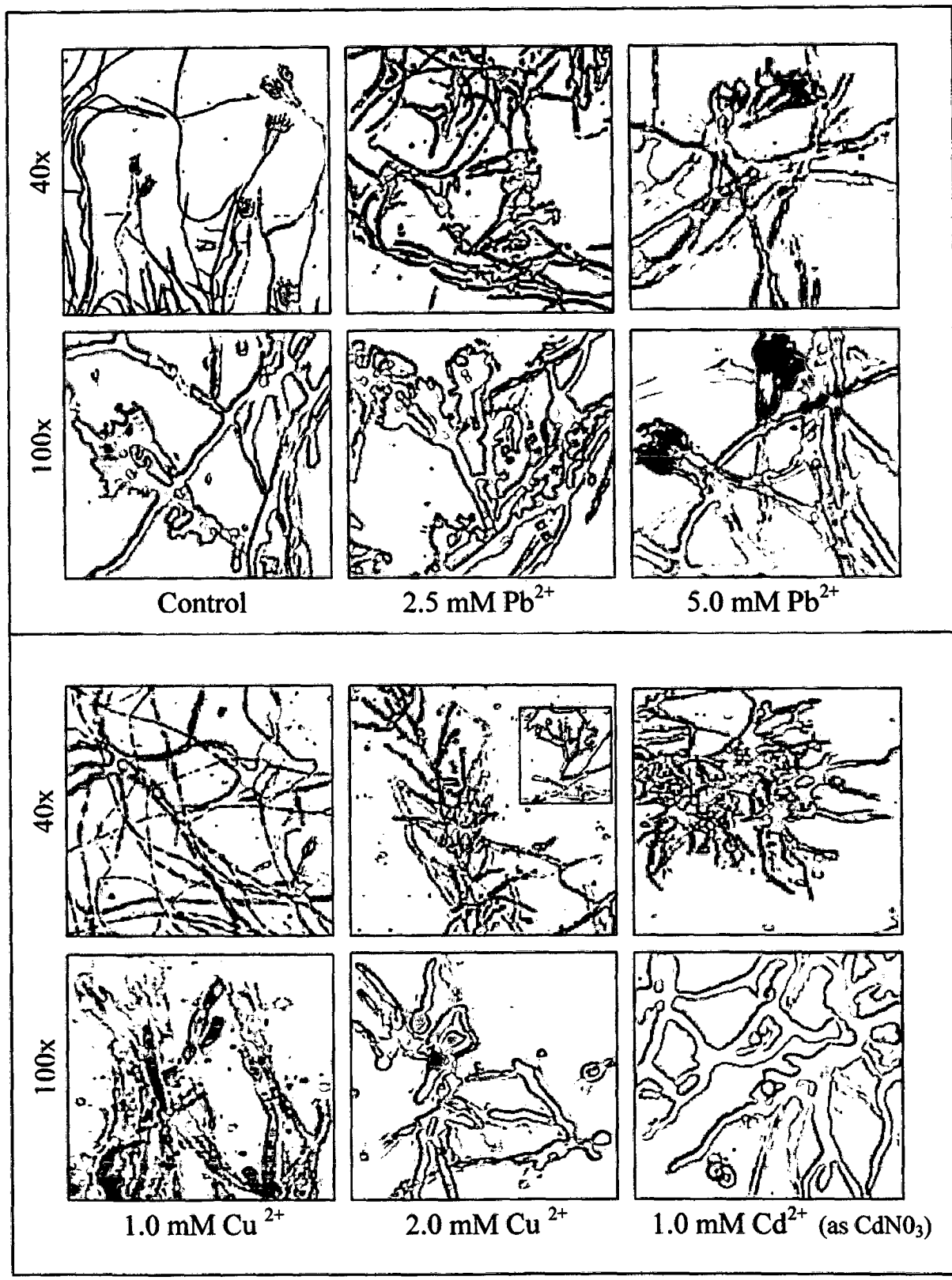


Fig 3.11A:Growth of WP1 at 0.0, 2.5 and 5.0 mM Pb^{2+}, 1.0 and 2.0 mM Cu^{2+}, 1.0 mM Cd^{2+} $(NO_3)_2$

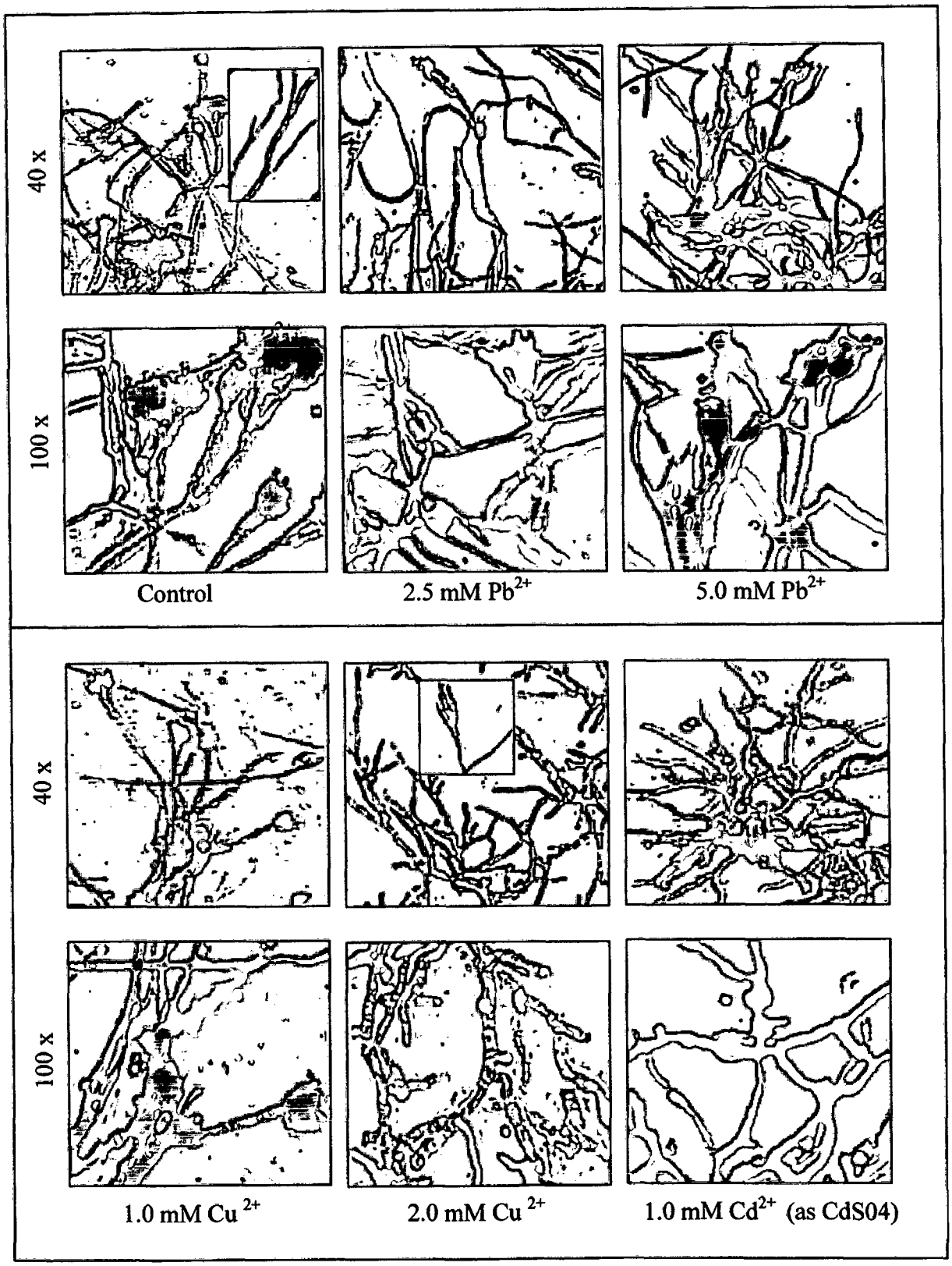


Fig 3.11B:Growth of MP2 at 0.0, 2.5 and 5.0 mM Pb^{2+}, 1.0 and 2.0 mM Cu^{2+}, 1.0 mM Cd^{2+} (SO_4)

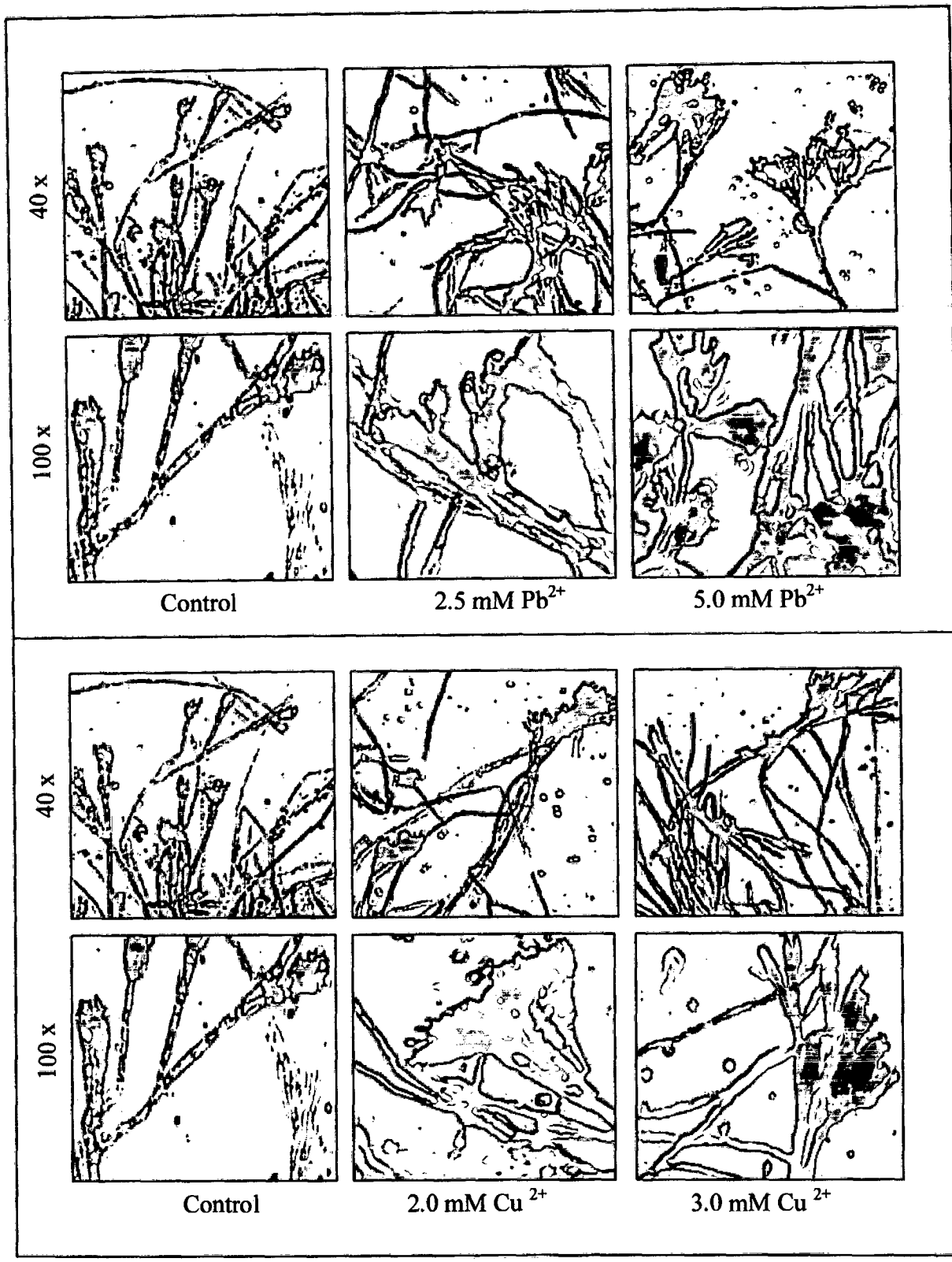


Fig 3.11C: Growth of MP4 at 0.0, 2.5 and 5.0 mM Pb^{2+}, 2.0 and 3.0 mM Cu^{2+}

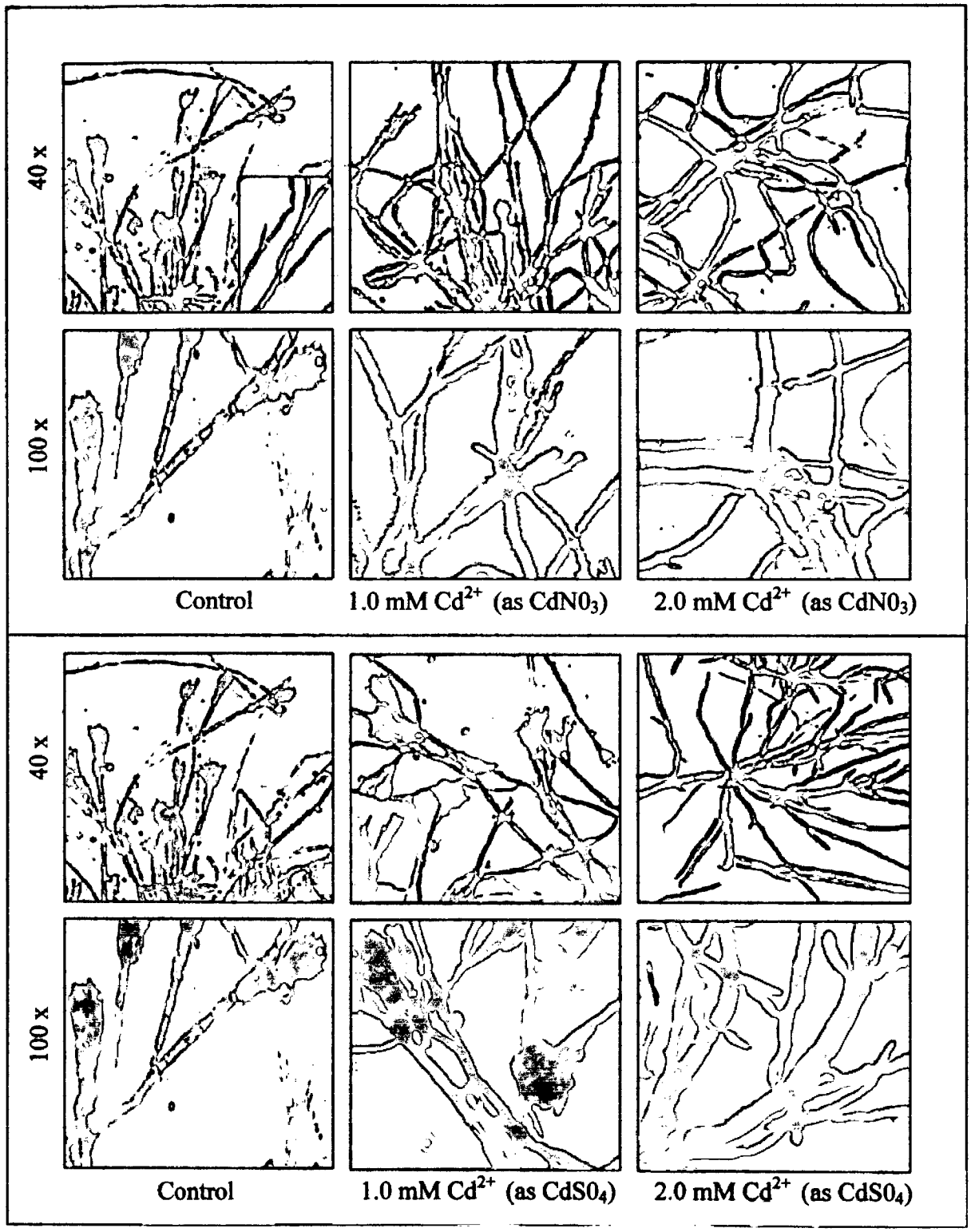


Fig 3.11D: Growth of MP4 at 0.0, 1.0 and 2.0 mM $Cd^{2+}(NO_3)_2$, 1.0 and 2.0 mM Cd^{2+} (SO_4)

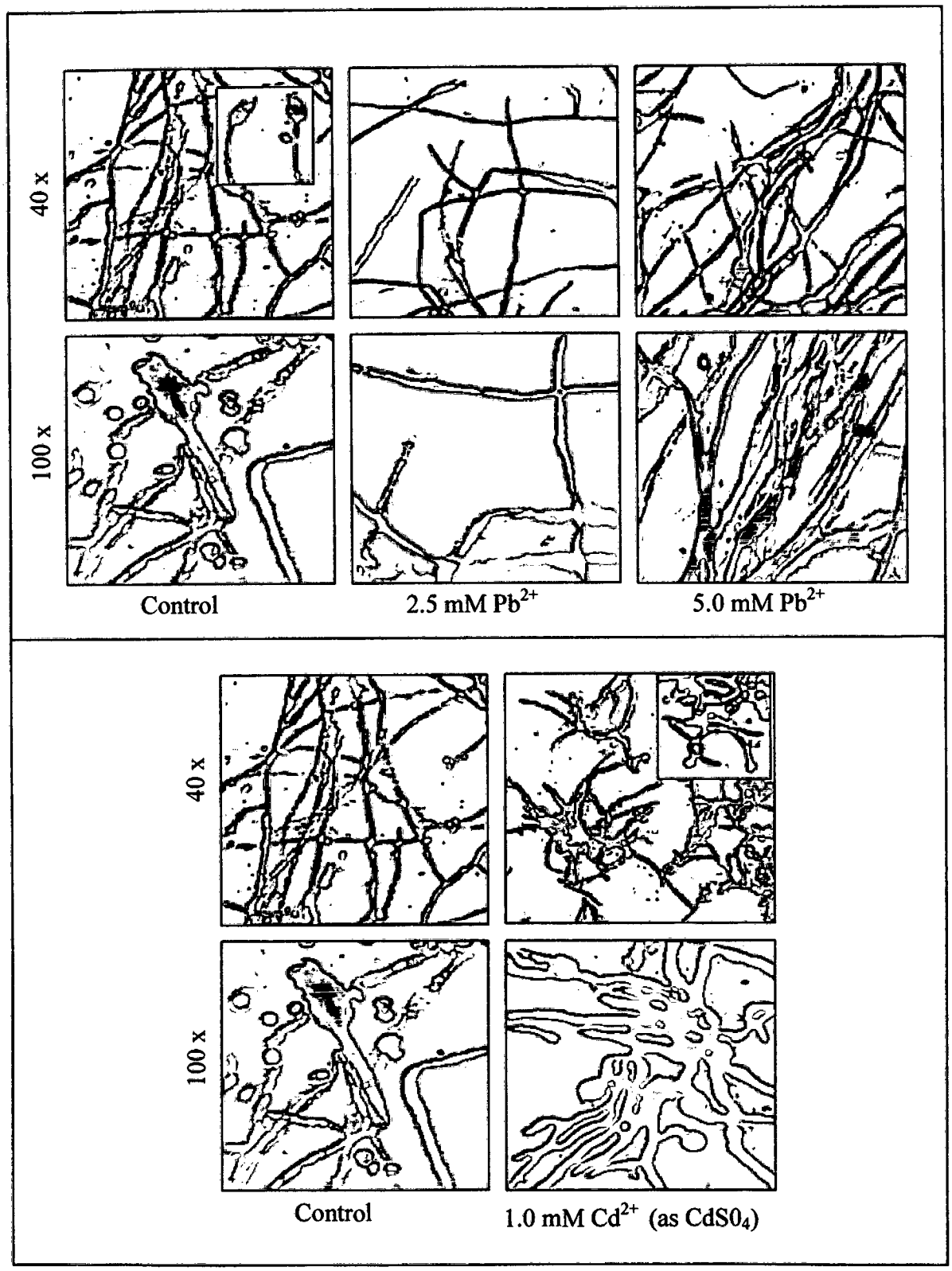


Fig 3.11E: Growth of SP10 at 0.0, 2.5 and 5.0 mM Pb^{2+}, 1.0 mM Cd^{2+} (SO_4)

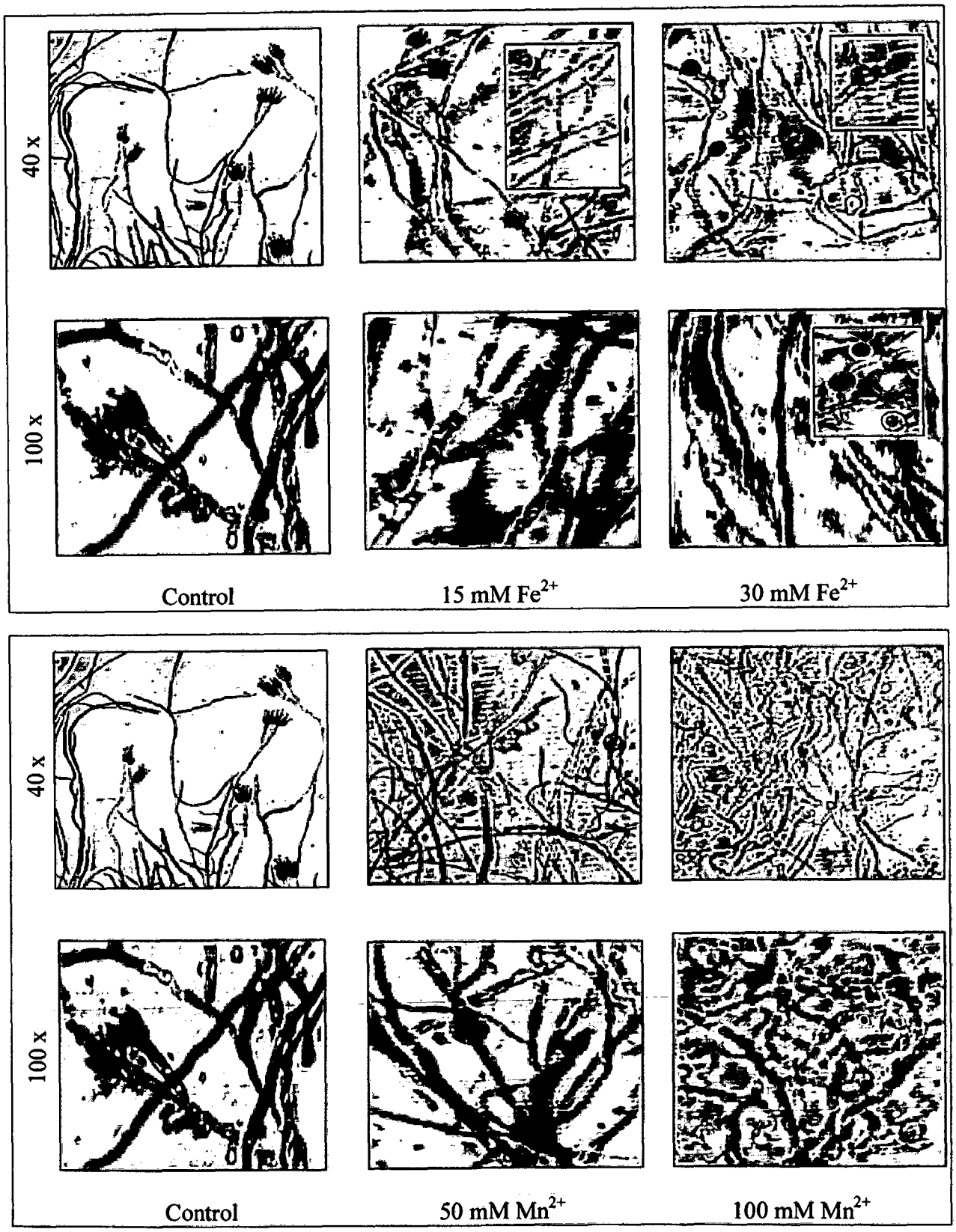


Fig 3.11 F: Growth of WP1 at 0.0, 15.0 and 30.0 mM Fe^{2+}, 50.0 and 100.0 mM Mn^{2+}

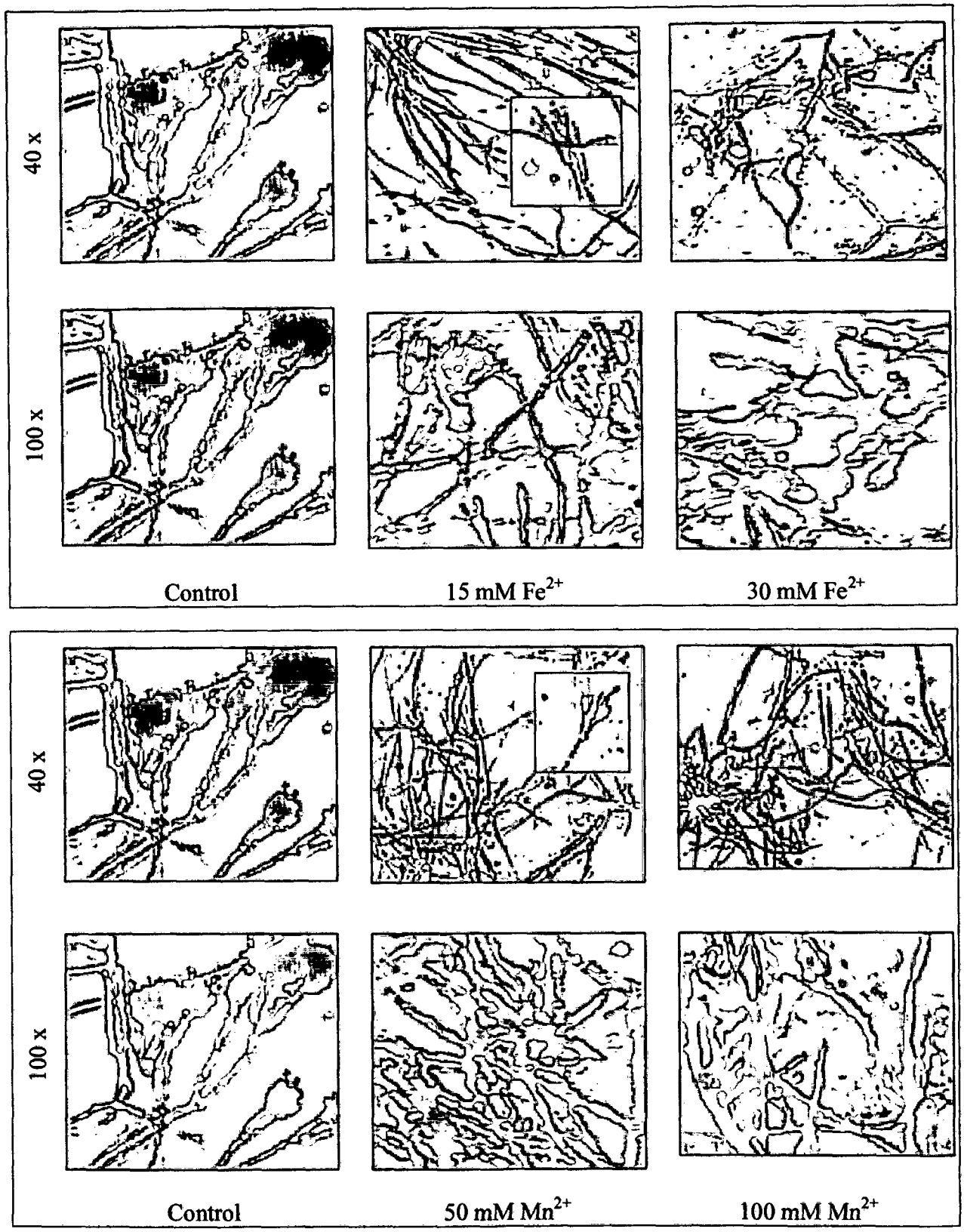


Fig 3.11 G: Growth of MP2 at 0.0, 15.0 and 30.0 mM Fe^{2+} and 50.0 and 100 mM Mn^{2+}

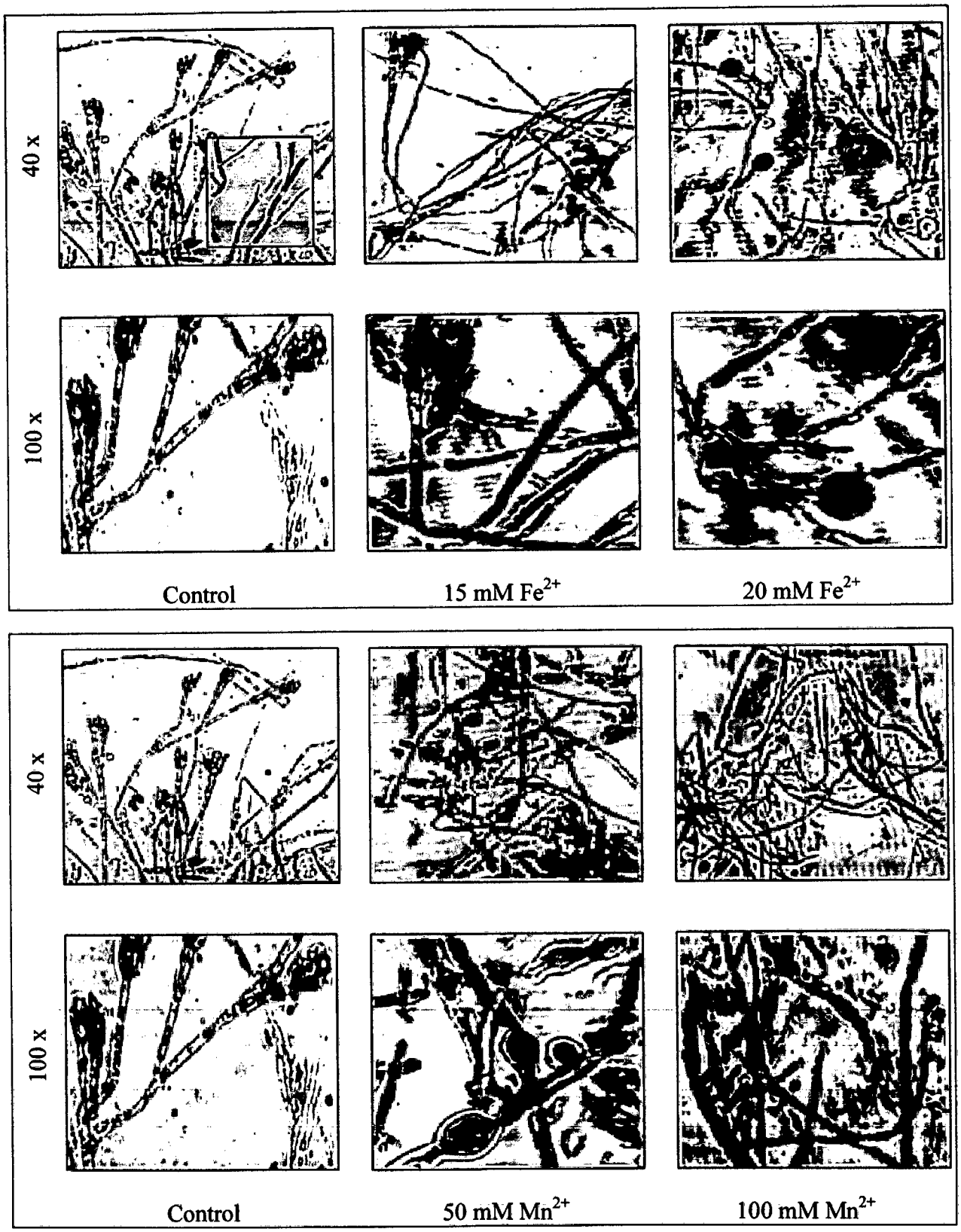


Fig 3.11 H: Growth of MP4 at 0.0, 15.0 and 20mM Fe^{2+}, 50.0 and 100 mM Mn^{2+}

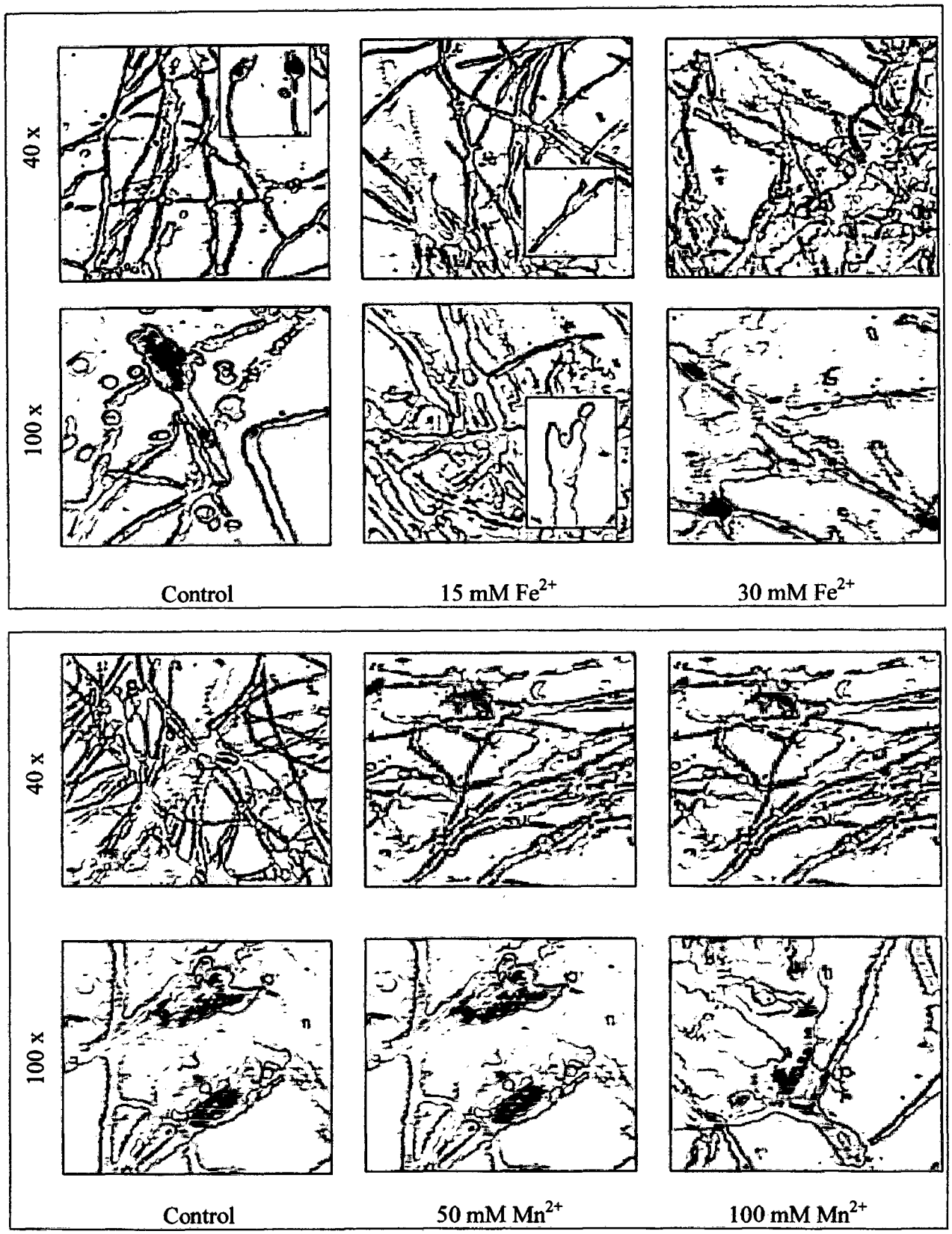


Fig 3.11 I: Growth of SP10 at 0.0, 15.0 and 30mM Fe^{2+}, 50.0 and 100.0 mM Mn^{2+}

3D. WHOLE CELL PROTEIN AND PLASMID PROFILES

3D.2. Materials and methods

3D.2.1 Whole cell protein profiles

Protein profiles of the cultures were obtained by SDS-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) of the cell lysate.

a) Growth of the culture and cell lysis

Cultures WP1, MP2, MP4 and SP10 were each inoculated in Czapek's Dox broth containing individual metal salts of $Pb(NO_3)_2$, $CuSO_4.5H_2O$, $Cd(NO_3)_2$ and $CdS0_4$ at concentration just below the MIC to cultures (Table 3.7) and incubated stationary at RT.

Table 3.7: Metal concentrations used during growth of the cultures for obtaining cell protein and plasmid patterns.

Culture	Metal (mM)			
	$Pb(NO_3)_2$	$CuSO_4.5H_2O$	$3CdSO_4.8H_2O$	$Cd(NO_3)_2.4H_2O$
WP1	3.0	0.1	0.1	nd
MP2	3.0	0.1	nd	0.1
MP4	3.0	2.0	3.0	3.0
SP10	3.0	ng	ng	0.1

ng: no growth

The grown culture was filtered and the mycelial mass washed with deionized water. This was centrifuged at 5000g for 15-20 min to obtain the pcv and the corresponding wet weight. The biomass was frozen at –20°C for 1h and ground to a paste with mortar and pestle in an ice bucket. Minimum water was then added and sonication was done with 10

pulses of 150mV for 20 sec each at an interval of 10 seconds. Centrifugation was then done at 4°C at 5000g for 10' to obtain the cell free extract (CFE) and cell debris.

b) Sample treatment

The CFE and cell debris were estimated for protein content by Folin-Lowry method and treated with 10 % SDS in a boiling water bath for 5 min and then with sample buffer (Appendix B) for another 5 min. The treated samples were cooled and stored at -20°C. Samples were run along with molecular weight markers (GENEI: Medium range marker).

c) Preparation of gel and electrophoresis

Solutions used for SDS-PAGE are detailed in Appendix B. The plates were clamped with spacers arranged between the plates and sealed with 1% agar on the two sides and bottom. The separating gel mixture was prepared and poured to cover 3/4th of the space between the plates. This was then gently overlayed with distilled water. A very sharp liquid-gel interface was visible when the gel polymerised. The overlay was then poured off and the stacking gel mixture was poured over the separating gel. A comb is inserted into the sandwich taking care not to trap any bubbles below the teeth of the comb. The gel is allowed to stand for about an hour. The plates were introduced into the chamber containing the tank buffer and pre-run for 30 min. The CFE samples (70μg protein), cell debris (70μg protein) and the marker (30 μg) were each loaded into the wells. Electrophoresis was carried out at 110-115 volts and a current of 20-25 mA till the tracking dye reached the end of the gel. The gel was then removed and immersed in the staining solution for 3h. It was then kept in destaining solution I for 1h and then transferred to destaining solution II till clear bands were observed.

3D.2.2 Plasmid profile

Agarose gel electrophoresis was used to study the plasmid pattern obtained in response of the cultures to different metals

a) Isolation of Plasmid

Solutions used for plasmid isolation are detailed in Appendix B). Cultures WP1, MP2, MP4 and SP10 were grown as detailed under 3D.2.1a for protein profile analysis. The grown culture was filtered and the mycelial mass washed with sterile deionized water. This was centrifuged at 5000g for 15-20 min to obtain the pcv and the corresponding wet weight. To 3 ml pcv mycelia, Tris - EDTA buffer, 10 ml, and 0.25 M EDTA (pH 8.0), 6.0 ml, was added and mixed gently. To this was added 20 % SDS, 2 ml, mixed by tapping the tubes and incubated for 10' in an ice bucket. 5M NaCl, 9 ml, was then added, mixed by inverting the tubes several times and incubated at 4°C overnight. The content was then centrifuged at 15,000 rpm at 4°C for 30'. The supernatant was carefully decanted and to it was added 2V of chilled ethanol and incubated at 4°C overnight. This was centrifuged at 15,000 rpm. at 4° for 20' and the pellet washed with 70% ethanol, and centrifuged as before. The centrifuge tube was then inverted on a clean pad of paper towels to drain out the alcohol and dried in a vacuum dessicator taking care not to allow the pellet to over-dry. The DNA was then dissolved in minimum TE Buffer.

b) Plasmid DNA quantitation

The ratio of A_{260} / A_{280} was taken for all the DNA samples to check the purity, a pure sample of DNA, substantially free of proteins having a high A_{260} / A_{280} ratio of 1.7 and above (Sambrook and Maniatis, 1989).

The DNA samples were quantitated, given that a sample of absorbance 1.0 at 260 nm contains 50 μg DNA ml^{-1}.

c) Agarose Gel Electrophoresis

The DNA samples (15 μg) along with molecular weight marker were electrophoresed in 0.8 % agarose gel in 0.5x TBE at 40 volts and 80mA current till the samples reached the end of the gel. The gel was viewed with UV transilluminator for presence of plasmid(s).

3D.3 Results

3D.3.1 Whole cell protein profiles

The protein profiles obtained by SDS-PAGE of the CFE and cell debris of the culture when grown in the presence and absence of metal salts are shown in Fig 3.12 and a summary of the analysis is given in Table 3.8. It was observed that in WP1, protein bands were more expressed when grown in presence of lead in comparison to the profile of the control culture and in presence of other heavy metals. Protein bands of 89.1, 80.5, 71, 66.1, 63.1, and 56.2 KDa were more expressed in presence of lead, 63.1 and 56.2 bands also expressed more in presence of copper and cadmium nitrate and additional bands of 44.7 and 35.5 KDa, absent in the control, were also expressed in presence of lead, copper and cadmium nitrate. A protein band of 40.5 KDa was expressed in presence of copper, which was absent in the others. Bands of 89.1, 80.5, 79.4, 71 and 66.1 KDa were under expressed in presence of copper and cadmium nitrate.

Culture MP2 could only grow in presence of Pb^{2+} and Cu^{2+}, the expression of 89.1, 80.5, 71, 68, 66 and 63.1 KDa proteins in presence of Pb^{2+} and Cu^{2+} was similar to the control. Also, an extra band of 55.5 KDa protein was observed when grown in the presence of both lead and copper, and an additional protein of 50 KDa in presence of copper.

The culture MP4 grown in presence of lead, copper, cadmium nitrate and cadmium sulphate showed extra protein bands in all cases both in the CFE as well as the cell debris. Extra protein bands of 56.1, 50.0, 39.8, 31.6 and 22.4 KDa were observed in presence of lead, copper and cadmium as its nitrate and sulphate forms, however protein bands of 56.1KDa was under expressed in presence of copper, 50.0 KDa in presence of copper and cadmium sulphate, while a 22.4 KDa protein was under expressed in presence

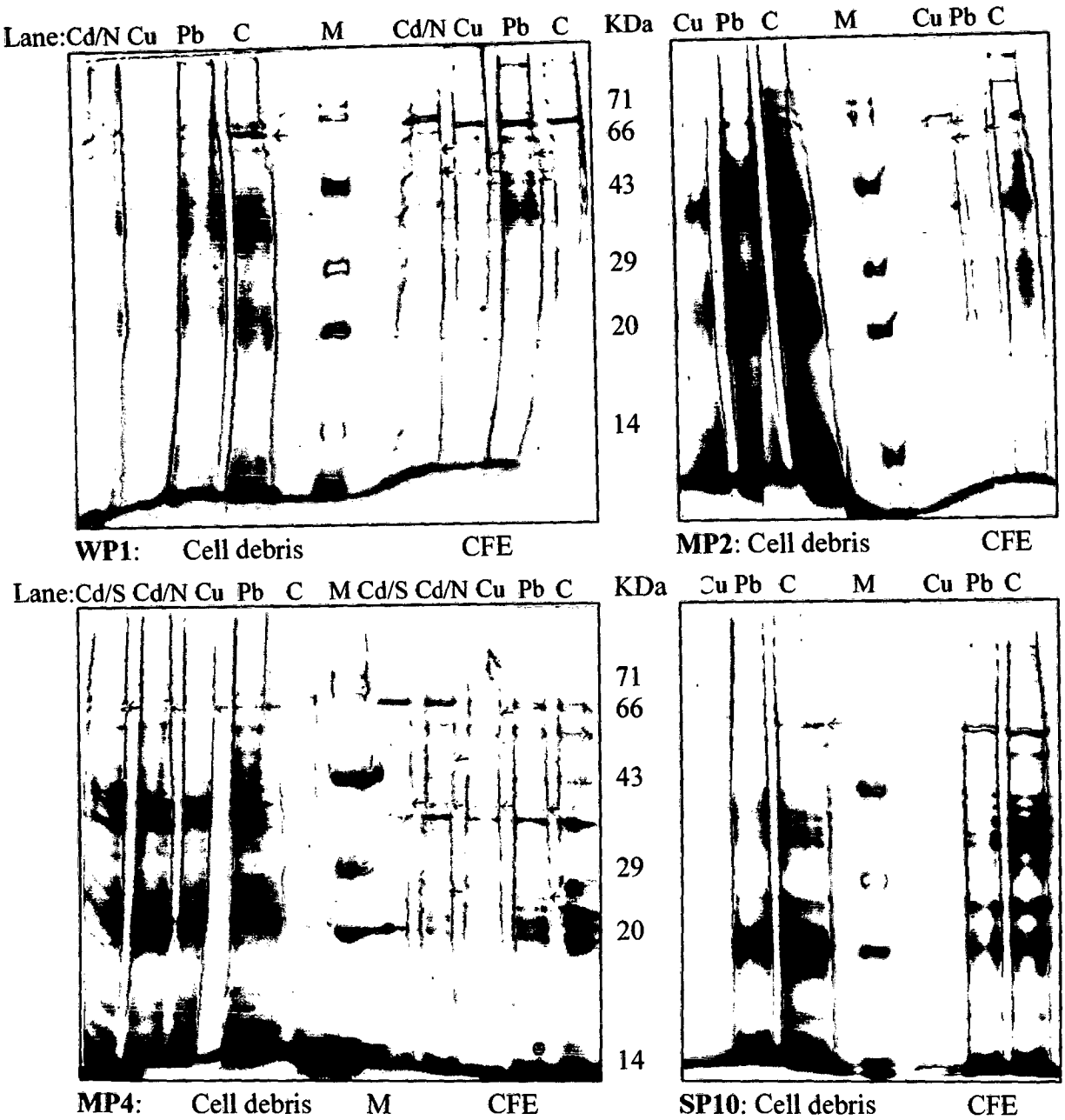


Fig 3.12: SDS-PAGE protein profiles of CFE and cell debris of WP1, MP2, MP4, SP10 grown in CDB containing individual metal salts:-
Pb : $Pb(NO_3)_2$; Cu : $CuSO_4$; Cd/N : $Cd(NO_3)_2$; Cd/S : $CdSO_4$.
C : Control; M : Molecular weight marker

of all three heavy metals. In addition, the expression of a 52.2 KDa protein in the presence of copper and a 43.3 KDa protein in the presence of cadmium nitrate was observed. A 35.5 KDa protein band, absent in the control, was also expressed in the presence of lead, copper, cadmium nitrate and cadmium sulphate.

Culture SP10 could only grow in presence of lead and showed very little growth in presence of cadmium sulphate. No change from that of the control was observed in the protein profiles of SP10 culture grown in the presence of metal.

Similar pattern of bands were observed in the cell debris of all cultures in all metals with a few extra bands of 70.8 and 63.1 KDa in the debris of control cultures of WP1 and SP10 respectively, while in MP4, a 56 KDa protein was expressed in presence of metals.

3D.3.2 Plasmid

The high A_{260}/A_{280} ratio is indicative that the DNA samples were pure (Table 3.2). A plasmid of 23.56 Kb was observed in the control culture of WP1, which was more prominent when grown under stressed conditions such as in presence of Pb^{2+} and more so in presence of $CdN0_3$; however, the band was repressed in the presence of copper. A plasmid of 23.566 Kb was observed in the control culture of MP2, which was more intense grown in presence of Cu^{2+}, but of lower intensity in the presence of lead. Similarly, a 22.06 Kb plasmid was detected in the control culture of MP4, which was more intense in presence of Cu^{2+} and $CdN0_3$; a very faint band was seen where the culture was grown in presence of Pb^{2+} and no plasmid was detected when grown in presence of $CdS0_4$. A 23.4 Kb plasmid was detected in SP10 control culture and when grown in presence of Pb^{2+} (Table 3.10 and Fig 3.13).

Table 3.8: Changes in protein bands when grown in presence of metals (KDa)

Culture	Control	Pb $(NO_3)_2$	$CuSO_4.5H_2O$	$Cd(N0_3)_2.4H_20]$	$3CdS0_4.8H_20$
WP1 (CFE)	89.1	Over exp	Under exp	Under exp	No growth
	80.5	Over exp	Under exp	Under exp	
	71.0	Over exp	Under exp	Under exp	
	66.1	Over exp	Under exp	Under exp	
	63.1	Over exp	As Control	Over exp	
	56.2	Over exp	As Control	Over exp	
	Absent	44.7	44.7	44.7	
	Absent	Absent	40.5	Absent	
	Absent	35.5	35.5	35.5	
WP1 Debris	70.8	Absent	Absent	Absent	
MP2 (CFE)	89.1	As control	Under exp	No growth	No growth
	80.5	As control	As control		
	71.0	As control	As control		
	68.0	As control	As Control		
	66.0	As control	As control		
	63.1	As control	As control		
	Absent	55.0	Absent		
	Absent	Absent	48.1		
	30.0	Under exp	As Control		
MP2 Debris	-	-	-		
MP4 (CFE)	56.1	As control	Under exp	As control	As control
	Absent	Absent	52.2	Absent	Absent
	50.0	As control	As control	As control	Under exp
	Absent	Absent	Absent	46.3	Absent
	39.8	As control	As control	As control	As control
	Absent	35.5	35.5	35.5	35.5
	31.6	As control	As control	As control	As control
	Absent	24.0	Absent	Present	Absent
	22.4	Under exp	Under exp	Under exp	Under exp
MP4 Debris	Absent	56.0	56.2	56.2	56.2
SP10 (CFE)	79.4	As control	No growth	No growth	No growth
	63.1	As control			
	56.0	As control			
	48.1	As control			
	39.8	As control			
	35.5	As control			
	31.6	As control			
	22.4	As control			
	17.8	As control			
	12.6	As control			
SP10 Debris	63.1	63.1			

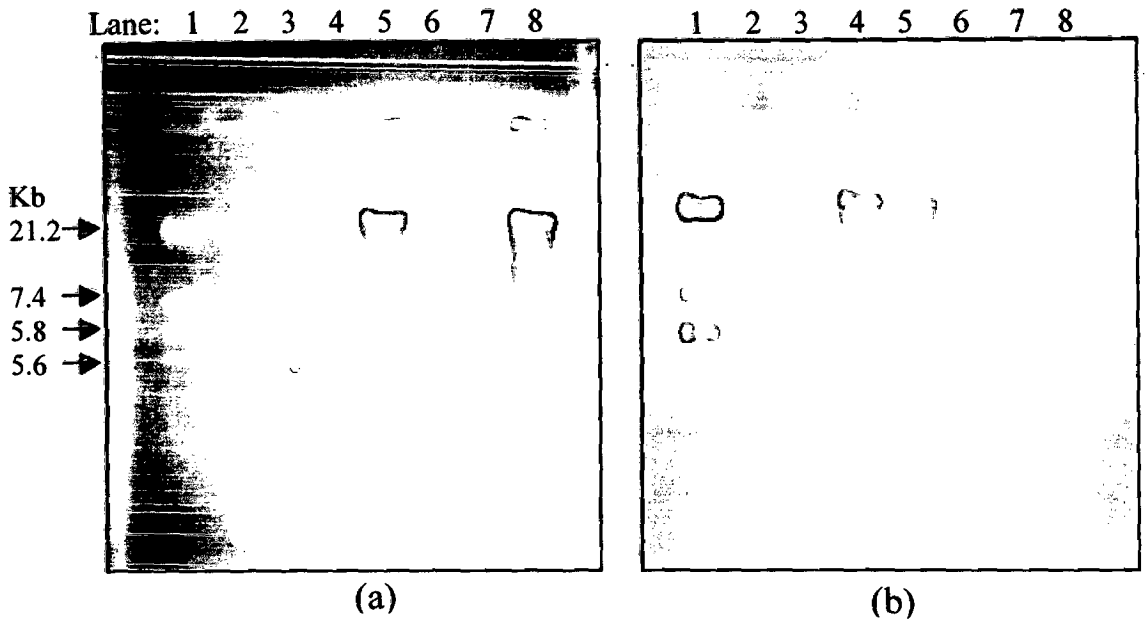
Table 3.9: Purity test of DNA samples

DNA samples from cultures grown in presence of metals				
Culture	Metal	A_{260}	A_{280}	A_{260} / A_{280}
WP1	control	0.438	0.221	1.98
	Pb $(NO_3)_2$	1.332	0.706	1.88
	$CuSO_4.5H_2O$	0.418	0.234	1.78
	$[Cd(N0_3)_2.4H_20]$	1.34	1.106	1.22
MP2	control	1.102	0.554	1.98
	Pb $(NO_3)_2$	1.083	0.450	2.4
	$CuSO_4.5H_2O$	1.095	1.10	0.995
MP4	control	1.325	1.205	1.09
	Pb $(NO_3)_2$	1.330	1.2	1.10
	$CuSO_4.5H_2O$	1.242	1.235	1.0
	$[Cd(N0_3)_2.4H_20]$	0.968	0.8	1.21
	3 $CdS0_4.8H_20$	0.746	0.495	1.5
SP10	control	0.722	0.425	1.7
	Pb $(NO_3)_2$	0.847	0.564	1.5

Table 3.10: Plasmid detection in cultures grown in presence of metals

Plasmid bands when grown in presence of metals				
Culture	WP1	MP2	MP4	SP10
Plasmid	23.56 Kb	23.56 Kb	22.06 Kb	23.4 Kb
Control	±	+	+±	+
Pb $(NO_3)_2$	+	±	±	+
$CuSO_4.5H_2O$	±	++	++	ng
$[Cd(N0_3)_2.4H_20]$	++	ND	++	ng
3 $CdS0_4.8H_20$	ND	ND	-	ng

ND: Not done; ng: no growth



Lane 1: Marker
Lane 2: WP1-control
Lane 3: WP1-Pb^{2+}
Lane 4: WP1- Cu^{2+}
Lane 5: WP1- Cd^{2+} ($CdN0_3$)
Lane 6: MP2-control
Lane 7: MP2- Pb^{2+}
Lane 8: MP2- Cu^{2+}

Lane 1: Marker
Lane 2: MP4- control
Lane 3: MP4- Pb^{2+}
Lane 4: MP4- Cu^{2+}
Lane 5: MP4- Cd^{2+} ($CdN0_3$)
Lane 6: MP4- Cd^{2+} ($CdS0_4$)
Lane 7: SP10-control
Lane 8: SP10 - Pb^{2+}

Fig 3.13: Plasmid profile of WP1, MP2, MP4, SP10 grown in presence of metals

3.4 Discussion

Fungi are present everywhere, and they often form a major and dominant component of the microbiota in soils and mineral substrates (Gadd, 1993). There are continuous changes occurring in the environment, and organisms under stress of the environment develop various mechanisms in order to cope with the adverse conditions. Fungal survival in 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 being difficult to determine (Gadd, 1993).

This chapter has given the mechanisms involved in metal sorption by the four *Penicillium* isolates selected: some factors affecting sorption of metals, their sorption isotherms and sequestration. It also describes the changes in cultural and morphological characteristics, the whole cell protein profiles, induction of proteins, and the plasmid bands in response to metals.

The physiological state of the organism, the age of the cells, the availability of micronutrients during their growth and the environmental conditions during the biosorption process, such as pH, temperature, and presence of certain cations, are important parameters that affect the performance of a living biosorbent (Cossich, et al., 2002). It is not possible to generalize the mechanisms of uptake, therefore, each of these factors need to be defined individually for each type of biomass and metal ion (Modak and Natarajan, 1995).

The representative culture, WP1, used in the first part of the study, showed distinct differences in the ability to sorb metals when grown in different growth medium and under different growth conditions. Lead sorption experiments indicated that the fungus grown in CDB and CDB*.G achieved maximum sorption capacity as compared to CDB + G or mineral salts glucose (MG) medium. CDB contains $NaNO_3$ as the nitrogen source while MG contains ammonium sulphate as nitrogen source. The nitrogen source in the form of $NaNO_3$ possibly increased the biosorption capacity of the biomass, while the carbon source in the form of glucose or sucrose showed not much difference as can be seen with CDB and CDB*.G having sucrose and glucose as the carbon source respectively. CDB contains sucrose and was therefore preferred as sucrose can be transported by some halotolerant and halophilic organisms, and this can enhance growth in higher NaCl, which is critical for stationary phase survival under salt stress conditions (Roberts, 2005).

The microbial growth conditions, such as growth media, significantly influences the composition of polysaccharides, thereby affecting metal removal (Tsezos, 1985; Muraleedharan et al., 1991; Dave, 1994; Andres et al., 2000; Zucconi et al., 2003). The cell walls of microorganisms consist mainly of polysaccharide groups such as carboxylates, hydroxyl, sulphate, phosphate and amino groups, which can bind metal ions (Gadd, 1993; Sayer et al., 1997). Ebner et al (2002) also showed that the type of nitrogen source in the cultivation media strongly influences the cell wall composition of fungi, the contents of chitosan and phosphorous in the cell wall being significantly higher with $NaNO_3$ as the nitrogen source, in comparison to the culture grown with peptone, and therefore the greater biosorptive capacity for chromium.

Sorption was higher when the SC production estimated in terms of the constituent protein, carbohydrate and lipid was higher. This indicated the involvement of the SC in metal sorption. Ianis et al (2006) showed that the polymers of cell walls are involved in metal binding. The cell-surface components that bind metals include chitin and chitosan and probably some other glycans of fungal cell walls (Gadd, 1990). The culture grown under stationary conditions achieved a relatively better sorption of metal where the SC production is more than that grown under shaker conditions at the same growth period. However, the sorption ml^{-1} pcv at 48h was comparable to that grown under shaker conditions.

The age at which the biomass is harvested is another significant parameter that affects the biomass metal uptake capacity (Tsezos, 1990). The 48h grown culture achieved the maximum metal sorption, the metal sorption capacity desreasing with further incubation under both stationary and shaker growth conditions. Ã‡eribasi and Yetis (2001) also selected the 41h cultivation period based on the findings of the previous studies which indicated that the maximum biosorption capacities were attained after 41 hours of incubation. However, it was also reported that the the maximum uptake of lead by the mycelial biomasses of *Paecilomyces lilacinus* was achieved during the lag periods or the early stages of growth and declines as the culture reach the stationary phase (Zucconi et al., 2003). Similarly, Mattuschka et al (1993) also found that younger cells in yeast and fungi accumulated approximately 80% of copper than the cells from the stationery growth phase.

Experimental results by different workers revealed that the pH of the solution greatly affects the uptake of ions (Maruthi Mohan et al., 1984; Luef et al., 1991; Muraleedharan et al., 1991; Mattuschka et al., 1993; Dave, 1994; Modak and Natarajan, 1995; Poole, 1995; Sag and kutsal, 1995; Ianis, et al., 2006). The sorption of lead by WP1 was maximum between pH 3.0 and pH 5.6 for removal of Pb^{2+} and Cu^{2+}, while removal of cadmium was maximum at pH 5.6. Vijayaraghavan et al., (2004) reported that the copper uptake was found to increase with increasing pH, to the maximum near pH 5.5, then decreased at higher pH value. Similarly, the largest amount of copper removed from solution was with initial pH of the solution at about 4.5 (Ianis, et al., 2006). Other results showed that pH 5 was optimum for the biosorption of lead (Fourest and Roux 1992; Niu, 1993; Ã‡eribasi and Yetis, 2001). Generally, metal uptake increases with the increase in pH, from pH 3 to 5, and an optimum was reached when the uptake is maximum, beyond which, from pH 5 to 7, a reduction in the uptake was observed, attributed to reduced solubility and precipitation of base metals. However, the uptake of precious metals and radionuclides are reported at alkaline pH of 8 to 10 (Modak and Natarajan, 1995).

The optimum pH selected for the experimental purpose was 5.6 as biosorption of heavy metals usually leads to the acidification of solutions (Volesky and Holan, 1995). Experimental results indicated a drop in metal removal when the biomass was incubated for 1h. As the pH is lowered, overall surface charge on the cells become positive which will inhibit the approach of positively charged metal cations (Sag and Kutsal, 1995). Extreme pH is not beneficial for cellular life and biomolecules. It affects the structure and function of proteins and enzymes. If proteins are exposed to low pH and high temperature for a long enough time, it will start to spontaneously hydrolyze, or breakdown to

component amino acids (Lodish et al., 1995). According to Sag and Kutsal (1995), the different pH binding profiles for heavy metals could be due to the nature of the chemical interactions of each metal with microbial cells and are related to the isoelectric point of the cell. At pH values above the isoelectric point, there is a net negative charge on the cells and the ionic state of the ligands such as carboxyl, phosphate and amino groups will be such as to promote reaction with the metal cations (Sag and Kutsal, 1995).

Metal precipitation in solution was observed at pH 7.0 and 9.0. Metals precipitate at neutral to alkaline pH making them unavailable for biosorption (Pethkar and Paknikar, 1998). Working over pH 6.0 is avoided to prevent the possible precipitation of metals (Yin, et al., 1999; Vijayaraghavan et al., 2004). The pH affects metal toxicity because many metal ions form complexes with various medium or buffer components or may be precipitated by phosphates, especially at pH near neutrality or higher. For example, Malakul et al (1998), had to buffer their medium with Tris-HCl instead of phosphate buffer, to avoid precipitation.

Metal sorption by the mycelia takes place rapidly within 1 min and therefore could be a physical process (Modak and Natarajan, 1995). Studies by Butter et al (1998) showed that the metal uptake reaction by *Penicillium cyclopium* was extremely rapid during the first 15 seconds of the copper biosorption, becoming progressively slower until equilibrium was achieved. Kowshik and Nazareth (1999) also reported that the maximum sorption of tested metals by *Fusarium solani* occurred within 1-4 min. However, at 60 min, metal removal dropped, which could be due to lowering in pH with time due to the acidification of the solution (Volesky and Holan, 1995). Lower pH leads to stripping off

of bound metal ions, which are strongly pH dependent. Studies by Ã‡eribasi and Yetis (2001) on *Phanerochete chryosporium* revealed that the pH decreased sharply in the first minutes upon contact with the solution, parallel to the fast metal uptake; some portion of the metal ions adsorbed to the surface of the bisorbent during the first rapid sorption phase were released to the solution till equilibrium was reached. Thus, the equilibrium for metal removal and sorption capacities were less than the maximum values reached during the first phase and the contact time required for the maximum total metal removal was generally 30 min.

The biosorption process involves a solid phase, that is the biological material which functions as the biosorbent and a liquid phase which is the solvent, normally water, containing a dissolved species to be sorbed or sorbate. such as the metal ions. Due to higher affinity of the sorbent for the sorbate species. the latter is attracted and bound there by different mechanisms. The process continues till equilibrium is established between the amount of solid-bound sorbate species and its portion remaining in the solution at a residual. final or equilibrium concentration (Cf). The degree of sorbent affinity for the sorbate determines its distribution between the solid and liquid phases (Volesky, 1999).

The metal sorption isotherms of the four selected *Penicillium* isolates. to determine the maximum metal uptake capacity. indicated that sorption continues till equilibrium is established between the amount of solid-bound sorbate species and its portion remaining in the solution. thereafter it reaches a plateau. The adsorption isotherm is often hyperbolic as the biosorbent uptake approaches saturation of the sorbing material (Nivogi et al.. 1998; Pinghe et al.. 1999). However. the affinity for each metal and sorptive capacities of

each isolates is significantly different. Sorption maxima for metals by WP1 decreased in the following order: $Cu^{2+} > Pb^{2+} > Cd^{2+}$ (nitrate), by MP2 it was: Cd^{2+} (sulphate) > Cu^{2+} > Pb^{2+}, by MP4 is: $Pb^{2+} > Cu^{2+} > Cd^{2+}$ (nirate) > Cd^{2+} (sulphate) and by SP10: Pb^{2+} > Cd^{2+} (sulphate), thus showing a difference in the sorption capacity between morphotpes of same genus. The fungus, *Cladosporium cladosporioides* 1 and *C. cladosporioides* 2 also showed different metal biosorption properties, where Strain 1 showed preferential sorption of gold and silver, while strain 2 could bind metals such as copper and cadmium in addition to gold and silver (Pethkar et al., 2001).

The uptake capacity for Pb^{2+} is similar for all cultures, and differs for other metals; interestingly, although the metal uptake capacity was more by WP1 and MP2, than for MP4 and SP10, the tolerance to the tested metals was higher in MP4. Studies suggested a correlation between tolerance and a decreased metal uptake. A metal resistant yeast strain also showed a decreased influx of copper, cadmium and lithium (Cervantes and Gutierrez-Corona, 1994). Similarly, reduced copper uptake was reported in a copper-tolerant strain of *A.pullulans* as compared to a sensitive strain. In *A.nidulans*, a restricted uptake of copper was proposed as a mechanism responsible for copper resistance (Cervantes and Gutierrez-Corona, 1994).

The mechanisms of fungal defense against metal toxicity are chemical, biochemical, and physiological, including extracellular metal sequestration and precipitation, metal binding to the walls, intracellular sequestration and complexation, and compartmentation or volatilization (Sayer et al., 1995). The sorbed metals may be cell bound and also accumulated intracellularly (Brierly, 1990; Kowshik and Nazareth, 1999).

All four species of halotolerant *Penicillium* achieved efficient removal of metals such as lead, copper, cadmium as nitrate and as sulphate, iron and manganese. *Penicillium* spp are well known to sorb heavy metals (Gadd, 1993; Niu, et al., 1993; Fourest, et al., 1994; Natarajan, et al; 1999; Skowronski, et al., 2001; Tan and Cheng, 2003; Cabuk, et al., 2005). As seen from the rate of reaction studies, maximum sorption is rapid and takes place within 1 min for all cultures, implicating a physical process that does not require the microorganism's active metabolism for sorption to take place (Volesky, 1994). Galun et al., (1983) also showed that the mechanism at work with certain *Penicillium* species is most probably a passive binding of metal ions, because of both the high speed of uptake, which would preclude metabolic activity, and the observation of no loss of uptake ability with the death of the cell. The cell wall sorption of the tested *Penicillium species* could be one of the biological mechanisms implicated in fungal survival (Gadd, 1993). Suh et al (1999) reported that the penetration time of Pb(II) accumulation in *Saccharomyces cerevisiae* associated with the intracellular region is 2 hours, while that of extracellular region takes place within 3 minutes, thus showing that a longer incubation period is probably required for intracellular accumulation by the cultures, given that at a sufficiently long enough contact time, living cells are expected to accumulate higher amounts of dissolved metal ions, since both active and passive mechanisms of metal uptake may operate simultaneously (Balakrishnan et al., 1994).

Metal removal by the four *Penicillium* species revealed that 85-93 % of the metal sorbed is by extracellular or cell-bound accumulation, with intracellular accumulation of heavy metals being only 7-15 % accumulation of the total sorbed metal. This explains the rapid removal of metal from solution indicated above. Studies on the total copper ions taken up

by *Penicillium cyclopium* revealed that upto 75 % were deposited on the cell surface during the first 5 min while the rest 25 % were bound to the cells during the next 50 min of the process (Ianis, et al., 2006). Similarly, investigations on the kinetics of copper ion biosorption by free cell suspensions of inactivated *Penicillium* biomass showed that the copper biosorption reaction reached approximately 90 % of the equilibrium position in one min (Butter et al., 1998), indicating a passive sorption to the cell wall.

The metal sorption is probably a physicochemical interaction between the metal and the functional groups present on the microbial cell surface, the microbial cell walls composing mainly of polysaccharide, proteins and lipids which have abundant metal binding groups such as carboxyl, sulphate, phosphate and amino groups (Tan and Cheng, 2003; Ianis et al., 2006). The carboxylic groups present on the fungal cell wall of different species of *Rhizopus, Mucor, Penicillium* and *Trichoderma* contributes from 30 to 70 % of the total heavy metal binding at the fungal wall, depending on the species (Ianis, et al., 2006). As seen from results of the optimisation studies for growth of the representative isolate, WP1, when the SC production consisting of carbohydrate, protein and lipid was more, metal sorption had also increased proportionately. The electrostatic attraction between groups also plays an important role in this process. The biological mechanisms implicated in fungal survival include biosorption to cell walls and extracellular polysaccharides are (Gadd, 1993). The relative affinity of different metals to bind to fungal cell walls also depends on the chemical composition of the cell wall, and thus, the fungal species involved (Kapoor and Raghavan, 1995). Since the chemical composition of fungal cell walls can vary considerably between different species, there may be considerable differences in adsorption capacity between species, strains and even

different cell types of the same organisms (Ilhan et al., 2004). This difference in the biosorption efficiency was also observed in the four *Penicillium* isolates for each metal.

Results showed that all four *Penicillium* isolates had a net preference for copper, followed by cadmium (sulphate). The ease of binding of the metals depends on the atomic weight, ionic radius and the charge of the metal, higher the charge, faster will be the binding due to greater ionic interaction (Volesky, 1994). Rama Rao, et al (2005) also reported that Cu^{2+} binds most efficiently (72%) to the biosorbent followed by Cd^{2+} (61%), Co^{2+} (49%) and Ni^{2+} (37%). The preference for copper could be related to the fact that fungal MTs were reported to contain exclusive copper ions, and studies indicated that the incorporated copper in fungi was bound to low molecular weight ligands in the mycelia. The copper resistance mechanisms can also include copper complexing by cell wall components and changes in membrane copper transport (Fourest et al, 1994).

The response to metal indicated high tolerance to lead in all four isolates of *Penicillium*, however, sorption of lead was the least by MP2 and MP4, while for WP1 and SP10, the minimum sorption was cadmium nitrate followed by lead, indicating a correlation between tolerance and an altered metal uptake. Gadd et al., (1984) demonstrated the decreased uptake of copper by a copper-tolerant strain of *S.cerevisiae* was due to changes in membrane transport properties rather than to alterations in cell wall permeability.

Fungal cell walls are mainly composed of mannoproteins, 1,3-β-glucan and chitin. Depending on the species, additional polymers such as 1,3-α-glucan or 1,6-β-glucan polymers may be present. It contains up to 10-30 % of the cell wall dry weight and chitin

contributes significantly to the mechanical strength of the cell wall. Both chitin and α1, 3-glucan biosynthesis are induced in response to cell wall stress most likely as a response mechanism of the fungal cell to change the composition of the cell wall, making it more resistant to cell wall disturbing compounds (Ram et al., 2004). In filamentous fungi the response to sub-lethal levels of cell wall stress can result in morphological abnormalities such as swollen apical tips (Vankuyk, 2004). Heavy metal contamination is one such stress condition which has increased sharply since 1900 (Nriagu, 1979) due to increasing industrialization. Cultural and morphological changes are also induced in response to metal stress (Babich and Stotzky, 1982; Venkateswerlu et al., 1989; Kowshik and Nazareth, 2000; Ram et al., 2004; VanKuyk et al., 2004; Fomina et al., 2005).

With increase in heavy metal concentration, the nature of growth of all four *Penicillium* isolates became more compact. This was also observed in *B. caledonica* where loose mycelium was observed in the control, while the morphological responses of the colonies to toxic metal after 3 weeks of growth at 25°C showed dense mycelial growth at the margins on media containing lead phosphate and cuprite (Fomina et al., 2005). Except for MP4, growth of the cultures in presence of very low concentrations of copper and cadmium reduced drastically; the appearance also turned filamentous with pronounced morphological changes. The biomass production is restricted by the continuous imposition of stress, while severe stresses may be tolerated by fungi which either possess appropriate physiological characteristics, or can adapt through a temporary alteration in their developmental pattern (Cooke and Whipps, 1993). The bioaccumulation of heavy metals is also closely connected with their toxicity which restrains metabolism and growth of the microorganisms (Zlatarov and Yakimov, 2001). The lag or inhibition of the

growth rate of the cultures in presence of metals may be due to the build up of toxic products of heavy metals, or probably due to the decrease or inhibition of spore germination. It is suggested that cadmium enter the spores, get associated with the particulate and soluble cytoplasmic components, reacts with cytoplasmic receptor sites and this reaction internally inhibits spore germination (Babich and Stotzky, 1978) which was evidenced on growth at increasing metal concentrations.

Microorganisms respond differently to heavy metals depending on the toxicity and the concentration of the heavy metal, and on the resistance mechanisms of the organism (Fourest and Roux, 1992). Tolerance to metal can be due to the different strategies employed by the organisms like adsorption on cytoplasmic membrane, sequestration by EPS, and chemical transformations from more toxic to less toxic forms (Starkey, 1973). The critical and threshold concentrations also depend on the type of the microorganism and the chemical form of the metal. In addition, the metal toxicity is also affected by the form in which they exist and the amount of cells in the medium (Dave, 1994; Zlatarov and Yakimov, 2001). Lead nitrate, lead oxide and tetraethyl lead have different affinities for microbes, thereby exerting different toxicities, for example, tetraethyl lead induced giant formation of multinucleate cells in a chrysophycean flagellate but lead nitrate had no such effect (Babich and Stotzky, 1982).

All four isolates resisted lead at a high concentration of 7.5 mM. Morphological changes in presence of lead was very less, while culturally, the changes in growth pattern, sporulation and pigment production was observed. In all tested metals, the increase in metal concentration increased the adverse microbial response, however the inhibitory

effects were reduced at a higher concentration of 5.0 mM lead in both MP4 and SP10. Similar results were observed in *Haematococcus capensis* whereby 0.1ppm lead inhibited its growth, but the inhibitory effects were reduced or eliminated at a higher concentration (Babich and Stotzky, 1982). The cultural response, such as pigment production, may have combated the metal stress by chelation, thereby increasing the tolerance to lead, hence not much changes in the morphological characteristics in presence of lead was observed.

Detoxification is one of the resistance mechanisms in microbial cells, which is achieved by production of extracellular organic material, for example, chelation of lead by citric acid secreted by *A. Niger*, and production of red pigments by *Pyrenophora avenae* that chelate phenyl-mercury thereby reducing its uptake (Babich and Stotzky, 1982). MP4 showed a higher tolerance to Cu^{2+} and Cd^{2+} both as its sulphate and nitrate form. The higher tolerance of MP4 may be related to an increase in pigment production diffusible into the medium, which probably chelated the metal ion, along with the significant morphological changes may have combated the metal stress to some degree. However, at high heavy metals concentration, and in presence of more toxic metals such as, copper and cadmium, growth was severely restrained which was observed as a prolonged lag-phase, along with significant morphological changes. Babich and Stotzky (1982) and Zlatarov and Yakimov (2001) also reported that growth at high heavy metals concentration is severely restrained, and can be observed as a prolonged lag-phase. In contrast to MP4, pigment production was arrested in the other three isolates in presence of copper and cadmium wherever growth was observed, resulting in lower tolerance to metal. The lag phase along with changes in the morphology could be a resistance

mechanism by intracellular detoxification, wherein cells try to repair the damage caused by the metal and redistribute the metal to the cell wall (Babich and Stotzky, 1982).

Kowshik and Nazareth (2000), reported that the *Fusarium* culture grown in mineral glucose medium produced an extracellular orange red pigment and could grow/tolerate higher amount of toxic metals, while that grown in sucrose nutrient medium where no pigment was produced showed lower tolerance or no growth was observed. Similarly, Pethkar and Paknikar (1998) obseved that the fungal biomass of *C. cladosporioides* harvested before pigment production adsorbed less than 30 % gold, while the biosorption efficiency after pigment production was 80 %. According to Babich and Stotzky (1982), one of the resistance mechanism is the prevention of intracellular uptake by plasmids conferring resistance to cadmium or copper by cell membrane impermeable to the metal.

The pronounced morphological changes observed in response to stress towards the maximum tolerance level by WP1, MP2, MP4 and SP10 were thickened and bulbous hyphal cell walls with frequent septation which was observed in presence copper and cadmium. As the metal concentration increases, penicilli were also not fully formed. The bulbous hyphal cells and stunted growth were more in presence of cadmium sulphate than cadmium nitrate at 1 mM concentration as seen in MP2 and SP10, probably because of the toxicity even at low concentrations, and consequent lower tolerance. However, these bulbous structures were not observed with the triverticillate culture, that is, MP4, possibly because of other mechanisms of resistance as seen by the increased thickening of the cell wall possibly by chitin accumulation. When chitin synthesis is affected, growing hyphae tend to lyse and form pronounced bulges unless the osmolarity of the

medium is increased. Cell wall damage caused by mutations in cell wall-related genes result in hyperaccumulation of chitin. Inability of the cells to respond to cell wall damage by increasing chitin levels, either by disrupting the major chitin synthase-encoding gene or by adding the chitin synthase inhibitor, results in cell lysis, indicating the importance of the chitin response to prevent cell death. In *A. niger* an increased chitin level in the cell wall in response to cell wall stress is accompanied by increased transcription levels, further indicating that both yeasts and filamentous fungi respond to cell wall stress by activating the chitin biosynthetic pathway (Ram, et al., 2004). Tsezos (1990) also reported that chitin, a structural acetylated aminopolysaccharide of the cell wall, is the most important cell wall component of the fungal biomass, with respect to uranium biosorptive uptake.

Similarly, scanning and transmission electron microscopy of *Cunninghamella blakesleeana* grown in the presence of toxic concentrations of copper and cobalt indicated that copper, but not cobalt or the control, induced both morphological and ultrastructural changes; the hyphae of copper-grown cultures (called "blue mycelia") were larger in diameter, had a rough and granular surface, and the cell wall was thicker. The cytoplasm of the blue mycelia was also abnormal and was in a compressed state (Venkateswerlu, et al., 1989). Cervantes and Gutierrez-corona, (1994) also reported the copper resistant strain of *Trichoderma viride* cultured in presence of toxic concentrations of copper produced about five-fold thicker cell walls.

These morphological changes indicate that the metals taken up are possibly cell bound, possibly some resistance mechanisms whereby the fungus is adapting the cell wall for metal sorption in order to prevent intracellular intake leading to toxicity of the cell.

Fusarium solani is also reported to show morphological changes, such as, increase in number of spores, thickened cell wall, bulbous hyphae and changes in the shape and size of the cultures in presence of metals (Kowshik and Nazareth, 2000). Similar observations were made by other workers, where the cells of a lignolytic white-rot fungus, *Phanerochaete chrysosporium* grown in presence of Pb(II) and Ni(II) have a somewhat spherical and bead-like shape with a diameter of about 1.5 to 2 mm (Ã‡eribasi and Yetis, 2001). Sub-lethal concentrations of Calcofluor-white (CFW) showed similar morphological effects of swollen apical tips in both *Aspergillus niger* and *Penicillium chrysogenum* (Vankuyk, 2004). Katarzyna, (2004) also reported that the morphology of mycorrhizal structures was significantly altered in presence of heavy metals. Arbuscules were often strongly septated or their branching was reduced and the accumulation of lipid like-bodies was observed.

All four isolates of *Penicillium* tolerated very high concentrations of Fe^{2+} upto 30 mM and Mn^{2+} upto 150 mM, showing a lag and significantly reduced growth with delayed sporulation at higher concentration. Heavy metals are known to inhibit the formation and germination of fungal spores (Babich and Stotzky, 1982). Contrasting to heavy metal tolerance where MP4 could resist higher concentrations as compared to WP1/MP2/SP10, MP4 resisted lower concentration of Fe^{2+} upto 20 mM only and delayed growth at 75 - 150 mM Mn^{2+}. The extended lag phase could be due to the cells repairing the damage

caused by the metal and redistributing the metal to the cell wall (Babich and Stotzky, 1982). As in heavy metals, pigment production was also observed in presence of both Fe^{2+} and Mn^{2+} upto high concentrations. The high tolerance to Fe^{2+} and Mn^{2+} may be the operation of one or more factors operative individually or in combination to combat metal toxicity.

Fe^{2+} and Mn^{2+} are required nutritionally by microbial cells in trace amounts, however, at elevated concentrations, can be highly toxic to microorganisms, hence their uptake must be regulated carefully by the cells. The uptake of metals can be harmless, toxic or even beneficial for the normal growth, development and reproduction of organisms. Copper, iron, manganese, and molybdenum can exist in more than one oxidation state in cells, for example Iron can exist as Fe (II) and as Fe (III), and can catalyse essential life processes that involve electron transfer (for example, photosynthesis, respiration and nitrogen fixation). Acidity or alkalinity of the medium can moderate the toxicity of metals, as a lower pH may increase the bioavailability of the metal ions resulting in increased toxicity, while an increase in pH could precipitate the metal making it unavailable for cellular use. In addition, changes in pH may also alter the oxidation state of the metal cations, making them unrecognisable to membrane chemoreceptors or transport proteins, and changing the oxidation state may also change their solubility, for instance, Fe^{2+} is soluble while Fe^{3+} is very insoluble.

Similarly, as with heavy metals, the morphological changes, such as, bulbous hyphal cells, unformed or even absence of penicilli heads, compact growth with thickened mycelia, were also observed in presence of Fe^{2+} and Mn^{2+}.

The protein profiles obtained by SDS-PAGE of the mycelia revealed that there are significant changes in the protein profiles when the cultures are grown in presence of metals. These changes are observed as additional protein bands and over/under expression of protein bands in comparison to the profile of the control culture grown in absence of heavy metals. Choudhury and Kumar (1998) also reported the synthesis of a 14-KDa periplasmic protein was increased when they were grown in presence of 10 mM Cu^{2+}. These changes in the protein profiles could be a resistance mechanism to the presence of toxic metals in the medium. The expression of new protein absent in the control cultures usually implies the synthesis of phytochelatins or metallothioneins, which was observed in fungi like *N.crassa, Dactylium dendeoides* and *S.cerevisiae* (Cervantes and Corona, 1994). However, the molecular weights of the new proteins synthesised, which was absent in the control of the four *Penicillium* isolates studied were more than 30 KDa, hence it possibly implies the synthesis of other metal binding proteins which conferred resistance to lead, copper and cadmium. A copper-resistant (10 mM-Cu) fungus identified as *Beauveria bassiana* also showed additional resistance towards zinc, cadmium and lead (Kameo et al., 2000). An organism under any other condition of stress causes the induction of stress proteins. Langlois et al (2003) studied the bacterial gene expression due to nutritional stress in the culture medium supplemented with *L. minor* fronds and observed that the proteins repressed under normal conditions were synthesized under starvation stress conditions, and reflects the bacterial adaptation to changes in the medium conditions or growth phase. Cooke and Whipps (1993) also reported that specific proteins are synthesized rapidly when cells are exposed to supraoptimal but non-lethal temperatures, anaerobiosis, ethanol, heavy metals and certain antibiotics. New

proteins also appeared in ascospores of *Neosartorya fischeri* in response to an increased heat tolerance, which develops with age.

Some of the main mechanisms involved in the resistance mechanism / metal detoxification are chelation of metal ions in the cytosol with thiol-containing compounds, such as glutathione (GSH), phytochelatins (PCs), or metallothioneins (MTs) (Courbot, et al., 2004). These MTs (including PCs in algae, plants and some fungi) plays a role in the detoxification of nonessential metals because of their high affinity for these metals (Mali and Bulow, 2001; Macaskie and Dean, 1990), and therefore the organisms show some degree of resistance or tolerance to the toxicity of metals and metalloids (Hamer, 1986). Fungal MTs contain exclusive copper ions, and reports indicated that the incorporated copper in fungi were bound to low molecular weight ligands in the mycelia (Vatamaniuk, et al., 1999) whereas PCs are reported to primarily bind Cd^{2+} with high affinity, and they play a pivotal role in Cd^{2+} tolerance in plants and some fungi by chelating these substances and decreasing their free concentrations (Vatamaniuk, et al., 1999). So far, the only fungus known to use both MTs and PCs for metal detoxification is *Candida glabrata*, which produces MTs when exposed to toxic concentrations of Cu but produces mainly phytochelatins in response to Cd stress (Gardea-Torresdey, et al., 1998; Hall, 2002; Courbot, et al., 2004).

MTs can be induced by Cu treatments and there is evidence for a role in heavy metal tolerance in fungi and animals (Hamer, 1986). The copper resistance mechanisms can include copper complexing by cell wall components, changes in membrane copper transport, synthesis of intracellular copper binding metallothioneins and phytochelatins

and productions of extracellular copper complexing or precipitating metabolites (Fourest et al, 1994).

The four *Penicillium* isolates may have evolved different mechanisms to tolerate excess metals and that even between morphotypes of the same genus more than one mechanism could be in operation. Microorganisms differ in their response to heavy metals depending on the toxicity, the concentration of the heavy metal and the resistance mechanisms of the organism (Fourest and Roux, 1992), and may rely directly and/or indirectly on several survival strategies, for example, metallothionein synthesis is a mechanism of copper resistance in *S.cerevisiae,* yet copper precipitation around the cell wall and intracellular transport are also components of the total cellular response (Gadd, 1993).

Microbial species generally express only that part of their genome that enables them to be structurally and functionally adjusted to a certain set of conditions. The plasmid isolated from the control cultures of WP1, MP2, MP4 and SP10 showed either increasing intensity or the intensity of the bands decreased under stressed conditions. The increase in intensity of the bands implies the amplification of the plasmids. WP1 and MP4 grown in presence of cadmium nitrate, and MP2 and MP4 in presence of copper showed increased intensity of the plasmid bands, indicating that the sorption of copper by MP2 and MP4 and cadmium nitrate by WP1 and MP4 could possibly be plasmid mediated, through the synthesis of metal binding protein. The plasmid was more intense in presence of lead in WP1 but of lower intensity in MP2 and MP4, hence the probable over expression of proteins in WP1 in presence of lead. No plasmid was detected in presence of cadmium sulphate in MP4. Culture SP10 showed no changes in the protein profile of the CFE, or in

the plasmid copy number in presence of lead, however, a thickening of the cell wall was observed in presence of increasing concentration of lead, which could probably be due to the increase in chitin level.

Plasmid mediated resistance to heavy metals is also seen in organisms like *Alcaligens eutrophus*; the resistance to copper being determined by large plasmids. The resistance to copper by *Pseudomonas* strains are also reported to be of probable plasmid origin. Amplification of genes in order to detoxify metal toxicity was observed in copper resistant strains of *S.cerevisiae*, whereas copper sensitive strains contained only one copy of the sequence (Cervantes and Corona, 1994).

3.5 Conclusions

The fungus grown in CDB as the growth medium at 48 h under shaker conditions achieved maximum sorption ml^{-1} pcv; biochemical analysis of the SC also indicated higher SC production in terms of protein, carbohydrate and lipid, contributing to higher sorption of metal. The maximum metal removal by grown mycelial mass was observed at pH 5.6. The age at which the biomass is harvested and pH are significant parameters that affect the biomass metal uptake capacity.

All four isolates of halotolerant *Penicillium* achieved efficient removal of metals such as lead, copper, cadmium as nitrate and as sulphate salt, iron and manganese. The maximum sorption took place within 1 min for all cultures, indicating a passive binding of metal ions which does not require the microorganism's active metabolism for sorption to take place, and is therefore a physical process.

The affinity of the mycelial mass for each metal, and the sorptive capacities of each isolates for each metal is also significantly different, thus showing a difference in the sorption between morphotypes of the same genus.

The grown cells of all four *Penicillium* isolates can sorb all the tested heavy metals from solution though some cultures could not tolerate either Cu^{2+} or Cd^{2+} (nitrate) or Cd^{2+} (sulphate), therefore it has its advantage in that the grown cells can be effectively used for metal sorption, though it cannot tolerate or when growth in presence of heavy metals is fairly low.

Though the tolerance to the tested metals was highest in MP4 as seen in the preceding chapter, the metal uptake capacity was more for WP1, MP2 and SP10 than for MP4 indicating a correlation between tolerance and a decreased metal uptake, whereby the decreased uptake by the metal-tolerant strain may be due to changes in membrane transport properties rather than to alterations in cell wall permeability.

The sorbed metals were mainly cell bound for all four *Penicillium* isolates, with very little intracellular accumulation of heavy metals, being only 7-15 % accumulation of the total sorbed metal. Biosorption to cell walls is one of the biological mechanisms implicated in fungal survival. Some intracellular accumulation of Pb^{2+} was observed in all four *Penicillium* isolates, while little / no intracellular uptake of Cu^{2+} was observed. Except for WP1, there is no intracellular uptake of Cd^{2+} (nitrate) in the other three isolates, while for Cd^{2+} (sulphate), some intracellular accumulation was observed only in WP1, MP4 and SP10.

Cultural and morphological changes were induced in presence of increasing metal concentration as compared to the control. All four isolates could resist Pb^{2+} at a much higher concentration as compared to Cu^{2+} and Cd^{2+}, while the tolerance level to the transition elements, Fe^{2+} and Mn^{2+} was very high. Distinct cultural changes in terms of growth pattern, sporulation, as well as pigment production and / or pronounced morphological changes such as, unformed or absence of penicilli heads, thickened and/ or bulbous hyphal cells were observed. Pigment production and the morphological changes could be mechanisms for external sorption of metals by the cells which could possibly

indicate the accumulation of chitin. These cultural and morphological changes in presence of toxic metals are survival mechanisms of the *Penicillium* isolates.

The protein profiles obtained by SDS-PAGE of the mycelia showed distinct changes when grown in the presence and absence of metal salts, characterised by new bands and the overexpression of protein bands in response to metal. The expression of new proteins absent in the control cultures could imply the synthesis of metal binding proteins. Similarly, an increase / decrease in intensity of plasmid bands were observed in presence of metal. These changes in the protein and plasmid profiles are also resistance mechanisms of the *Penicillium* isolates to respond to the harmful effects of heavy metals; the plasmid mediated resistance being determined by the amplification of genes inorder to detoxify metal toxicity as seen with the increase in the inensity of the plasmid.

WP1 in presence of all the heavy metals tested showed some intracellular accumulation. Morphologically, very little change was observed in presence of Pb^{2+}, while distinct changes such as unformed or absence of penicilli heads, thickened and/or bulbous hyphal cells were observed in presence of Cu^{2+} and Cd^{2+}(nitrate). An increase in diffusible pigment was observed in presence of Pb^{2+}, while sporulation and pigment production was inhibited in presence of Cu^{2+} and Cd^{2+}(nitrate). However, the protein and plasmid response differs in presence of the different heavy metals. The culture in presence of Pb^{2+} showed over expression of proteins and new bands which appear to be plasmid mediated, as observed with the increased in intensity of the plasmid, while in presence of copper the proteins showed under expression of bands with the synthesis of new proteins which may not be plasmid mediated as there was no amplification of the plasmid in presence of

copper. When the culture was grown in presence of Cd^{2+}(nitrate), some proteins showed over expression, while some showed under expression in addition to new protein bands which appear to be plasmid mediated. WP1 could not tolerate cadmium as its sulphate salt.

The culture MP2 showed very little intracellular accumulation only in presence of Pb^{2+}, with not much morphological changes and an increase in diffusible pigment was observed in presence of Pb^{2+}. However, in presence of Cu^{2+} and Cd^{2+}(sulphate), pronounced changes such as unformed or absence of penicilli heads, thickened and/or bulbous hyphal cells were observed along with inhibition of spores and pigment. As with WP1, the protein and plasmid response differs in presence of each of the heavy metals. The culture grown in presence of Pb^{2+} showed the expression of new proteins with a reduced intensity of the plasmid band, and therefore may not be plasmid mediated, while in presence of copper the expression of new proteins may be plasmid mediated, the amplification of the plasmid being observed. The protein/plasmid response in presence of Cd^{2+}(sulphate) could not be studied as the amount of mycelial mass produced was too little. MP2 could not tolerate cadmium as its nitrate salt.

The culture MP4 showed some intracellular uptake in presence of Pb^{2+}, Cu^{2+} and Cd^{2+} (sulphate). Morphologically, not much changes was observed in presence of all four heavy metals tested. Culturally, the presence of diffusible pigment with decreased spores was observed in presence of Pb^{2+} and an increase in diffusible pigment in presence of Cu^{2+}. A non-diffusible pigment with inhibition of spores was observed in presence of Cd^{2+}(nitrate), while in presence of Cd^{2+} (sulphate) inhibition of spores/pigment was

observed. The protein profiles of MP4 in presence of the different heavy metals showed similar expression of protein bands as compared to the control, in addition to new proteins which differs in presence of each metal. The culture in presence of Pb^{2+} showed repression of some proteins. Amplification of the plasmid was not observed, hence the expression may not be plasmid mediated, while in presence of Cu^{2+} and Cd^{2+} (nitrate), it may be plasmid mediated as observed with the increase in intensity of the plasmid.

The culture SP10 could only tolerate Pb^{2+} and Cd^{2+} (sulphate) with some intracellular accumulaion observed in presence of both these metals. Morphologically, not much changes was observed in presence of Pb^{2+}, while distinct changes such as unformed or absence of penicilli heads, thickened and bulbous hyphal cells were observed in presence of Cd^{2+}(sulphate). A non-diffusible pigment with decrease in sporulation was observed in presence of Pb^{2+}, while sporulation and pigment production was inhibited in presence of Cd^{2+}(sulphate). No difference from the control was observed in the protein or plasmid profiles of the culture in presence of Pb^{2+}. The protein/plasmid response in presence of Cd^{2+}(sulphate) could not be studied as the mycelial mass produced was too little.

From the above, it is seen that each isolates react differently in presence of the heavy metal studied, and the isolates react differently to a given metal. WP1 and MP2 both are biverticillate-symmetric *Penicillium* isolates and showed similar cultural and morphological response in presence of heavy metal but differs in the protein and plasmid copy number, while MP4, a triverticillate and SP10, a monoverticillate behaved differently from the rest of the *Penicillium* isolates.

CHAPTER 4

TREATMENT OF AN INDUSTRIAL EFFLUENT SUCH AS MINE TAILINGS

CHAPTER 4: TREATMENT OF AN INDUSTRIAL EFFLUENT SUCH AS MINE TAILINGS

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4.1. Introduction

Goa has rich resources of iron ores, and mining has formed the major industry of Goa for decades. Mining activities release a large amount of wash waters or mine tailings which form a thick slurry and if left untreated would take months to sediment. These tailings also have high amount of suspended material and metal complexes, which are sedimented and clarified routinely by addition of lime; the wash water is then recycled for washing of the ore. A more ecofriendly alternative proposal is to use microbial biomass to achieve sedimentation / clarification and sorption of metals. The use of biological measures in the sedimentation of industrial effluent has been gaining importance, both for its cost effectivity and the absence of adverse side effects.

The fourth chapter deals with the application of two representative isolates of *Penicillium* to treatment of an industrial effluent such as mine tailings for sedimentation, clarification and metal removal from different samples / grades of iron ore mine tailings as a cost effective approach.

4.2. Materials and methods

4.2.1 Cultures

Two isolates of *Penicillium*, that is WP1 and MP2, selected on basis of higher tolerance to iron and manganese were used for sedimentation, clarification and metal removal from different samples / grades of iron ore mine tailings.

4.2.2 Samples of cheap, easily available carbon sources

Molasses and crude sugar samples were obtained from the sugarcane factory at Ponda.

4.2.3 Mine tailing samples

Samples of High grade [HG] and two types of Low grade [LG] ore tailings, that is LG1 and LG2 tailings (Fig 4.1) were obtained from the mining site at Sesa Goa, Codli, Ponda.

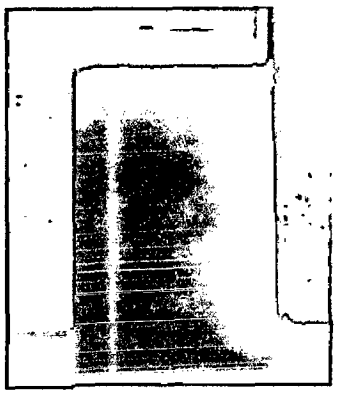

Fig 4.1: Mine tailings

HG tailings have larger particle size hence sedimentation of the heavier particles was observed. The LG1 and LG2 mine tailings were both obtained from different sites; the LG2 tailings were thicker which may be due to more of clay and a higher percentage of fine particles which had led to increased turbidity. Natural sedimentation was not observed for both LG1 and LG2. These differences in the tailings is possibly due to the nature and type of the ore.

4.2.4 Optimization studies for growth of the cultures on cost effective carbon source and parameters for biosedimentation of mine tailings

4.2.4.1 Growth of the cultures on cost effective carbon source and biosedimentation and metal removal from mine tailings by the biomass.

Spore suspensions were inoculated into mineral salts medium (Appendix A) with glucose / molasses / crude sugar / refined sugar as carbon source (1.0 %) and incubated at room temperature (RT) of 30°C on a rotary shaker at 160 rpm for 2 days. The mycelial mass was obtained by filtration through muslin cloth and washed with saline. The mycelial mass was then centrifuged at 1000g equivalent of 3260 rpm for 20 min to obtain the pcv and corresponding wet weight. Biomass, 2 % pcv/v, was used for sedimentation. The mycelial mass [M^+] was lightly pressed and added to 50 ml of mine tailings taken in measuring cylinders (50 ml capacity). The contents of the cylinder were mixed well and the sedimentation rate (SR) monitored for 60 min. The absorbance at 670 nm of the clarified supernatant and the sediment volume was noted after 1h from the start of sedimentation as a quantification of clarification. A control of the mine tailings without culture was maintained.

The contents of Fe^{3+}, Mn^{2+} and Pb^{2+} from the clarified supernatant were estimated as follows:

Estimation of Fe^{3+} - A standard solution of iron was prepared (Vogel, 1978), by taking Fe_2O_3, 5 mg which was digested on a sand bath on low flame with 1:1 HCl and evaporated to dryness to expel excess of acid. To this was added 2 ml of water, 1-2 drops of 1: 5 H_2SO_4 and a dilute solution of $KMnO_4$ until a slight pink colouration remained after stirring well. This was diluted to 100 ml to obtain a working solution of 0-0.35 mg Fe^{3+}. A standard curve was obtained.

Iron (III) reacts with thiocyanate to give a series of intensely red-coloured compounds, which remains in solution, iron (II) does not react.

Sample - 1ml of the supernatant digested as above was diluted to 100 ml and estimated for its iron content.

Estimation procedure - 20 ml standard/sample was treated with 1.5 ml of 4 M HNO_3 and 2.5 ml of 2 M K-thiocyanate to obtain a red coloured complex. The absorbance was measured immediately at 480 nm on a schimadzu spectrophotometer.

Estimation of Mn^{2+}- The filtrate, 2 ml was added to deionised water, 25 ml taken in a flask. Hydroxlyamine hydrochloride, 0.25 gm and ammonium nitrate buffer, 10 ml (Appendix B) were added to the solution. The contents were mixed well by shaking, and then titrated using 0.02 N EDTA and 0.1gm of 0.5% Eriochrome black T as indicator. The colour change was from red to blue (Vogel, 1978).

1.0 mole EDTA = 1.0 mole Mn^{2+}
1 ml of 0.02 N EDTA = 2.1994 mg Mn^{2+}

Estimation of Pb^{2+} - as in 3A.2.1c

4.2.4.2 Parameters for biosedimentation of mine tailings:

Spore suspensions were inoculated into mineral salts medium with molasses (1%) as the carbon source and incubated as per the experiment. The saline washed mycelial mass of each culture was centrifuged to obtain the pcv. Sedimentation and clarification were examined as detailed above.

a) Age of the culture

The culture was grown for 24, 48 and 72 h and a pcv of 1.0 ml was used for sedimentation.

b) Amount of mycelial mass.

The culture was grown for 48h; washed mycelia of 0.00, 0.25, 0.50, 0.75, 1.0, 1.5 and 2.0 ml pcv was added to 50 ml of mine tailings.

c) Storage age of the culture

The 48h grown culture was stored at 4°C upto 3 days and used for sedimentation.

d) Treatment of mycelia

Heat dried: The culture was grown for 48h, 1ml pcv washed and heat dried at 50°C for 1 h, then homogenized and used for sedimentation.

Acid / Alkali treatment: Washed biomass, 1 ml pcv, was acid treated with 0.1N HCl kept at RT on a rotary shaker at 150 rpm for 15 min, and alkali treated with 5 % KOH at 100°C for 10-15 min. The mycelial mass was then filtered and washed with distilled water to neutral pH. This was used for sedimentation of mine tailings.

The untreated mycelium was used as the control.

e) Culture filtrate

The culture filtrate, 5 ml, obtained after filtering off the 48h grown mycelia was added to 45 ml of mine tailings. The uninoculated medium, 5 ml added to 45 ml of mine tailings was kept as the control.

f) Effect of saline versus distilled water for wash treatment of mycelia and extraction of [SC] on sedimentation, clarification and metal removal.

Growth of the cultures and extraction of [SC]: The culture was grown as in 4.2.3.1 with glucose / molasses as carbon source. The [SC] was extracted from the grown mycelia by adding saline / distilled water to the mycelial mass ml^{-1} pcv and 8-10 glass beads in a flask shaken at 100 rpm for 15 min. The mycelial mass with the [SC] removed, $[M^-]$, was filtered to obtain the filtrate, 2ml, containing the [SC], to be used for sedimentation and clarification of mine tailings.

Sedimentation / Clarification and metal removal: The mycelial mass washed in saline / distilled water and the [SC] extracted both in saline / distilled water were used for sedimentation, clarification and metal removal studies as in 4.2.3.1.

4.2.5 Sedimentation by chemical treatment

Sedimentation by increasing concentrations of lime: 0.00, 0.25, 0.50, 0.75, 1.0, 1.5, 2.0 % lime was added to 50 ml of mine tailings, monitored as above. This was compared with sedimentation by increasing concentrations of mycelial mass as given in 4.2.3.2b.

Lime / NaOH at a given pH: After sedimentation by lime, the pH of the mine tailing where best sedimentation was obtained was noted.

Mine tailings were treated with addition of NaOH to 50 ml of mine tailings to obtain the pH at which maximum sedimentation by lime was obtained; the SR was compared with that achieved by lime.

4.2.6 Sedimentation of high grade [HG] / low grade [LG] ore mine tailings by the fungal cultures and the involvement of the cell surface compound [SC].

The cultures were tested for their sedimentation efficiency, clarification and metal removal from three types of mine tailings, that is, HG tailings, and two types of LG tailings obtained from two different sites at two different times, that is LG1 and LG2 tailings, by using the whole mycelial mass [M^+], the extracted surface compound [SC] to check the involvement of the [SC], and the efficiency of the biomass after removal of the [SC], that is, [M^-].

4.2.6.1 Growth of the cultures and extraction of [SC]

The growth of the culture and extraction of the [SC] was done as in 4.2.4.2f with glucose / molasses as carbon source; the [SC] was extracted in saline.

4.2.6.2 Sedimentation / clarification

[M^+], [M^-] lightly pressed, and the [SC], 2 ml, were each added to 50 ml of mine tailings taken in measuring cylinders (50 ml capacity), mixed thoroughly and the rate of sedimentation and clarification monitored for 30 min. The absorbance was noted after 1 hour of sedimentation to note the clarification.

A control was maintained as earlier.

4.2.6.3 Metal removal

Fe^{3+}, Mn^{2+}, Pb^{2+} and Cu^{2+} content of samples from the clarified supernatant at 1h from the start of sedimentation were determined as in 4.2.4.1, the amount of metal removed by the culture during sedimentation was calculated from the amount of residual metal obtained.

4.2.7 Biosedimentation of tailings in larger volume using cost effective carbon source

4.2.7.1 Growth of the cultures

Culture was grown as detailed in 4.2.4.1 in flasks of 400 ml mineral medium with molasses as the carbon source.

4.2.7.2 Extraction and precipitation of the surface compound [SC]

The [SC] was extracted as in 4.2.4.2f from 20 ml pcv of the grown mycelia taken in a 200 ml conical flask to which 60 ml saline was added. To the filtrate two volumes of cold absolute alcohol was added and kept overnight at 4°C. This was then centrifuged at 13,000 rpm for 45 min at 4°C. The precipitate obtained was dissolved in distilled water and used for sedimentation.

4.2.7.3 Sedimentation / clarification and metal removal

Biomass [M^+] and [M^-], 1%, washed and lightly pressed were added to 2 litres of mine tailings taken in graduated chambers, mixed thoroughly and the rate of sedimentation and clarification monitored at 1min interval for 10 min, then at 30 and at 60 min. The

absorbance at 600 nm of the clarified supernatant was noted after 1 h of sedimentation to examine clarification. A chamber containing only the mine tailings was kept as the control.

The content of Fe^{3+}, Mn^{2+} and Pb^{2+} from the clarified supernatant were estimated as in 4.2.4.1

4.3 Results

4.3.1. Optimization of growth conditions

4.3.1.1 Growth of the cultures on cheap, easily available carbon source / media and biosedimentation and metal removal from mine tailings by the mycelial mass

Cultures WP1 and MP2 achieved good sedimentation and metal removal from mine tailings (Fig 4.2a - 4.2b). A study with WP1 and MP2 grown for 2d in mineral medium with glucose / molasses / crude sugar / refined sugar (1 %) showed that molasses could indeed support a good growth of the fungi. Sedimentation by the mycelial mass (Fig 4.3) was also efficient in all cases.

Removal of Fe^{3+} was 93 - 94 % by both WP1 and MP2 mycelial mass grown in all four carbon sources; removal of Mn^{2+} was at 100 % by WP1 grown in glucose / refined sugar as the carbon source followed by mycelial mass grown in molasses at 84 % removal; while 100 % removal of Mn^{2+} was obtained by MP2 grown in all four carbon sources (Fig 4.3). Removal of Pb^{2+} was highest at 97.4 % by both WP1 and MP2 grown in molasses as the carbon source, not much difference being obtained by the culture grown

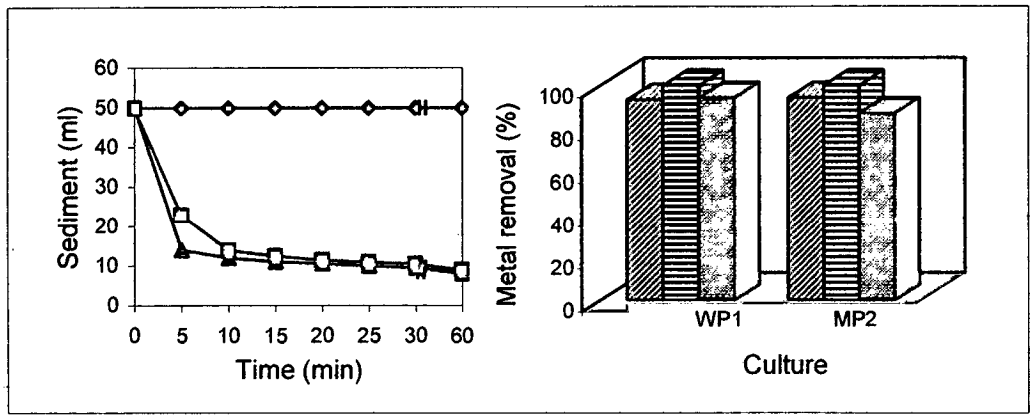

Fig 4.2a Sedimentation of LG1 tailings by: ▲ WP1, ◻ MP2; ◇ control tailings and Metal removal during sedimentation: ▨ Fe, ▤ Mn, ▩ Pb

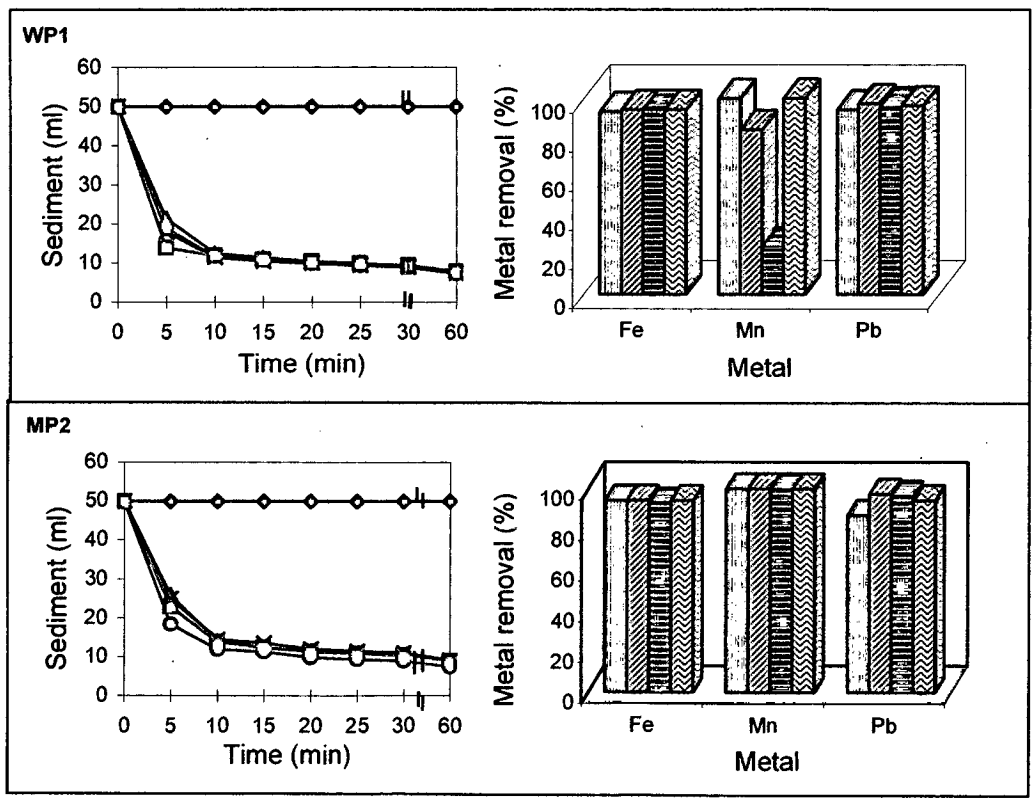

Fig 4.3: Sedimentation of LG1 tailings by WP1 and MP2 grown on: ◻ glucose, ▲ molasses, x crude sugar, ○ refined sugar; ◇ control tailings, and Metal removal during sedimentation by cells grown on: ▩ glucose, ▨ molasses, ▤ crude sugar, ▧ refined sugar

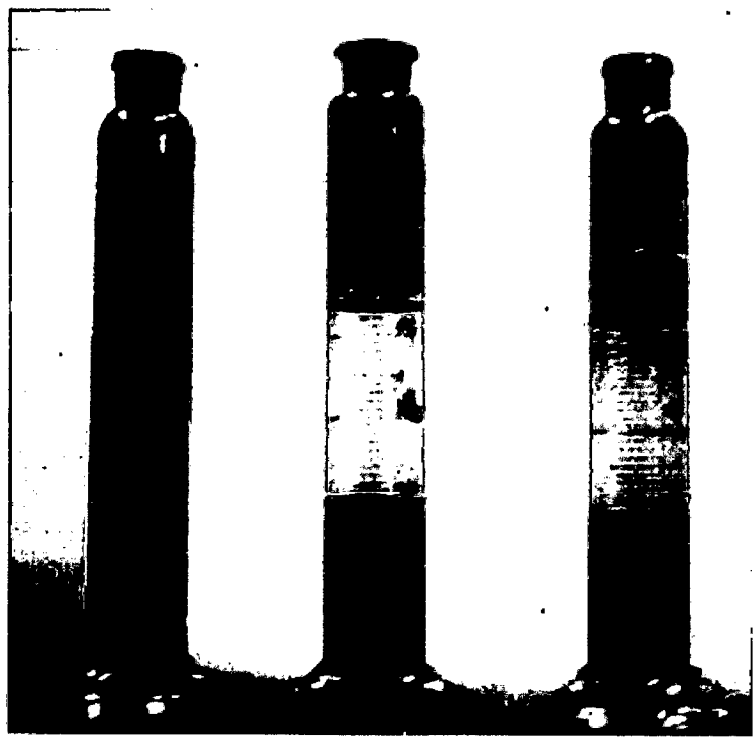

Fig 4.2 b: Sedimentation of mine tailings by WP1 and MP2

in crude sugar and refined sugar, with both the cultures grown in glucose achieving the least removal of Pb^{2+} at 94.3 and 87 % by WP1 and MP2 respectively.

4.3.1.2 Parameters for biosedimentation of mine tailings

Age of the culture

The cultures grown for 48 and 72h gave comparable results better than the 24h grown culture with OD of the clarified supernatant being equal to 0.02 and 0.03 as compared to the 24 h grown culture with an O.D of 0.11 and 0.47 for WP1 and MP2 respectively (Fig 4.4A).

WP1 and MP2 achieved 100 % removal of Fe^{3+} by the cultures grown for 24h/48h/72h. Removal of Mn^{2+} by WP1 and MP2 was highest with the 48h grown culture, while Pb^{2+} was sorbed maximally by the 48h grown culture of WP1 and 72h grown culture of MP2 (Fig 4.4A) as compared to the supernatant of the control after 1h of sedimentation.

Amount of mycelial mass

Sedimentation of LG by increasing concentrations of mycelial mass of 0.00, 0.25, 0.50, 0.75, 1.0, 1.5 and 2.0 ml pcv in 50 ml of mine tailings showed that the SR was highest with 0.5 % pcv, decreasing with increasing concentrations of mycelia, not much difference being obtained with 2 % pcv and above of both cultures (Fig 4.4B). However, the degree of clarification was inversely proportional to the SR, being highest with 2 % biomass for both WP1 and MP2, the OD at 670 nm of the clarified supernatant after 1h

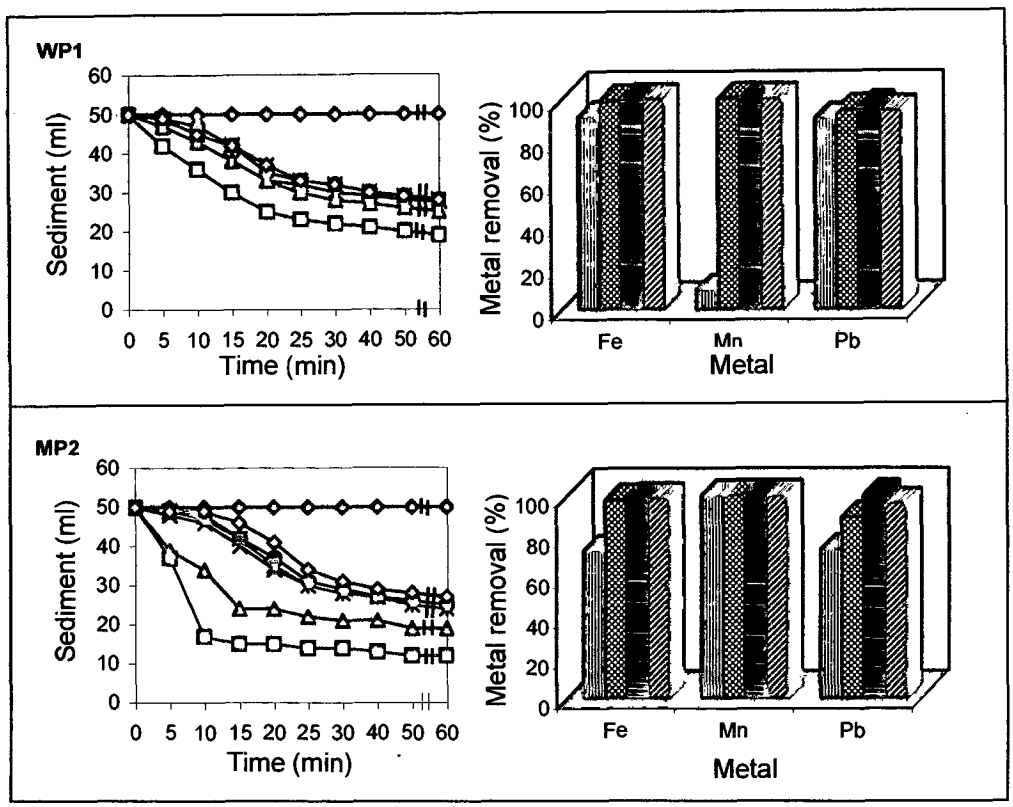

Fig 4.4B:Sedimentation of LG tailings (50ml) by WP1, MP2 :-
pcv: □0.25, △0.5, x 0.75, ✱1.0, ○1.5, ◇2.0ml; ◇control tailings,
and Metal removal during sedimentation by
pcv: ▥0.5, ▩1.0, □1.5 and ▨2.0ml

being equal to zero for both cultures. The control with no mycelia added, had an OD of 0.75×10^2.

About 95-100 % removal of Fe^{3+}, Mn^{2+} and Pb^{2+} was obtained with 2 % biomass for WP1. Similar results were obtained by MP2 for removal of Fe^{3+}, and Mn^{2+}, however at least 3 % biomass was needed for complete removal of Pb^{2+} (Fig 4.4B).

Storage age of the culture

Not much difference was noted in the extent of clarification with respect to the storage age of the mycelia upto 3d for WP1, while clarification by the stored biomass of MP2 decreased slightly, the O.D. values at 1h from the start of sedimentation being 0.02 and 0.03 by the fresh and stored mycelia respectively for WP1, and 0.03 and 0.09 for MP2. Metal removal was also equally efficient by the 3d stored mycelia of both cultures (Fig 4.4C).

Treatment of mycelia

i) Heat dried

Not much difference in the SR by the untreated and heat dried biomass of both *Penicillium* isolates was observed, however, the untreated mycelia achieved a slightly better clarification than the heat dried biomass, the O.D values being 0.02 and 0.05 for WP1, and 0.03 and 0.11 for MP2 by the untreated and heat dried biomass respectively (Fig 4.4 C).

Metal removal during clarification by the heat-dried biomass was also as efficient as the untreated mycelia with slight variations. For WP1, complete removal of Fe^{3+} was achieved by both the heat dried as well as the untreated biomass, however removal of Mn^{2+} and Pb^{2+} was a little more efficient by the untreated mycelia: 100 % for Mn^{2+} and 95 % for Pb^{2+}, than the heat dried biomass: 92 % for Mn^{2+} and 91 % for Pb^{2+}. In case of MP2, removal of Fe^{3+} and Mn^{2+} was slightly better by the heat dried biomass: 100 % for Fe^{3+} and 94 % for Mn^{2+} than the untreated biomass: 98 % for Fe^{3+} and 89 % for Mn^{2+}, while for Pb^{2+} complete removal was achieved by both the heat dried as well as the untreated biomass.

ii) Acid / Alkali treatment

Both the acid and alkali treated mycelia of both isolates showed rapid sedimentation but with very little clarity as compared to the untreated control, the alkali treatment of WP1 and MP2 yielding a more rapid SR than the acid treated mycelia. However, the untreated biomass obtained very good clarification with an O.D of 0.03 by WP1 and 0.02 by MP2 as compared to that by the acid treated mycelia with an O.D.of 0.352×10^2, 0.192×10^2 and 0.3×10^2, 0.2×10^2 of the alkali treated mycelia of WP1 and MP2 respectively (Fig 4.4 C).

Metal removal studies indicated that in both isolates of *Penicillium*, alkali treatment of WP1 and MP2 obtained better removal of metals: 90 % Fe^{3+}, 98 % Mn^{2+} and 40 % Pb^{2+} by WP1, and 99.7 % Fe^{3+}, 100 % Mn^{2+} and 73.3 % Pb^{2+} by MP2, than the acid treated

mycelia: 11 % Fe^{3+}, 93% Mn^{2+} and 6 % Pb^{2+} by WP1 and 95 % Fe^{3+}, 100% Mn^{2+} and 4 % Pb^{2+} by MP2.

Culture filtrate

The uninoculated medium obtained equally good sedimentation and clarity as compared to the test, that is the culture filtrate, with an O.D. of 0.02 and 0.01 at 1h from the start of sedimentation for the control and the test respectively. Both the uninoculated medium and the culture filtrate also showed equal efficiency in metal removal (Fig 4.4 D).

Effect of saline versus distilled water for wash treatment of mycelia and extraction of [SC] on sedimentation, clarification and metal removal

The [M^+] and [M^-] of both *Penicillium* isolates grown either on glucose or molasses and washed in distilled water (d/w) showed a faster rate of sedimentation but with a lesser clarity than that washed in saline as compared to the control (Fig 4.5 A). A similar result was obtained with the [SC] extracted in saline and in d/w (Fig 4.5A and Table 4.1).

The [SC] extracted in saline gave good clarification; however, the control with saline also gave as good clarification. The test done with [SC] extracted in d/w yielded a better clarification than the control although incomplete. The [SC] extracted in d/w also achieved efficient removal of metals by both isolates though it was not as effective as the [SC] extracted in saline in the removal of metals.

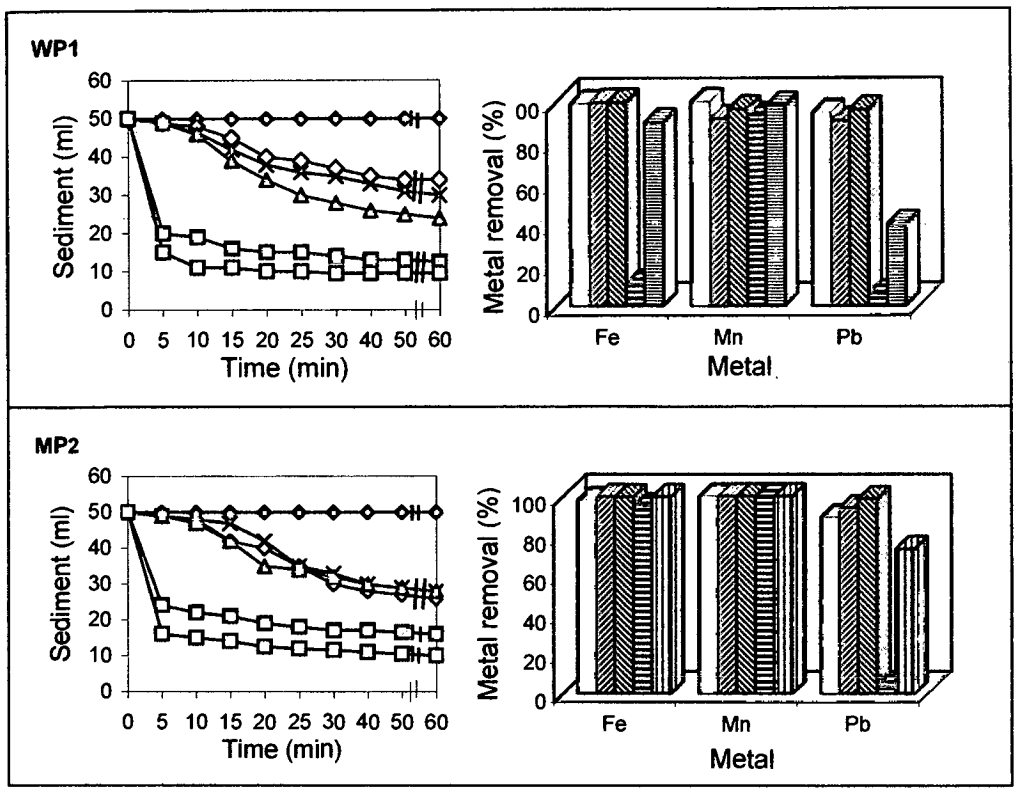

Fig4.4C :Sedimentation of LG tailings by WP1 and MP2 mycelia:
◇ untreated, ▲3d storage x heat dried, □ acid, □alkali; ◇control tailings and Metal removal during sedimentation by:
□ untreated, ▨ heat dried, ▧ storage, ▤acid, ▥ alkali biomass

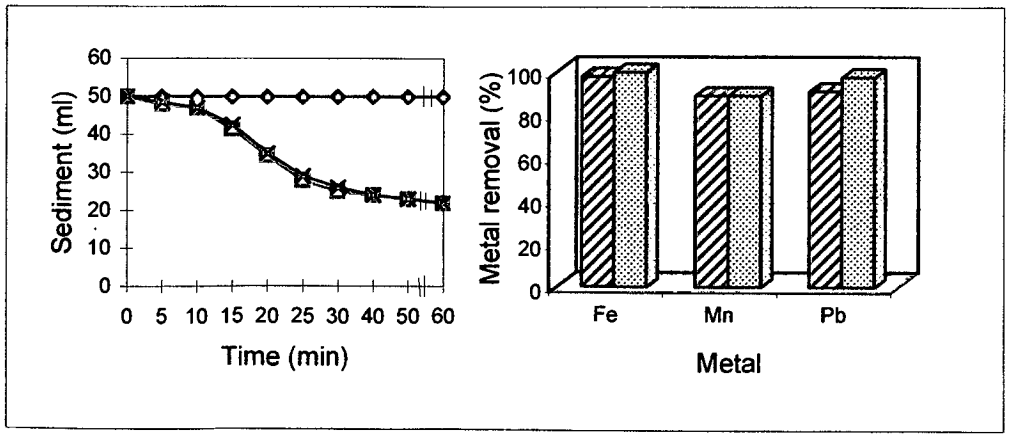

Fig4.4D:Sedimentation of LG tailings :◇ control[C],□ medium[C], by x culture filtrate and Metal removal during sedimentation by:□ medium[C], ▨ culture filtrate

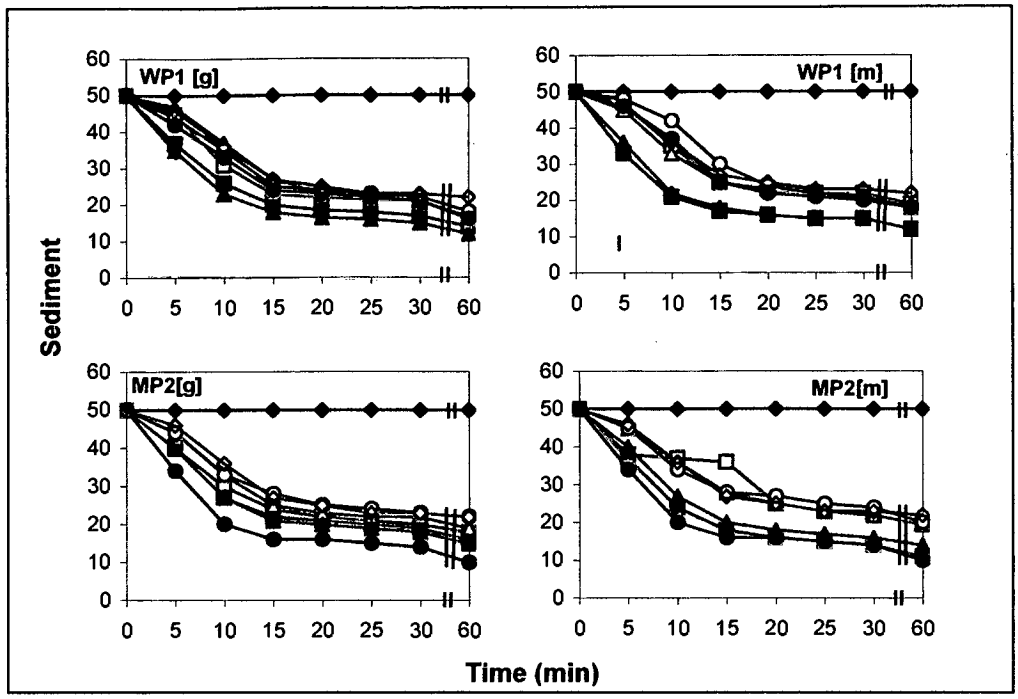

Fig 4.5A: Sedimentation of LG2 tailings by WP1 and MP2 grown in,
[g]: glucose and [m]: molasses:
i) saline washed [M+] (□); [M-] (△); [SC] (○) and saline control (◇)
ii) d/w washed [M+] (■); [M-] (▲); [SC] (●) and d/w control (◆)

Table 4.1: A $_{670}$ at 60 min sedimentation by [SC]: extracted in saline / dw

Control saline	Control d/w	WP1		MP2	
		saline	d/w	saline	d/w
0.06	0.82×10^2	0.03	0.17×10^2	0.07	0.28×10^2

d/w: distilled water

Effective removal of Fe^{3+}, Mn^{2+} and Pb^{2+} by the fungal cultures grown either on glucose or molasses and washed in distilled water or in saline was observed, removal being more for Fe^{3+} than for Mn^{2+} and Pb^{2+} (Fig 4.5B). Also, the efficiency of removal for Fe^{3+} is almost the same, both in saline or in distilled water wash treatment of both isolates, removal of Mn^{2+} and Pb^{2+} was more efficient by saline washed mycelia than with that washed with distilled water for both WP1 and MP2.

4.3.2 Sedimentation by chemical treatment

Maximum sedimentation and clarity was obtained with 0.25 % lime (Fig 4.6A) with an O.D of the supernatant equal to zero, no difference being observed upon addition of increasing concentrations of lime. Similar values were obtained by 1.0-2.0 % pcv of WP1 and of MP2. The control where no lime was added showed an O.D of 0.85×10^2. Sedimentation by increasing concentrations of mycelial mass showed that the SR was fastest with 0.5 % pcv decreasing with increasing concentrations of mycelia, becoming near constant with 2 % pcv and above of both cultures (Fig 4.6B). The degree of clarification was inversely proportional to the SR, becoming more efficient with 2 % and above of biomass for both WP1 and MP2, the OD at 670 nm of the clarified supernatent after 1h equal to zero for both cultures, while the control with no mycelia added, had an OD of 0.75×10^2.

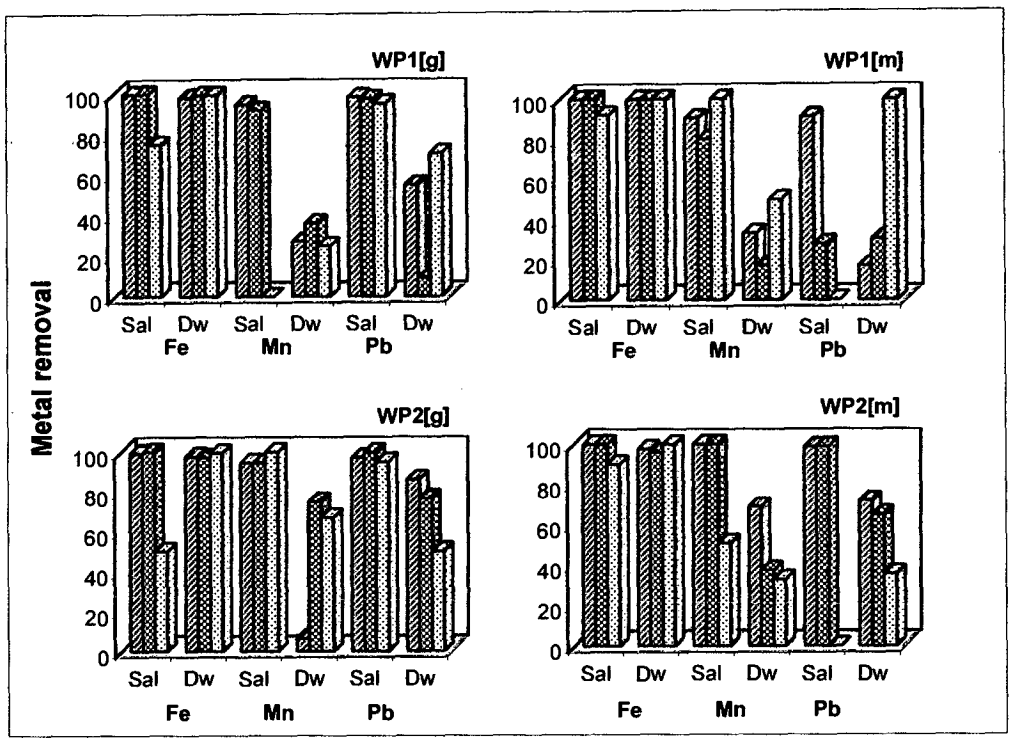

Fig 4.5B: Metal removal from LG2 tailings by WP1 and MP2 grown in,
[g]: glucose and [m]: molasses:
saline / d/w washed [M+] (▨); [M-] (▩); [SC] (▦)

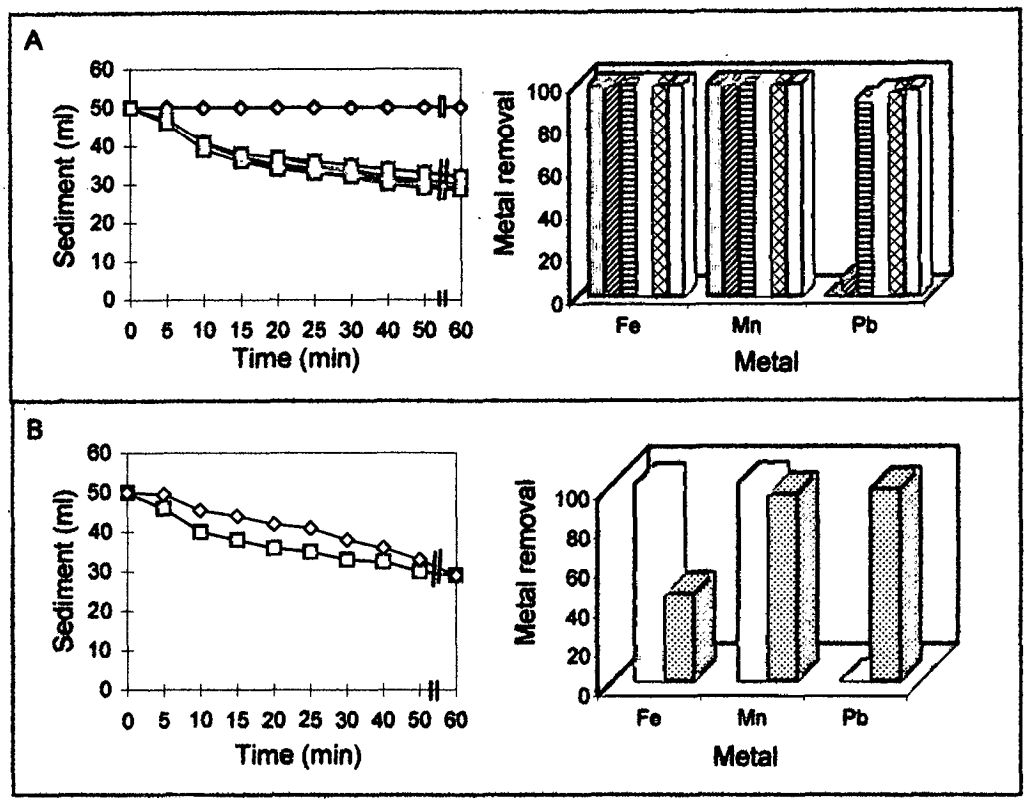

Fig 4.6A: Sedimentation of LG tailings, ◇ [C] control, by lime:
□ 0.25, △ 0.5, k 0.75, x1.0, ○ 1.5, ◇ 2 %
and Metal removal during sedimentation by :
▨ 0.25, ▨ 0.5, ▬ 0.75, □ 1.0, ⊠ 1.5, □ 2 % lime.

B: Sedimentation of LG tailings by:
□ lime(0.25 % = pH:7), ◇ NaOH (0.25ml of 2N NaOH = pH 7)
and Metal removal during sedimentation by: □ lime, □ NaOH.

Complete removal of Fe^{3+} and Mn^{2+} was maximum with 0.25 % lime, while 0.75 % lime was needed for 93 % removal of Pb^{2+} (Fig 4.4A). In comparison, maximal removal of metals by WP1 and MP2 was achieved by 2 % pcv (Fig 4.6B).

Sedimentation was efficiently achieved by 0.25 % lime, which brought about a pH of 7.0; the control tailings showed a pH of 5.0. Sedimentation by 0.25 ml of 2 N NaOH at the same pH of 7.0 was however less efficient than that by lime (Fig 4.6B). The O.D at 670 nm after 1h of sedimentation of the lime treated mine tailings being equal to zero as compared to the NaOH treated tailings with an O.D value of 0.57 x10.

Metal removal was also more efficient by the lime treated tailings, 100 % of Fe^{3+} and Pb^{2+} but no sorption of Mn^{2+}, as compared to the NaOH treated tailings with 45 % removal of Fe^{3+}, 95 % Pb^{2+} and 97 % Mn^{2+} (Fig 4.6).

4.3.3 Sedimentation of High grade [HG] / Low grade [LG] ore mine tailings by the fungal cultures and the involvement of the cell surface compound [SC]

The sedimentation and metal removal from mine tailings of High grade [HG], Low grade ore sample 1 [LG1] and Low grade ore sample 1 [LG2], by [M^+], [M^-], [SC] of WP1 and MP2 grown in glucose and in molasses as the carbon source is shown in Fig 4.7-4.9 and Table 4.2. Results showing sediment volume (SV) at 60 min and O.D of the clarified supernatant at 670 nm at 1 hour from the start of sedimentation arranged in the decreasing order of clarity are shown in Chart 1.

Table 4.2: A $_{670}$ of clarified tailings after 60 min sedimentation

Tailings	Control	Control saline	WP1			MP2		
			M+	M-	[SC]	M+	M-	[SC]
HG	1.90	0.04	0.10	0.02	0.07	0.01	0.06	0.04
LG1	0.224 x10	0.05	0.11	0.17	0.03	0.12	0.31	0.03
LG2	0.375 x10^2	0.06	1.39	1.01	0.03	0.06	0.02	0.07

Key - HG: High grade ore tailings; LG1/ LG2: Low grade ore tailings

Table 4.3: Metal concentration in the supernatant of the control samples (mg ml^{-1}) after 1 h of natural sedimentation

Tailings	Fe^{3+}	Mn^{2+}	Pb^{2+}
HG	0.055	0.137	0.025
LG1	0.30	0.866	0.175
LG2	7.58	0.247	0.312

Key - HG: High grade ore tailings; LG1/ LG2: Low grade ore tailings

<u>As seen in Chart 1, Table 4.2 - 4.3 and Fig 4.7 - 4.9:</u>

a) Effective sedimentation by both *Penicillium* isolates was observed, the cultures grown both in glucose and in molasses as the carbon source obtained comparable sedimentation and clarification. Sedimentation volume and clarification by the fungal isolates are inversely proportional to each other.

b) For glucose grown cells of both *Penicillium* isolates, the sedimentation rate (SR) by [M^+] was slightly faster than by [M^-]. However, in the case of molasses grown cells, the SR by [M^+] was almost the same as [M^-]. A better clarification was obtained with [M^-] of both isolates when grown in glucose as the carbon source. Results indicate that the [SC] also achieved sedimentation and clarification of mine tailings.

c) Sedimentation and clarification by the cultures was also affected by the nature of the ore sample, namely HG / LG ore, and the sampling site: a faster rate of sedimentation and clarification was observed in HG than in LG mine tailings. Between LG mine

tailings also, it was observed that LG1 sedimented and clarified faster than LG2 mine tailings, both obtained from different sites.

d) The metal content (mg ml^{-1}) of the control tailings of HG, LG1 and LG2 after 1 h of natural sedimentation are indicated in Table 4.3. Fe^{3+} and Pb^{2+} content were highest in LG2 followed by LG1. Mn^{2+} content was more in LG1 than in LG2 and HG showed the least content of all three metals. It was also seen that HG and LG1 contained a higher amount of Mn^{2+} than Fe^{3+}, followed by Pb^{2+}, while LG2 contains a higher amount Fe^{3+} followed by Pb^{2+} and Mn^{2+}.

e) Removal of Fe^{3+}, Mn^{2+} and Pb^{2+} from all samples of tailings during sedimentation by either M^+, M^- or [SC] of both *Penicillium* isolates was observed, the efficiency being almost the same. Overall, 80 - 100 % removal of Fe^{3+}, Mn^{2+} and Pb^{2+} were observed.

f) HG tailings have large particle size; hence the control has a high SR equivalent to the test, but clarity was much lower than the test. Removal of Fe^{3+}, Mn^{2+} and Pb^{2+} from HG at 1h of sedimentation by [M^+], [M^-] or [SC] of both fungi was observed (Fig 4.5A). MP2 achieved removal of Pb^{2+} better than WP1 (Fig 4.5B).

g) Sedimentation of the control was not observed for both LG1 and LG2 tailings (Fig 4.6A-4.7A). Removal of Fe^{3+}, Mn^{2+} and Pb^{2+} by [M^+], [M^-] or [SC] at 1h during sedimentation by both isolates, from LG1 (Fig 4.6B) and LG2 tailings (Fig 4.7B) was observed. No copper was detected in the supernatant of all the mine tailing samples.

h) Further, all the test samples of mine tailings achieved complete sedimentation in 30 min and complete clarification with a zero absorbance within 2 h for HG tailings, and overnight for LG1 and LG2 samples. The controls however required a much longer period: HG tailings took 30 min to sediment and 8 days to clarify; LG1 required 8 d

Chart 1: Sedimentation and clarification by $[M^+]$, $[M^-]$, [SC] grown in glucose (G) / molasses (m).

<u>HG tailings:</u> [Fig 4.7]

$[M^+ (G)]$: **WP2** SV=13ml OD=0.16 > **MP1** SV=13ml OD=0.19 > **C** OD=1.90

$[M^+ (m)]$: **MP2** SV=15ml OD=0.01 > **WP1** SV=16ml OD=0.10 > **C** OD=1.90

$[M^- (G)]$: **WP1** SV=16ml OD=0.07 > **MP2** SV=16ml OD=0.09 >> **C** OD=1.90

$[M^- (m)]$: **WP1** SV=18ml OD=0.02 > **MP2** SV=17ml OD=0.06 > **C** OD=1.90

[SC (G)] : **MP2** SV=10ml OD=0.13 > **WP1** SV=10ml OD=0.16 > **Cs** SV=10ml OD=0.08

[SC (m)] : **MP2** SV=15ml OD=0.04 > **WP1** SV= 16ml OD=0.07 **Cs** SV=15ml OD=0.04

LG1 tailings : **[Fig 4.8]**

$[M^{+}(G)]$: **MP2** SV=10.5ml OD=0.12 > **WP1** SV=10.5ml OD=0.13 > **C** OD=1.58(x10)

$[M^{+}(m)]$: **WP1** SV=9.0ml OD=0.11 > **MP2** SV=9.5ml OD=0.12 > **C** OD=0.224(x10)

$[M^{-}(G)]$: **MP2** SV=10.5ml OD=0.13 > **WP1** SV=10.5ml OD=0.21 >> **C** OD=1.58 (x10)

$[M^{-}(m)]$: **WP1** SV=8ml OD=0.17 > **MP2** SV=8ml OD=0.31 > **C** OD=0.224(x10)

[SC (G)] : **MP2** SV=10.4ml OD=0.05 = **WP1** SV=10.4ml OD=0.05 **Cs** SV=10.4ml OD=0.05

[SC (m)] : **W P1** SV=10.0ml OD=0.03 = **MP2** SV= 10ml OD=0.03 **Cs** SV=10ml OD=0.05

<u>LG2 tailings :</u> [Fig 4.9]

$[M^{+}(G)]$: **MP2** SV=17ml OD=1.2 > **WP1** SV=17ml OD=1.61 > **C** OD=0.375(x10^2)

$[M^{+}(m)]$: **MP2** SV=19.5ml OD=0.06 >>**WP1** SV=19ml OD=1.39 > **C** OD=0.375(x10^2)

$[M^{-}(G)]$: **WP1** SV=17ml OD=0.29 > **MP2** SV=19ml OD=0.30 >> **C** OD=0.375(x10^2)

$[M^{-}(m)]$: **MP2** SV=19.5ml OD=0.02 >>**WP1** SV=18ml OD=1.01 >> **C** OD=0.375(x10^2)

[SC (G)] : **WP1** SV=18ml OD=0.05 > **MP2** SV=22ml OD=0.11 >> **Cs** SV=22ml OD=0.22

[SC (m)] : **WP1** SV=18ml OD= 0.03 > **MP2** SV=20ml OD=0.07 > **Cs** SV=22ml OD=0.06

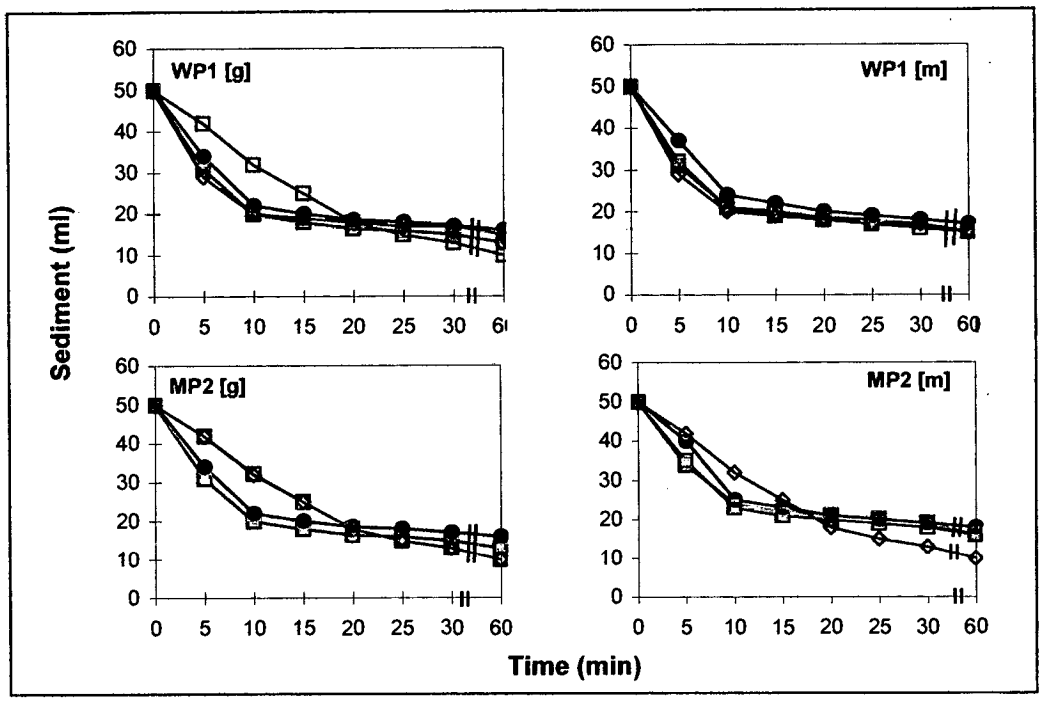

Fig 4.7A:Sedimentation of HG tailings by WP1 and MP2 grown on,
[g]: glucose and [m]:molasses:▩ [M+],● [M-] □ [SC] ◆[C], ◇[Cs].

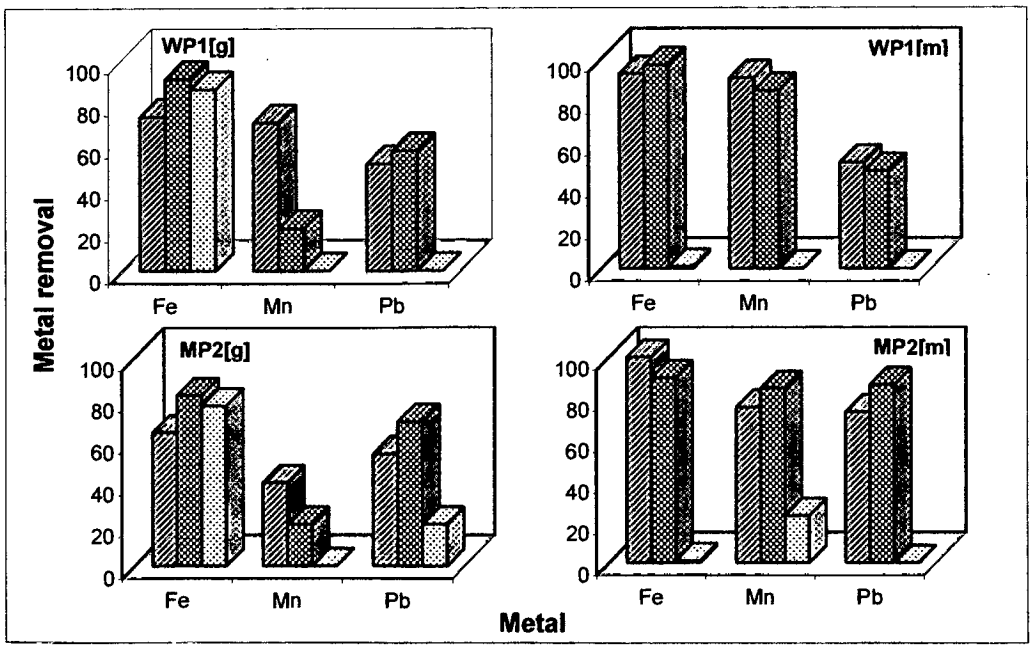

Fig 4.7B:Metal removal from HG tailings by WP1 and MP2 grown on,
[g]: glucose and [m]:molasses:▨ [M+],▩ [M-] ▩[SC]

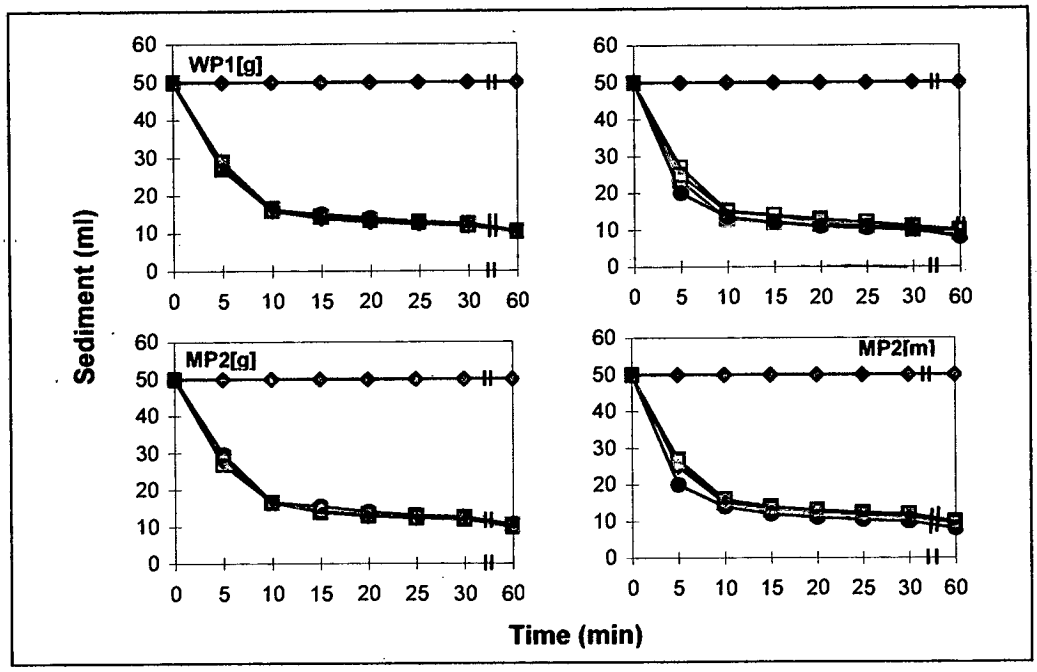

Fig 4.8A: Sedimentation of LG1 tailings by WP1and MP2 grown on,
[g]: glucose and [m]:molasses:□ [M+],● [M-] □[SC] ◆[C], ◇[Cs].

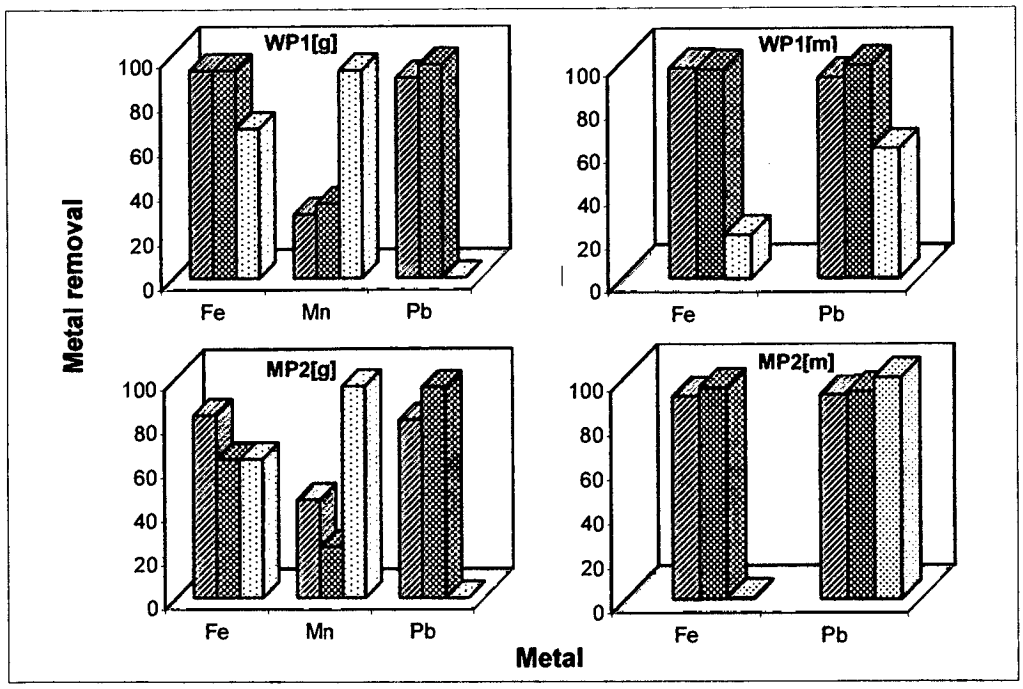

Fig 4.8B:Metal removal from LG1 tailings by WP1 and MP2 grown on,
[g]: glucose and [m]:molasses:▨ [M+],▩ [M-]▧ [SC]

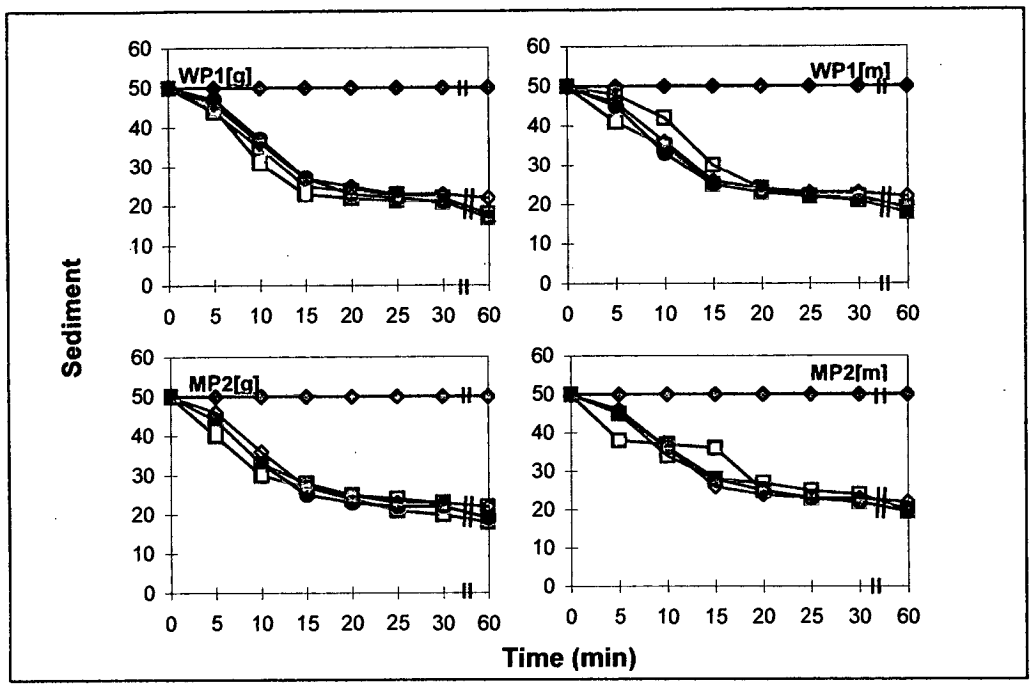

Fig 4.9A: Sedimentation of LG2 tailings by WP1 and MP2 grown on,
[g]: glucose and [m]: molasses:□ [M+],● [M-]□ [SC] ◆[C], ◇[Cs]

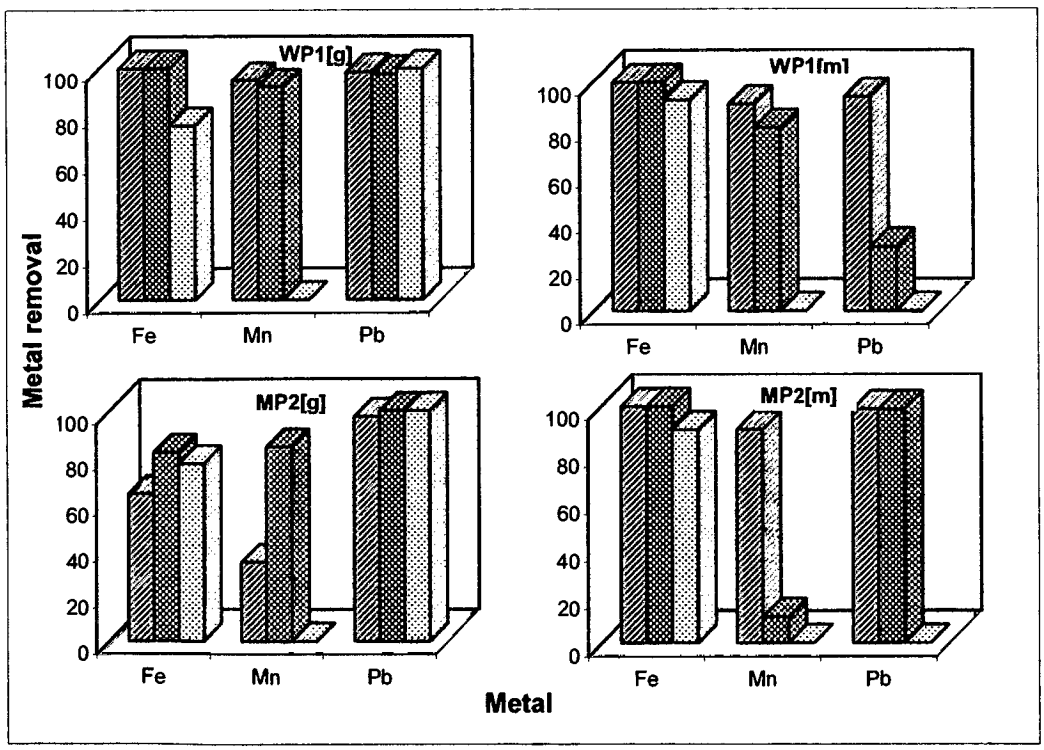

Fig 4.9B:Metal removal from LG2 tailings by WP1 and MP2 grown on,
[g]: glucose and [m]:molasses:▨ [M+],▩ [M-] ▣ [SC]

to sediment and 13 d for clarification, while LG2 required 9 d for sedimentation and 15 d for clarification.

4.3.4. Biosedimentation and metal removal of tailings in larger volume using cost effective carbon source

Sedimentation by both the [M^+] and [SC] treated tailings (Fig 4.10) achieved efficient sedimentation and clarification of mine tailings. The [M^+] treated tailings achieved the fastest SR as compared to the [M^-] treated tailings and the control (Fig 4.11). Little sedimentation being observed by the [M^-] treated tailings, while the control showed no sedimentation till 1h from the start of sedimentation. The O.D at 600 nm of the clarified supernatant at 1h from the start of sedimentation is 0.14 by the [M^+] treated tailings, 0.23 by the [M^-] treated tailings and 0.93 of the control.

The 2nd experiment using the purified [SC] obtained by precipitation with alcohol and dissolved in distilled water, showed that the [SC] added tailings achieved very good clarification with an O.D at 600 nm equal to 0.04 as compared to 0.93 of the control.

Metal removal experiments indicated that [M^+] sorbed 57 % Fe^{3+}, 38 % Mn^{2+}, 3.4 % Pb^{2+}; [M^-] showed 38 % removal of Mn^{2+} and no sorption of Fe^{3+} and Pb^{2+}, while [SC] sorbed 80 % Fe^{3+}, 53 % Mn^{2+}, 3.2 % Pb^{2+}. Results showed that the [SC] was more effective than [M^+] and [M^-] both for clarification and for metal removal from 2 liters of mine tailings followed by [M^+], then [M-].

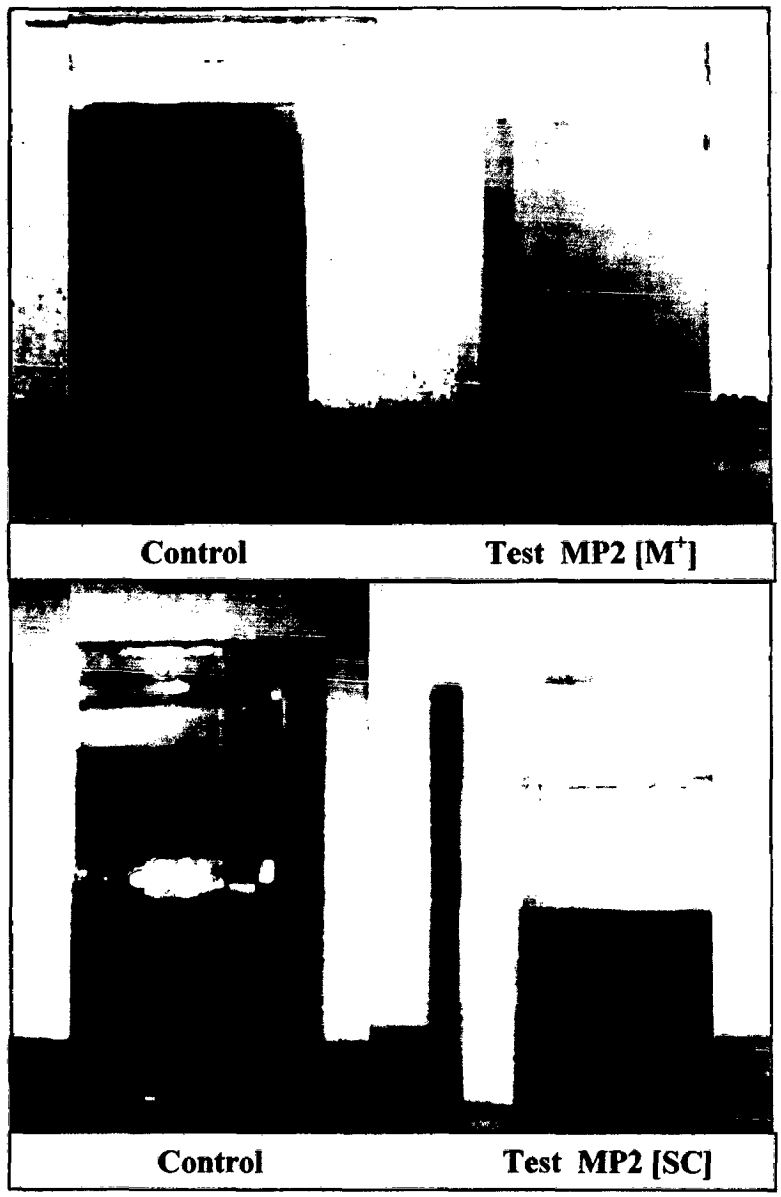

Fig 4.10: Sedimentation of tailings by MP2 [M^+] and [SC]

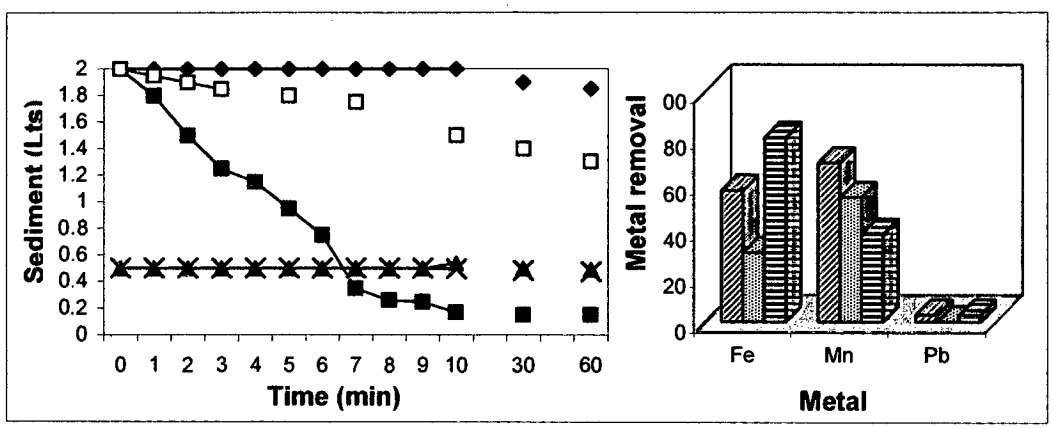

Fig 4.11B: Bulk sedimentation of LG3 tailings by :
◆, x control tailings, ■ M+, □M-, ▲SC of MP2,
and Metal removal during sedimentation by:
▨M+, ▩M-, ▤ SC.

4.4 Discussion

Mining activities, agricultural run off, industrial and domestic effluents are mainly responsible for the increase of the metallic species released into the environment (Cossich et al., 2002). In Goa, mining has formed the major industry, giving rise to large amounts of mine tailings or wash waters obtained from washing of the ores. These mine tailings form thick slurries of earth and metal fines, which require time for sedimentation and gets released into the natural waters. To combat this, chemical flocculation such as lime and salt are used, which however would have an adverse effect on aquatic life and man (Kowshik and Nazareth, 2001). Contrary to toxic organics, that in many cases can be degraded, the metallic species that are released into the environment tend to persist indefinitely, accumulating in living tissues throughout the food chain (Cossich et al., 2002). Hence, an ecofriendly cost effective approach using microbial biomass is of immense importance.

The fungal isolates, WP1 and MP2 achieved efficient sedimentation and clarification of mine tailings. However, the sedimentation rate (SR) and clarification are inversely proportional, that is, a faster sedimentation is due to quick sedimentation of the heavier particles, while the fine particles remain suspended giving turbidity. On the other hand, a slower sedimentation is due to sedimentation of the finer particles as well, therefore a slower sedimentation is observed, which also gives better clarity.

A study of the cultures grown on glucose, molasses, crude sugar and refined sugar showed that molasses could support a good growth of the fungi, as well as sedimentation

and metal removal by the grown mycelial mass. Molasses can thus be used as a cost effective carbon source for growth of the culture for sedimentation, clarification and metal removal experiments. Cost effectiveness is the main attraction of environmental bioremediation; the nutrients from readily available and inexpensive sources such as carbohydrate-rich industrial wastewaters have been conveniently used (Vieira and Volesky, 2000; Volesky and Naja, 2005).

The age at which the biomass is harvested affects sedimentation, clarification and metal removal. As was observed in the experiment, the younger cells of WP1 and MP2 at 48h growth achieved good sedimentation and about 100% clarity including efficient metal removal. The 24h grown culture may have been too early in the growth stage of these isolates, and was therefore not as efficient as the 48h grown culture. Ã‡eribasi and Yetis, (2001) also indicated that the maximum biosorption capacities were attained after 41 hours of incubation. However, it was also reported that the younger cells were more efficient than the stationery growth phase for metal sorption (Mattuschka et al., 1993; Zucconi et al., 2003).

The SR decreases with increasing concentrations of mycelia, the degree of clarification being inversely proportional to the SR, being highest with 2 % biomass for both WP1 and MP2, which could possibly be the increase in interaction between the biomass and the mine tailing components with more number of reactive sites when the biomass is more. However, this is in contrast to that reported by Kowshik and Nazareth (2001) where the SR increases with increasing concentration of the biomass, which could be attributed to the nature of growth of the cultures; the *Penicillium* cultures growing as pellets as

compared to the *Fusarium* culture which grows as a uniform suspension. A ratio of 2 % biomass to mine tailings (pcv/v) yielded efficient sedimentation, clarification and metal removal from mine tailings, which is better than *Bacillius* and *Arthrobacter* as reported by Deshpande (1990). Removal of Fe^{3+}, Mn^{2+} and Pb^{2+} was also maximal at 95-100 % with 2 % biomass of both the cultures, and as seen in the earlier chapter, the removal of metals possibly may be by passive sorption at the cell surface.

Sedimentation efficiency by the 3d stored mycelia at 4°C was comparable to the fresh biomass. Little difference was noted in the extent of clarification with respect to the storage age of the mycelia, metal removal being also equally efficient by the stored mycelia of both cultures. The fungal biomass can thus be stored and used for longer period. Kowshik and Nazareth (2001) reported a decrease in SR by *Fusarium solani* with storage.

The SR and metal removal by the untreated and heat dried biomass of both *Penicillium isolates* was equally efficient, not much difference being observed; the untreated mycelia achieving a slightly better clarification than the heat dried biomass, while that reported for *Fusarium solani* was lowered when the heat-treated powdered mycelium was used (Kowshik and Nazareth, 2001). Thus, the heat-dried biomass can be efficiently used as a safer method with no adverse effect resulting from live biomass. The dried mycelium is also more attractive in that it has longer shelf life, the toxicity of the metal in solution cannot affect the adsorptive function of the biomass, nutrient and other biomass growth requirement need not be satisfied and maintenance of purity is not a concern (Tsezos, 1990).

Though the acid and alkali treated mycelia of both isolates showed rapid sedimentation, however acid/alkali treatment of the mycelia reduced drastically the clarification efficiency as compared to the untreated mycelia, the alkali treatment yielding a more rapid SR and metal removal than the acid treated mycelia. Similar results were observed by Yan and Viraraghavan, (2000), where the acid pretreatment of *Mucor rouxii* significantly decreased the bioadsorption of heavy metals, which is also in agreement with the observation of Kapoor and Viraraghavan (1998) in the case of *A.niger*. Ã‡eribasi and Yetis (2001) revealed that when the fungi were subjected to acid treatment, some portion of the sorbed metal was rapidly released. This is attributed to the binding of H^+ ions to the biomass after acid treatment which may be responsible for the reduction in adsorption of heavy metals. The polymeric structure of biomass surface exhibits a negative charge due to the ionization of organic and inorganic groups. The higher the biomass electronegativity, the greater the attraction and adsorption of heavy metal cations. Ahalya (2003) reported that the H^+ ions on the acidic pretreated *M.rouxii* biomass may change the biomass electronegativity, resulting in a reduction in bioadsorption capacity. However, when non-viable biomass is used in the removal of heavy metals, alkali pretreatment is an effective method to improve the bioadsorption capacity for metal ions (Yan and Viraraghavan, 2000).

1.0 - 2.0 % pcv biomass of both isolates achieved equal efficiency as with lime in sedimentation and clarification of mine tailings; therefore it serves as a very eco-friendly alternative to the use of chemical treatment, besides sorbing metals. Deshpande (1990) reported that the sedimentation of mine tailings using biomass is more efficient than the

lime sedimentation as well as natural sedimentation. Microbial biomass eventually gets degraded, while the use of lime on the other hand adversely affects the environment by introducing chemicals in addition to the pre-existing iron and manganese. These waters are let out into the rivers, and also find their way into the surrounding agricultural land and wells, thus posing a serious threat to aquatic life and man through the adverse side effects on agriculture (D'Souza, 1995). Biological systems to remove metal ions from polluted waters could emerge as the potential alternative to chemical treatments (Deshpande, 1990; Volesky, 1990; Maeda and Sakaguchi, 1990).

The use of $CaCO_3$ is better than NaOH as observed with $CaCO_3$ giving better sedimentation, clarity and metal removal as compared to NaOH at the same pH. The divalent cation, Ca^{2+} possibly contributed to increased biosorption than the monovalent cation, Na^{+}.

Addition of the mycelial biomass of both the *Penicillium* isolates has helped to speed up the sedimentation process and clarification with removal of Fe^{3+}, Mn^{2+} and Pb^{2+} from all three types of tailings; the SR being very rapid as compared to the control of uninoculated tailings. This rapid process was comparable to earlier results (Kowshik and Nazareth, 2001). Therefore, the cultures can be effectively used for bioremediation, using molasses as a cost effective carbon source comparative with that grown on glucose as the carbon source; the sediment volume and clarity by the fungal isolates are inversely proportional to each other; the faster SR with lesser clarity being due to incomplete sedimentation.

The extracted cell-surface compound [SC] of both the *Penicillium* isolates played a role in sedimentation, clarification and metal removal from mine tailings, in addition to the whole mycelial mass [M^+] indicating that the [SC] components have probably contributed to the metal sorption by the surface compound present on the microbial biomass. However, removal of the [SC] did not affect sedimentation, clarification and metal removal, indicating that other than the cell surface compound, there are other factors involved in the sedimentation of mine tailings, the microbial cell walls being mainly composed of polysaccharide, proteins and lipids which have abundant metal binding groups such as carboxyl, sulphate, phosphate and amino groups (Ianis, et al., 2006). The cell-surface components that bind metals include glycans of fungal cell walls (Gadd, 1990).

Sedimentation and clarification of mine tailings with addition of the fungal biomass was a very rapid process achieved within 30 min, and complete clarification was observed within 2 h for HG tailings, and overnight for LG1 and LG2 samples. However, the controls with no biomass added required a much longer period: HG tailings took 30 min to sediment and 8 d to clarify; LG1 required 8 d to sediment and 13 d for clarification, while LG2 required 9 d for sedimentation and 15 d for clarification. Earlier results showed that *Fusarium solani* also achieved rapid sedimentation and clarification within 30 min, while the control took 7 d to sediment (Kowshik and Nazareth, 2001). Therefore, the addition of the fungal biomass can help speed up the rate of sedimentation which would otherwise require days to sediment and clarify.

The sedimentation and clarification by the cultures was affected by the nature of the ore sample and the sampling site: a faster rate of sedimentation and clarification was observed in HG than in LG mine tailings. Between LG mine tailings also, it was observed that LG1 sedimented and clarified faster than LG2 mine tailings, both obtained from different sites, the LG2 tailings containing more of clay and a higher percentage of fine particles which had led to increased turbidity, hence the differences in sedimentation and clarification between LG1 and LG2 tailings. HG tailings have large particle size; hence the control has a high SR, equivalent to the test, but clarity was much lower than the test. Sedimentation of the control was not observed for LG1 and LG2. Metal content also differed in each of the different tailings, the high grade ore [HG] tailings showed lesser metal content as compared to the low grade ore [LG] tailings, which could possibly be due to the nature and type of the ore.

The lightly pressed mycelial mass increased the SR of the tailings in comparison to the untreated mycelia. Kowshik and Nazareth, (2001) reported homogenization of the biomass significantly increased the sedimentation efficiency of the mycelia. Light pressing by mortar and pestle may have increased the exposure of the active sites of the fungal biomass enhancing the metal binding capacity.

Mineral salts play a role in the sedimentation and clarification of mine tailings, which was observed when the uninoculated medium obtained equally good sedimentation and clarity as the culture filtrate. Results indicated that the mycelial mass of both the *Penicillium* isolates washed in distilled water showed a faster SR than that washed in saline but with little clarity; the SR and clarity being inversely proportional. A similar result was obtained with the [SC], however the [SC] extracted in distilled water was as

effective as the [SC] extracted in saline in metal removal by both species. Saline has contributed to the sedimentation and clarification of mine tailings possibly by giving a positive charge by binding of the Na^+ ions onto the mycelial surface or on the [SC]. However, it may be noted that the mycelial mass washed in d/w and the [SC] extracted in d/w also showed sedimentation and clarification of mine tailings as compared to the control. This is indicative that the fungal biomass in itself achieved sedimentation and metal removal.

Culture MP2 is a more promising isolate in that it achieved overall better clarification and metal removal. This isolate grown in molasses as the cost effective carbon source indicated equal efficiency when tested for bulk sedimentation, clarification and metal removal with the same percentage of biomass, that is, 2 % (pcv/v) by the whole mycelium, and by the purified surface compound. Sorption of Fe^{3+} and Mn^{2+} was equally effective. Therefore, bioremediation of mine tailing using fungal isolates offers much potential for future use.

4.5 Conclusions

Molasses can be used as a cost effective carbon source for the growth of the cultures, as it gave good mycelial yield, which were also effective in sedimentation / clarification and metal removal. 2% biomass of both cultures gave optimum results for sedimentation and clarification, as well as optimal metal removal from mine tailings. Both *Penicillium* isolates achieve efficient sedimentation of mine tailings from all samples of high-grade ore, low-grade ore mine tailings and biosorption of Fe^{3+}, Pb^{2+} and Mn^{2+} for the purpose of bioremediation. Variations were also seen between the *Penicillium* isolates, with MP2 giving greater efficiency than WP1. Sediment volume and clarification by the both the isolates are inversely proportional. Sedimentation of mine tailings was observed by the whole mycelia [M^+] as well as the mycelial mass without the surface compound [M^-] of both the isolates. In addition, the cell surface compound also played a role in sedimentation of mine tailings.

Heat dried biomass showed comparable sedimentation capacity and metal removal, but obtained lesser clarity of mine tailings as compared to the fresh biomass. The acid and alkali treated mycelia obtained good sedimentation but with very little clarity as compared to the untreated mycelia. Acid treatment also lowered the metal removal efficiency. Stored mycelia can be effectively used for sedimentation, clarification and removal of metals.

The SR was faster by the mycelial biomass as compared to the chemical treatment, although the clarity obtained was not as good as lime. 0.25 % lime was needed to achieve

100 % clarity, while 2 % biomass of *Penicillium* grown for 48 h was needed to achieve about 100 % clarity of mine tailings.

The mineral salts played a role in the sedimentation of mine tailings. Both saline and distilled water wash treatment of mycelia can be effectively used for sedimentation / clarification and metal removal. A faster sedimentation rate was obtained by the distilled water wash treatment but better clarification was obtained by the saline wash treatment of mycelia.

Sedimentation rates and extent of clarification varied with the ore grade, the site and / or period of sampling, a faster sedimentation rate and clarification was observed in HG than in LG tailings. Between LG mine tailings also, it was observed that LG1 sedimented and clarified faster than LG2 mine tailings.

Metal removal is near or equal to 100 % of the original concentration after 1hour of settlement: 0.055 mg in HG mine tailings, 0.30 mg in LG1 mine tailings and 7.58 mg in LG2 mine tailings.

SUMMARY

Salient features

- Sampling was done from the mangroves and solar salterns, Ribander, of Goa, out of which 10 *Penicillium* isolates were obtained. In addition, the cultures include two previous isolates, that is, from well water samples and from mangroves.
- The cultures were termed as WP1, from well (W) water and MP2, from mangrove (M) area, additional to the present isolates from mangroves (M), MP3 - MP4, and eight isolates from salterns (S), SP5 - SP12.
- All the cultures showed extreme halotolerance upto 10.0 % NaCl. Heavy metal resistance was checked against Pb^{2+}, Cu^{2+} and Cd^{2+}. All cultures were resistant to Pb^{2+} at a high concentration of 7.5 mM, while most could also tolerate either Cu^{2+} and / or Cd^{2+} as sulphate or as nitrate salt, with MP3 and MP4 showing resistance to all the heavy metals tested. The lag in growth and decrease in sporulation with respect to increasing metal concentrations were also seen in presence of metals.
- Further, 50 % of the isolates could resist upto 30 mM Fe^{2+} (MP4 upto 20 mM only) while the remaining 50 % showed tolerance level upto at least 50 mM Fe^{2+}. The cultures also showed extreme tolerance, upto a minimum of 150 mM Mn^{2+}. Metal precipitation occurred at 50 mM Fe^{3+} and 150 mM Mn^{2+}, hence the tolerance levels beyond these concentrations could not be obtained.
- Out of the twelve *Penicillium* cultures used for the screening work, four representative isolates, that is, WP1, MP2, MP4 and SP10 were selected based on morphological characteristics such as monoverticillate, biverticillate symmetric, biverticillate asymmetric, triverticillate, for investigations on these and their high level of halotolerance and resistance to metals.

- The MTC of the metals (lead, copper and cadmium) on growth of the selected cultures revealed that the MTC in liquid medium of Pb^{2+}, Cu^{2+}, Cd^{2+} as $CdS0_4$ and as $CdN0_3$ for WP1 / MP2 / MP4 / SP10 ranged from 0.5 mM – 4.0 mM.
- A representative isolate WP1 was used for optimisation studies with regard to the factors affecting sorption by the mycelial biomass, such as growth media, age of the culture, stationary and shaker growth conditions and pH of the solution.
- The fungus grown in S-CDB as the growth medium at 48 h under shaker conditions achieved maximum sorption per ml^{-1} pcv, which also produced higher amount of the [SC] estimated in terms of protein, carbohydrate and lipid. This indicates the involvement of the [SC] in metal sorption. Metal sorption along with [SC] production decreased with increase in age of the biomass after 48h. The optimum pH was 5.6 and the maximum rate of metal removal was observed at 1min for all four cultures indicating a passive binding of metal ions which does not require the microorganism's active metabolism for sorption to take place, and is therefore a physical process.
- All four isolates could resist Pb^{2+} at a much higher concentration as compared to Cu^{2+} and Cd^{2+}, while the tolerance level to the transition elements, Fe^{2+} and Mn^{2+} was very high. The tolerance to the heavy metals tested was highest in MP4, while for Fe^{2+} was highest for WP1 and MP2.
- The harvested mycelium of all four *Penicillium* isolates can sorb all the tested heavy metals from solution though some cultures could not tolerate either Cu^{2+} or Cd^{2+} (nitrate) or Cd^{2+} (sulphate) during growth, therefore it has its advantage in that the grown cells can be effectively used for metal sorption, though growth in presence of heavy metals is fairly low or nil.

- The sorbed heavy metals are mainly extracellular for all four *Penicillium* isolates, with very little intracellular accumulation of only 7-15 % if any. Some intracellular accumulation of Pb^{2+} was observed in all four *Penicillium* isolates, while very little / no intracellular uptake of Cu^{2+} was observed. Except for WP1, there is no intracellular uptake of Cd^{2+} (nitrate) in the other three isolates, while for Cd^{2+} (sulphate), some intracellular accumulation was observed only in WP1, MP4 and SP10.
- The metal uptake capacity was slightly more for WP1/ MP2 than for MP4 indicating a correlation between tolerance and a decreased metal uptake, whereby the decreased uptake by the metal-tolerant strain could be due changes in the membrane transport permeability due to increased chitin accumulation as observed with the increased thickening of the cell wall of MP4.
- Different isolates showed different responses to heavy metal, for example, WP1 could tolerate Pb^{2+}, Cu^{2+} and Cd^{2+} (nitrate), while MP2 could tolerate Pb^{2+}, Cu^{2+} and Cd^{2+} (sulphate). MP4 tolerated all four tested heavy metals, while SP10 tolerated only Pb^{2+} and Cd^{2+} (sulphate). The affinity for each metal and sorptive capacities of each isolate for each metal is also significantly different, thus showing a difference in the sorption capacity between morphotypes of the same genus.
- Cultural and/or morphological changes were induced in presence of increasing metal concentration as compared to the control. The cultures in presence of Pb^{2+} showed distinct cultural changes, such as, growth appearance, pigment production and decreased spores, while morphological changes was very little. However, in presence of Cu^{2+} and/or Cd^{2+}, pronounced morphological changes such as, unformed or absence of penicilli heads, thickened and/or bulbous hyphal cells were observed. These cultural and

morphological changes in presence of toxic metals are survival mechanisms of the *Penicillium* isolates.

- The protein profiles obtained by SDS-PAGE of the mycelia showed distinct changes when grown in the presence of metal salts, characterised by new bands and the overexpression of protein bands in response to metal. The expression of new proteins absent in the control cultures could be related to metal uptake. Similarly, an increase / decrease in intensity of plasmid bands were observed in presence of metal. These changes in the protein and plasmid profiles are also resistance mechanisms of the *Penicillium* isolates to respond to the harmful effects of heavy metals; the plasmid mediated resistance being determined by the amplification of genes as observed with the increase in intensity of the bands which could be related to protein synthesis as observed with the over expression of protein bands or new proteins.
- The symmetric and asymmetric biverticillate isolates, WP1 and MP2, showed similar cultural and morphological responses to heavy metal as compared to the monoverticillate, SP10 and triverticillate isolates, MP4 but differs in the protein and plasmid expression.
- The application potential of the isolates in treatment of an industrial effluent such as mine tailings, for sedimentation, clarification and metal removal were tested. WP1 and MP2 showed a good tolerance to Mn^{2+} and Fe^{2+} and were selected for this study. The cultures were effective in sedimentation and clarification, also in removal of metals from mine tailings.
- Molasses can be used as a cost effective carbon source for the growth of the cultures, as it gave good mycelial yield, which were also effective in sedimentation / clarification and metal removal. Biomass, 2 %, of 48h grown cultures gave optimum results for sedimentation and clarification, as well as optimal metal removal from mine tailings.

Mycelia, stored upto 3d, can be effectively used for sedimentation, clarification and removal of metals.

- Sedimentation of mine tailings was observed by the whole mycelia [M^+] as well as the mycelial mass without the surface compound [M^-] of both the isolates. In addition, the [SC] also played a role in sedimentation of mine tailings.
- Heat dried biomass showed comparable sedimentation capacity and metal removal, but obtained lesser clarity of mine tailings as compared to the fresh biomass.
- SR and extent of clarification varied with the ore grade, the site and / or period of sampling, a faster SR and clarification was observed in HG than in LG tailings. Between LG mine tailings also, it was observed that LG1 sedimented and clarified faster than LG2 mine tailings. Metal removal is near or equal to 100 % of the original concentration after 1hour of settlement: 0.055 mg in HG mine tailings, 0.30 mg in LG1 mine tailings and 7.58 mg in LG2 mine tailings.
- Both *Penicillium* isolates achieved efficient sedimentation and biosorption of Fe^{3+}, Mn^{2+} and Pb^{2+} from all samples of HG, LG1 and LG2 tailings for the purpose of bioremediation. Variations were also seen between the *Penicillium* isolates, with MP2 giving greater efficiency than WP1.
- Culture MP2 achieved overall better clarification and metal removal. This isolate grown in molasses as the cost effective carbon source was used to demonstrate the efficiency when tested for clarification and metal removal from 2 liters of tailings.
- Bioremediation of mine tailing using fungal isolates offers much potential for future use.

PUBLICATIONS

Journals

1. Marbaniang, T and Nazareth, S. Isolation of halotolerant *Penicillium* species from mangroves and salterns and their resistance to heavy metals. Current Science (Accepted).

Papers presented

International:

1. Nazareth S and Marbaniang T (2005). Isolation of salt tolerant, metal resistant fungi from mangroves and salterns of Goa, India, at International Marine Biotechnology Conference, Newfoundland, Canada. 7-12 June.
2. Nazareth S, Marbaniang T, Gaitonde S and de Souza C (2005). Halotolerant fungi with heavy metal resistance and exhibiting alkalo-thermotolerant enzyme activity. International conference on microbial diversity. Current Perspectives and Potential Applications, New Delhi, 16-18 April.

National:

1. Marbaniang T and Nazareth S (2004). Screening for cost effective carbon source for growth of fungi and their application in bioremediation of mine tailings. *Oral presentation* at National Seminar on Recent Trends in Mycology and 31st Annual meeting of the M.S.I. Mangalore, 2-3 Dec.
2. Marbaniang T and Nazareth S (2004). Fungal diversity in bioremediation. 26th Annual Conference and Symposium of Indian Society of Mycology and Plant Pathology (ISMPP) organized by UGC-SAD, Department of Botany, Goa University, Goa, 7-9 October.
3. Marbaniang T and Nazareth S (2004). Fungal sorption of iron by mine tailings. National Symposium on Recent Trends in Biology, Biodiversity and Biotechnology of Fungi and 30th Annual Meeting of the M.S.I, Chennai, 5-6 Feb.
4. Marbaniang T and Nazareth S (2003). Clarification of mine tailings by fungal isolates. National Symposium on Prospecting of Fungal Diversity and Emerging Technologies and 29th Annual Meeting of M.S.I, Pune, 6-7 Feb.

Appendices

Appendix – A

Media composition

1. Czapek's Dox Broth (CDB) - g / L

Sucrose*	-	30.0
$NaNO_3$	-	3.0
K_2HPO_4	-	1.0
$MgSO_4 . 7H_2O$	-	0.5
KCl	-	0.5
$FeSO_4$	-	0.01

2. Czapek's Dox Agar (CDA) - g / L

CDB	-	As above
Agar	-	3.0

3. Modified Czapek's Dox Broth with glucose (CDB*G) -g / L

Glucose*	-	10.0
$NaNO_3$	-	3.0
K_2HPO_4	-	1.0
$MgSO_4 . 7H_2O$	-	0.5
KCl	-	0.5
$FeSO_4$	-	0.01

4. Czapek's Dox Broth with glucose (CDB+G) - g / L

CDB	-	As above
Glucose*	-	10.0

5. Mineral salts media with glucose (MG) - g / L

$FeSO_4$	-	10 mg
$MgSO_4 . 7H_2O$	-	0.2
KH_2PO_4	-	0.7
Na_2HPO_4	-	0.1
$CaCl_2.2H_2O$	-	0.1
NaCl	-	0.05
$(NH_4)_2 SO_4$	-	0.5
*Glucose / Molasses / Crude sugar / Refined sugar	-	10.0
Malt extract	-	0.5
Micronutrient solution	-	1.0 ml
Distilled water	-	Liter

Note: *Sterilized separately at 121°C for 15 min

Micronutrient solution – g / 100 ml

$CuSO_4$	-	0.0007
H_3BO_3	-	0.005
$CoSO_4$	-	0.005
Na_2MoO_4	-	0.2
$MnSO_4 . H_2O$	-	0.5
$ZnSO_4$	-	1.1

Appendix - B

Reagents and Buffers

I. SDS PAGE Reagents

1. Monomer Solution

Acrylamide - .29.2g

Bis - 0.8g

H_2O - to 100ml

Stored at 4°C in the dark

2. 4x Running Gel Buffer

(1.5 M Tris –Cl pH 8.8)

Tris - 18.15g

Adjusted to pH 8.8 with HCl

H_2O - to 100 ml

3. 4x Stacking Gel Buffer

(0.5 M Tris-Cl pH 6.8)

Tris - 3.0g

Adjust to pH 6.8 with HCl.

H_2O - to 50ml

4. 10 % SDS

SDS - 5.0g

H_2O - to 50ml

5. Initiator (10 % ammonium persulphate, fresh)

Ammonium persulphate - 0.5g

H_2O - to 5.0 ml

6. 2x Treatment Buffer

Tris - 2.5 ml Solution (3)

SDS - 4.0 ml Solution (4)

Glycerol - 2.0 ml

2-Mercaptoethanol - 1.0 ml

H_2O - to 10ml

7. Tank Buffer

Tris - 3 g

Glycine - 14.4 g

SDS - 10 ml Solution (4)

H_2O - to 1 Liter

8. Stain

Coomassie Blue-250 -0.2g

Methanol - 80 ml

Stirred untill dissolved

Acetic acid - 14 ml

H_2O - to 200 ml

9. Destaining Solution I

Methanol - 100 ml

Acetic acid - 20 ml

H_2O - to 200 ml

10. Destaining Solution II

Acetic acid - 700ml

Methanol - 500ml

H_2O - to 10 L

II. PLASMID ISOLATION Reagents

1. TE Buffer

Tris HCl	-	50 mM (pH 8.0)
EDTA	-	10 mM (pH 8.0)
pH	-	8.0 with HCl

2. TBE - 5x

Tris HCl	-	27 g
Boric acid	-	13.75 g
0.25M EDTA	-	10 ml (pH 8.0)
pH	-	8.0

Diluted to 500 ml with distilled water

3. 0.8 % Agarose Gel

Agarose	-	0.8 g
0.5x TBE	-	10ml
Distilled water	-	90ml

4. Tracking dye

Glycerol	-	2.7 ml
TBE - 5x	-	0.3 ml
SDS 1%	-	1.0 ml
EDTA (0.5 M)	-	1.0 ml
Bromophenol blue	-	0.25 %

5. Ethidium Bromide - 0.1 - 0.5μg / ml

III. PROTEIN ESTIMATION Reagents

1. Folin Reagent A

Na_2CO_3	-	2 g
NaOH	-	0.4 g
Distilled water	-	100 ml

2. Folin Reagent B

$CuSO_4$	-	0.3 g
Na-K-tartarate	-	1g
Distilled water	-	100 ml

3. Reagent C (Reagent A + Reagent B)

Reagent A	-	50ml
Reagent B	-	1ml

(Mixed just before use)

4. Folin Ciocalteau Reagent

Diluted with Distilled water in 1: 3 ratio

5. Aqueous Phenol (5 %)

Distilled phenol	-	5 ml
Distilled water	-	95 ml

IV. Buffers

1. Ammonium Nitrate Buffer

Ammonium Nitrate	-	8.0 gm
D/W	-	65 ml
Ammonia solution	-	35 ml

Appendix - C

METAL STOCK SOLUTIONS

1.	$Pb(NO_3)_2$	-	50 mM		
	331mg salt (207 mg Pb^{2+})	-	1000 ml	-	1 mM
2.	$CuSO_4 . 5H_2O$	-	50 mM		
	250 mg salt (64 mg Cu^{2+})	-	1000 ml	-	1 mM
3.	$Cd (NO_3)_2 . 4H_2O$	-	50 mM		
	308 mg salt (112 mg Cd^{2+})	-	1000 ml	-	1 mM
4.	3 $Cd (SO_4) . 8H_2O$	-	50 mM		
	768 mg salt (336 mg Cd^{2+})	-	1000 ml	-	1 mM
	256 mg salt (112 mg Cd^{2+})	-	1000 ml	-	1 mM
5.	$FeSO_4 . 7H_2O$	-	500 mM		
	278 mg salt (56 mg Fe^{2+})	-	1000 ml	-	1 mM
6.	$MnSO_4 . H_2O$	-	500 mM		
	169 mg salt (55 mg Mn^{2+})	-	1000 ml	-	1 mM

Note: Sterilized at 121°C for 15 - 20 min

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