

**BIOACCUMULATION OF HEAVY METALS BY THE YEAST  
*S. cerevisiae* AND THE BIOREMEDIATION OF INDUSTRIAL  
WASTE WATER**

**THESIS**

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## ABSTRACT

Water is an essential element in all aspects of life and is vital for both domestic and industrial purposes regarding both the quality and quantity thereof. Similar to many other drought stricken countries, South Africa requires water for the socio-economic growth of the country, yet is faced with the problem of maintaining the quality of its drinking water as well as protecting the dwindling supplies. In an attempt to prevent the deterioration of South African water supplies, the treatment, purification and recycling of industrial and mining waste water has recently become of prime importance.

Many industrial and mining waste waters contain heavy metals in toxic quantities. The conventional processes that have been used till recently to address this problem, are often expensive or contain chemical agents which compound the environmental problem. As an alternative biological methods of metal accumulation appear to offer an economic and efficient alternative to these methods. An advantage to the South African scenario is the commercial production of the yeast, *S. cerevisiae* as a readily inexpensive by-product from some fermentation industries. Yeast cells, and in particular *S. cerevisiae* have proven to be capable of accumulating heavy metals, and therefore exhibit potential application in the bioremediation of waste water.

The aim of this project was twofold. The initial part of this work attempted to define the mechanisms of metal accumulation by the yeast cells and cellular components. The information obtained from these initial studies provided a data base required for the development of a bioremediation system.

Initial contact with the metal ions occurs at the wall interface of the yeast cell. Metal accumulation appears to be a function of all the cell wall components. The isolated cell wall components are better metal chelators than the intact cell walls. An apparent affinity series of mannan > chitin > glucan > intact cell walls exists. However, these components differ in their affinities for metal ions. Storage of metal ions within the cell occurs predominantly in the vacuole. The present study concluded that metal accumulation by the vacuole could be related to size. Metal accumulation occurred in the order of  $\text{Cu}^{2+} > \text{Co}^{2+} > \text{Cd}^{2+}$  with a corresponding decrease in atomic radii of  $\text{Cd}^{2+} > \text{Co}^{2+} > \text{Cu}^{2+}$ . Vacuolar ion deposition occurs at an early stage during the internalization of metal ions within the yeast cells. At the onset of vacuolar saturation, depositions of metal ions as granules within the cytosol occurs. In the presence of heavy metal cations viable yeast cells can be shown to exhibit two

types of cellular responses. Uptake of  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  causes the loss of intracellular physiological cations from within the yeast cell. In comparison, uptake of  $\text{Co}^{2+}$  into the cell does not have this effect. All three heavy metal cations initiate plasma cell membrane permeability, thus the  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  induced loss of the intracellular cations, occurs as a result of ion-exchange mechanisms and not due to cation leakage brought about by membrane permeabilization.

Uptake of heavy metals by viable yeasts appears to be generally non-selective though the amount of metals accumulated are largely affected by the ratio of ambient metal concentration to biomass quantity. In addition, the energy dependent nature of internalization necessitates the availability of an external energy source for metal uptake by viable yeast cells. For these reasons metal removal from industrial waste water was investigated using non-viable biomass.

By immobilizing the yeast cells additional mechanical integrity and stability was conferred upon the biomass. The three types of biomass preparations developed in this study, viz. polyvinyl alcohol (PVA) Na-alginate, PVA Na-orthophosphate and alkali treated polyethylenimine (PEI):glutaraldehyde (GA) biomass pellets, all fulfilled the necessary physical requirements. However, the superior metal accumulating properties of the PEI:GA biomass determined its selection as a biosorbent for bioremediation purposes. Biosorption of heavy metals by PEI:GA biomass is of a competitive nature, with the amount of metal accumulated influenced by the availability of the metal ions. This availability is largely determined by the solution pH. At low pH values the affinity of the biomass for metals decreases, whilst enhanced metal biosorption occurs at higher pHs, eg. pH 4.5 - 6.0.

PEI:GA biomass pellets can be implemented as a biosorbent for the bioremediation of high-concentration, low-volume metal containing industrial waste. Several options regarding the bioremediation system are available. Depending on the concentration of the metals in the effluent, the bioremediation process can either be used independently or as part of a biphasic remediation system for the treatment of waste water. Initial phase chemical modification may be required, whilst two types of biological systems can be implemented as part of the second phase. The PEI:GA biomass can either be contained within continuous-flow fixed bed tanks or continuous-flow stirred bioreactor tanks. Due to the simplicity of the process and the ease with which scale-up is facilitated, the second type of system shows greater application potential for the treatment of this type of industrial waste water than the fixed-bed systems.

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## ABBREVIATIONS

(ATP)	Adenosine triphosphate
(BEI)	Backscattered electron images
(BIO-FIX)	Biomass-foam immobilized extractant
(BV)	Breakthrough volume
(DCCD)	N,N - dicyclohexylcarbodiimide
(DES)	Diethylstilbestrol
(DNP)	2,4 Dinitrophenol
(EDAX)	Energy dispersive X-ray analysis
(EDTA)	Ethylenediaminetetraacetic acid
(FDA)	Fluorescein diacetate
(GA)	Glutaraldehyde
(ID)	Internal diameter
(MRA)	Metal recovery agent
(MT)	Metallothionien
(MW)	Molecular weight
(PEI)	Polyethylenimine
(PIPES)	1,4 piperazinediethanesulphonic acid
(PST)	Post settling tank
(PVA)	Polyvinyl alcohol
(SEI)	Secondary electron images
(SEM)	Scanning electron microscopy
(SOB)	Sorbitol containing sodium citrate buffer
(TEM)	Transmission electron microscopy
(TMAH)	Tetramethylammonium hydroxide

## **1. GENERAL INTRODUCTION**

Water is an important, though often underrated resource, with the availability and quality thereof being of paramount importance. This fact was recognized by the United Nations during the 1980's, resulting in the designation of that decade to maintaining the quality of international drinking water. With such a high demand for potable water, it is essential to prevent, or at least to limit the pollution of this resource. A commonly encountered group of pollutants in water is the heavy metals. The concentration of these metals in natural waters varies greatly with locality and time. Since the onset of the industrial revolution, anthropogenic sources have contributed to such variations, though natural fluctuations often predominate depending on the underlying geology of the area (1).

If not processed correctly, these metals can be extremely toxic and therefore become a health hazard to both humans and the environment. In addition, metals are a non-renewable resource and expensive to locate, mine and refine. Reclaiming heavy metals downstream of their source will minimize the hazard that they pose to the environment and depending on the species of metal being recovered, the financial rewards of such an operation could be vast.

Biological based metal-recovery may provide a possible solution to metal removal from waste or natural waters. Microorganisms in metal-contaminated environments have been forced to cope and utilize the inorganic world around them to ensure their survival (2, 3). These microorganisms play an important role in the solubilization, accumulation, transport and deposition of metals in the environment. To achieve this they participate in a number of metal-interactions, eg. bioleaching, biotransformations and bioaccumulation (2, 4, 5).

The incorporation of these natural phenomenon into biological metal removal processes in polluted or waste water will result in the development of a potential bioremediation tool. Such a process would offer an alternative to the existing conventional metal removal technologies (6).

## **PART 1: METAL BIOACCUMULATION**

### **1.1. HISTORICAL OVERVIEW**

Microorganisms and plants have been depositing and decomposing minerals in the earth crust and oceans since geologically ancient times. Because microbes serve as the basis of all ecosystems they will influence the concentration of metal elements in the environment which will have a significant impact on the food chains and food webs. For example, cells of algae, bacteria, plants, yeasts and other fungi can all accumulate metal ions, often at levels up to 25% of cell dry weight. Ingestion of these microbes and plants result in the cumulative effect of metals at the higher levels of the food chain.

Metal-microbial interactions can however, be beneficial and have been used throughout human history to the benefit of mankind. As early as 1000 BC mine workers in the Mediterranean basin recovered the copper that was leached into mine drainage waters by bacteria. Metal recovery using bacterial leaching was also practiced by the Romans in the first century, the Welsh in the sixteenth century and the Spanish who worked the Rio Tinto mine during the eighteenth century (7).

The ability of a specialized species of the nettle family to grow on toxic soil and subsequently accumulate the copper from the soil enabled archaeologists to unearth the ancient Kabambian culture which flourished in Central Africa. The soil at the sites of the Kabambian copper smelters remained extremely toxic, preventing all plant growth bar the small nettle plant. By following the trail of these plants, the culture of these ancient people could be elucidated (8).

During the 200 years following the beginning of industrialization, changes in the distribution of elements at the surface of the earth have occurred and due to elevations in the levels of many metals in the environment, microbes and other living organisms have had to adapt. This has necessitated enhanced metal accumulation by the microbes and subsequently these microbes have had to develop metal tolerance and metal resistance for their survival.

## 1.2. MICROBIAL BIOACCUMULATION OF METAL CATIONS

The bioaccumulation mechanism of heavy metal cations such as  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$  by the yeast *Saccharomyces cerevisiae* was first reported by Norris and Kelly (4). The accumulation occurs as a biphasic process. The initial phase involves a rapid, metabolism and temperature independent stage, which is followed by slower, progressive metabolism dependent uptake (4). The uptake system which allows for the accumulation of  $\text{Cd}^{2+}$  and  $\text{Co}^{2+}$  appears to be a general one with only limited specificity, since competition for the uptake of cations occurs. Further investigations (5) proved that yeasts are capable of accumulating other cations, eg.  $\text{Mg}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Mn}^{2+}$  (2 - 5). In addition this process is not limited to yeasts, with similar uptake mechanisms being exhibited by bacteria (5) and fungi (9).

### 1.2.1. EXTRACELLULAR ADSORPTION

The first stage of the biphasic metal uptake process involves cation binding to the cell surface, eg. to the extracellular microbial polysaccharide layers of slime produced by the alga *Zoogloea*, or to the cell wall matrix of *S. cerevisiae* or *Rhizopus arrhizus*. Biosorption of metal ions to microbial cell walls is a rapid, reversible process. The overall net negative charge of microbial cell walls facilitates physico-chemical reactions between the cell wall and the positively charged metal cations. This process has been compared to an ion exchange phenomenon, with the reaction chemistry of both the receptors sites and metal species subject to environmental conditions (2, 10 - 12).

Microorganisms are able to accumulate metal ions from solution up to 25% of their dry weight. However, the number of binding sites available on the cell wall for biosorptive complexations are insufficient to account for the amount of metal accumulated. This suggests that associated binding phenomenon occur whereby the deposition of insoluble metal cations at the microbial cell surfaces can proceed. In some cases microbial transformations of the metals is necessitated. Beveridge (cited by 13), noted the formation of gold deposits on the cell walls of *Bacillus subtilis*. He proposed that the metal ions reacted initially with the carboxyl groups of the cell walls, then subsequently acted as nucleation sites for the crystallization of additional gold molecules. A similar phenomenon was observed by Strandberg (cited by 13), regarding the crystalline deposits of uranium on the surface of *S. cerevisiae* cells. Large quantities of insoluble lead (490 mg/g dry weight) have also been observed

on the surface of *Micrococcus luteus* (2).

Precipitation of metal residues in the microbial wall may also explain the elevated levels of cell wall associated metal ions, eg. uranium is precipitated as uranyl hydroxide within the wall of *R. arrhizus* (11).

### 1.2.2. INTERNALIZATION OF METAL CATIONS BY *S. cerevisiae*

Secondary uptake of metal ions is responsible for the accumulation of large quantities of metal ions. The process is usually metabolism-dependent (4), although intracellular metal deposition can also occur via non-metabolically mediated processes (13). Internalization involves the movement of the metal cations from the external surface, across the cell membrane or envelope into the cell interior. Diverse transport mechanisms can be associated with these actions: diffusion through pores, carrier mediated diffusion, uphill thermodynamically active transport, group translocation and pinocytosis (14). A distinction is usually drawn between the uptake of monovalent and divalent cations (2, 15, 16).

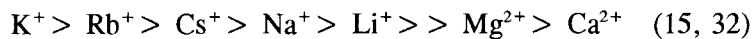
#### 1.2.2.1. TRANSPORT OF MONOVALENT CATIONS

The transport of monovalent cations in *S. cerevisiae* is regulated by the action of the plasma membrane-bound H<sup>+</sup>-ATPase (15, 17 - 24). The H<sup>+</sup>-ATPase enzyme system is based on a K<sup>+</sup>/H<sup>+</sup> antiport action, whereby protons are expelled from the cell. In doing so a transmembrane electrochemical proton gradient, which has both a chemical component (pH) and electrical component (membrane potential), is created. It is the electrical component, *ie.* the membrane potential, which is predominantly responsible for monovalent cation transport into the cell.

The mechanism of uptake of monovalent metal cation into the cell is governed by complex kinetic interactions. Initial kinetics (25) suggested that uptake of monovalent cations adhered to Michaelis-Menton principles. However, these kinetics are influenced by the number of binding sites involved in the translocation process. The kinetics are further influenced by indirect effects of the surface potential (pH) and in addition the membrane potential of the yeast cell across the plasmalemma may also alter the transport kinetics (14, 16, 19, 26).

Sustained transport of cations into the cell requires a metabolizable energy source to maintain ATPase activity and to sustain H<sup>+</sup> efflux from the cell. Addition of glucose (27) and other sugar monosaccharides (28) to the yeast cell stimulate H<sup>+</sup>-ATPase activity, through two processes: glucose can act as an energy source for the biosynthesis of both cytoplasmic and membrane transport proteins, or alternatively the H<sup>+</sup> efflux pump is activated, thereby stimulating accumulation (15).

The majority of information on the monovalent cation system focusses on K<sup>+</sup> accumulation, since the affinity of the transport system for this ion is high. Other cations which can be translocated via this process, include Ca<sup>2+</sup> (29, 30) and Mg<sup>2+</sup> (31). The relative order of affinities of cations for the monovalent cation transport system is:



Heavy metal cations and organometallic compounds inhibit the ATPase antiport system. This results in the reduced ability to maintain the electrochemical gradients, though the H<sup>+</sup> efflux is affected to a lesser extent than the K<sup>+</sup> influx. H<sup>+</sup> efflux is inhibited by a toxicity sequence of divalent cations of Cd<sup>2+</sup> > Cu<sup>2+</sup> > Ni<sup>2+</sup> > Zn<sup>2+</sup> > Co<sup>2+</sup> > Mn<sup>2+</sup> whilst inhibition of K<sup>+</sup> uptake occurs in order of Cd<sup>2+</sup> > Cu<sup>2+</sup> > Ni<sup>2+</sup> > Co<sup>2+</sup> > Mn<sup>2+</sup> > Zn<sup>2+</sup> (15).

The variation in K<sup>+</sup> and H<sup>+</sup> inhibition suggests that K<sup>+</sup> uptake is not limited to H<sup>+</sup>-ATPase activity, but that K<sup>+</sup> accumulation can occur via alternative routes, eg. membrane permeability (14, 15).

#### **1.2.2.2. DIVALENT METAL CATION UPTAKE**

Although certain divalent metal cations, eg. Mg<sup>2+</sup>, Ca<sup>2+</sup>, Zn<sup>2+</sup>, Mn<sup>2+</sup> and Cu<sup>2+</sup>, are essential for yeast cell growth and metabolism, and therefore need to be accumulated from the external environment, many metal divalent cations are toxic to yeast cells above certain concentrations. The most toxic include the group of heavy metal cations, many of which do not fulfill any essential functions in the yeast cell, yet are still sequestered within the cell.

The uptake of divalent cations into the yeast cell is complex. Kelly *et al.* (2), reviewed numerous transport systems and intracellular accumulation patterns of divalent and heavy metal cations, but hesitated to identify any accumulation mechanisms.

Divalent cation uptake in yeast is an energy dependent mechanism (25), which exhibits apparent saturation kinetics (16, 33). The involvement of either a dual mechanism consisting of two simultaneously operating single-site transport processes, or that of a single site transport mechanism (16, 33), results in deviations occurring from Michaelis-Menton kinetics. Addition of glucose, especially in the presence of inorganic phosphate, enhances the uptake of divalent metal cations (25).

Early work by Fuhrmann and Rothstein (25) demonstrated an apparent affinity series of accumulation of  $Mg^{2+} > Co^{2+} > Zn^{2+} > Mn^{2+} > Ni^{2+} > Ca^{2+} > Sr^{2+}$  by yeasts. Subsequent work (34 - 37) has demonstrated uptake of  $Cd^{2+}$  and  $Fe^{2+}$ .  $Co^{2+}$ ,  $Zn^{2+}$  and  $Ni^{2+}$  appear to be transported into the cell by the same mechanism responsible for the uptake of  $Mn^{2+}$  and  $Mg^{2+}$  (25), which are accumulated to a greater extent than  $Ca^{2+}$  and  $Sr^{2+}$ . The difference in accumulation is not due to differences in the affinity for the transport system, but can be ascribed to the leakage and efflux of  $Ca^{2+}$  and  $Sr^{2+}$  from the cells due to increased cell membrane permeabilization (15, 16, 25).

Active divalent cation transport is dependent on plasma membrane  $H^+$ -ATPase activity, although non-competitive inhibition of  $Co^{2+}$  and  $Ni^{2+}$  by  $K^+$  implicates that they are not translocated by the monovalent cation carrier (25). Similarly  $Ca^{2+}$  and  $Sr^{2+}$  uptake is inhibited in a non-competitive way by  $Rb^+$  (38). The major role of the plasma membrane  $H^+$ -ATPase in the uptake of divalent cations is one of energizing the cell membrane thereby sustaining an electrochemical gradient. Enhancement of the membrane potential, eg. increased  $K^+$  efflux, results in increased divalent cation accumulation.

Mutual interaction between divalent cations can also occur. In the presence of specific metal cations, uptake of other divalent cations may be enhanced. For example the uptake of  $Zn^{2+}$  is increased specifically by  $Cu^{2+}$ , whereas the uptake of  $Mn^{2+}$  remains unaffected (16). Similarly  $Co^{2+}$  enhances  $Zn^{2+}$  uptake, with the reciprocal also being true (25). In comparison  $Ca^{2+}$  inhibits  $Zn^{2+}$ ,  $Co^{2+}$  (25) and  $Cd^{2+}$  (4) uptake. The presence of  $Mg^{2+}$  results in a minimal reduction in  $Cd^{2+}$  accumulation (4).

Active divalent metal cation uptake into the cell is inhibited by metabolic inhibitors. Inhibitors of the plasma membrane  $H^+$ -ATPase, eg. DES (diethylstilbestrol) and DNP (2,4 dinitrophenol) block the uptake of divalent cations whilst monovalent cation uptake remains unaffected.

Intracellular divalent cation deposition can also occur via non-metabolically mediated processes. In

the presence of  $\text{Cu}^{2+}$  the permeability barrier of the yeast cell plasma membrane is lost within 2 min. The addition of copper ions to the yeast cells causes increased permeability of the plasma membrane, with massive and rapid release of 70% of the cellular  $\text{K}^+$ . The  $\text{Cu}^{2+}$  causes lesions in the plasma membrane but does not appear to alter the permeability of the vacuole membrane (39). The ability to permeabilize the yeast cell membrane is limited to specific cations, Addition of  $\text{Zn}^{2+}$  does not result in  $\text{K}^+$  efflux, while  $\text{Cd}^{2+}$  does. For every  $\text{Cd}^{2+}$  ion accumulated within the cell, approximately 4  $\text{K}^+$  ions are released. Though responsible for  $\text{K}^+$  efflux from yeast cells,  $\text{Cd}^{2+}$  treatment stimulates  $\text{Ca}^{2+}$  uptake in yeast by increasing the cation permeability of the cell membrane (36).

Internalized heavy metal cations are distributed between the soluble (cytosol) and insoluble (vacuole and mitochondrial) fractions of the cell. Those divalent cations which have not been sequestered within the cell remained complexed to the yeast cell walls.

### 1.3. LOCALIZATION OF ACCUMULATED HEAVY METAL CATIONS

#### 1.3.1. CELL WALL

Rapid, yet reversible binding of metal ions occurs at the cell surface. The interaction between the cell surface and metal ions has been observed in several fungal, bacterial and algal species and has been directly observed in transmission electron micrographs.

##### 1.3.1.1. METAL BINDING BY MICROBIAL CELL WALLS

Sequestration of metal cations by microbial cell walls has been documented for yeast cells (40 - 44), bacteria (45 - 49) and algae (50, 51). Microbial cell walls tend to have an overall net negative charge enabling interaction with counter ions in the environment (46). Metal-microbial wall interaction can be accounted for by cation exchange with negatively charged groups (41, 52), whilst other microbial wall ligands allow coordination of metal ions (52).

The cell envelopes of *Escherichia coli* K-12 are capable of binding large amounts of Hf and Os, intermediate levels of the group IV and transition elements, but only small amounts of the alkali and alkali-earth metals (47). In comparison *B. subtilis* accumulates substantial amounts of the alkali and

alkali-earth metals, viz.  $Mg^{2+}$ ,  $K^+$ , and  $Na^+$ , and intermediate amounts of the higher atomic numbered elements ( $Mn^{2+}$ ,  $Zn^{2+}$ ,  $Ca^{2+}$ ,  $Au^{3+}$  and  $Ni^{2+}$ ). Only small quantities of  $Hg^{2+}$ ,  $Sr^{2+}$ ,  $Pb^{2+}$  and  $Ag^+$  are complexed by the cell walls (48). Yeast cell surfaces are also capable of binding a wide range of metal ions (40, 41, 43).

The ranging affinities of microbial cell walls for specific metal ions can be ascribed to differences in cell wall composition and chemistry, eg. the cell walls of *B. subtilis*, a gram-positive bacterium consists primarily of techoic acid and peptidoglycan. A galactosamine polymer and bound protein may also be present in this structure (49). The chemical and structural composition of gram-positive bacteria differ completely to their gram-negative counterparts. *E. coli*, a gram-negative bacteria contains a minimal amount of peptidoglycan in the cell wall, whilst the outer membrane possesses a lipopolysaccharide (46, 47). In comparison, the rigid cell wall of *S. cerevisiae* consists predominantly of polysaccharides and a lesser amount of protein. Due to the nature of this study, the composition of bakers yeast cell walls and their metal binding properties are discussed in greater detail.

#### **1.3.1.2. COMPOSITION OF THE YEAST CELL WALL**

Due to its abundance, *S. cerevisiae* has been used intensively to study cell wall morphology. The yeast cell wall which is approximately 70 +/- 10 nm thick (53), has been described as one of the most tough and rigid of all microbial cell walls, imparting mechanical strength to the cells. The portion of the dry cell weight represented by the cell wall is large and can account for between 25 - 30% of the dry weight of the cell (54).

The wall fraction of the yeast cell has a layered structure. This bilayered structure consists mainly of intermeshed polysaccharide microfibrils separated into an inner amorphous net of microfibrils (7.5 - 10.0 nm thick) (55) and an interwoven fibrillar outer layer (54). In addition to the polysaccharide components, proteins and lipids occur within the wall (56 - 58) (Table 1.1). A small percentage of the cell wall may be comprised of inorganic ions such as  $Ca^{2+}$  and  $Mg^{2+}$  (59).

Table 1.1. Chemical composition of the cell wall of *S. cerevisiae* (from 57, 58)

Component	% Total dry mass
Glucan	28.8
Mannan	31.0
Protein	13.0
Chitin	1.0
Chitosan	1.0
Lipid	8.5
Ash	3.0
Unknown	13.7

The predominant structural component of the cell wall is glucan, whilst the outer layer is represented mainly by mannoproteins. Chitin which occurs in small amounts, constitutes the primary septum, acting as an indicator of bud scars.

#### 1.3.1.2.1. GLUCANS

Yeast cell wall glucan is a homopolymer of glucose linked through either  $\beta(1-3)$  or  $\beta(1-6)$ -D-glycosidic bonds. It forms part of the inner layer of the bilayered cell wall and is responsible for cell rigidity and subsequent maintenance of cell integrity. Two species of glucans, either alkali-soluble or alkali-insoluble fractions occur. The alkali-soluble fractions constitute the minor component, whilst the major glucan component (a  $\beta(1-3)$  linked backbone containing 3%  $\beta(1-6)$  linkages) is insoluble in alkali (54, 55, 57, 58; 60, 61).

#### 1.3.1.2.2. CHITIN AND CHITOSAN

Closely associated with glucans are the chitin polymers. Recent evidence has stated that chitin, a linear polymer of  $\beta(1-4)$  linked N-acetylglucosamine, may be covalently linked to glucan. The glucan chains appear to be directly linked to chitin through a glycosidic linkage between position one of the

first residue of the glucan chain and position six of the intra-chain N-acetylglucosamine residue (54,62).

#### **1.3.1.2.3. MANNANS**

Mannan is a polymer of mannose monomers forming a main chain linked by  $\alpha(1-6)$  bonds and  $\alpha(1-2)$  and  $\alpha(1-3)$  linked mannose sidechains (53). In yeast, mannans are not found as a separate entity within the wall but are covalently linked via serine, threonine or asparagine residues to form a mannoprotein complex of 25 - 500 kDa. The complex has a mannan : protein ratio of 12 : 1, forming a molecule with a slightly larger dimension than pure mannan.

The mannan contains approximately 1% phosphate which appears to be linked to the 6 position of the non-reducing mannose residue of the main chain (57). The phosphate moiety is diesterified, and therefore capable of bonding with another C-mannose residue, thereby fulfilling a cross linking function (53).

#### **1.3.1.2.4. PROTEINS**

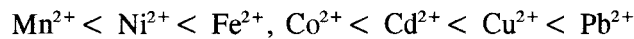
The exact number of cell wall proteins is unknown (54), although different types of proteins varying in molecular mass between 30 - 410 kDa have been extracted from the wall. Though these proteins are found throughout the cell wall they occur more prominently in the outer mannoprotein layer (58).

#### **1.3.1.3. YEAST CELL WALL - METAL INTERACTIONS**

Cell walls of yeast have been proven to bind  $\text{Ca}^{2+}$  (44) and  $\text{UO}_2^+$  (40) forming stable, yet reversible complexes. Subsequent work has established an affinity of metal ions for cell wall components, eg.  $\text{Cu}^{2+}$  and  $\text{Ca}^{2+}$  for chitosan (63, 64),  $\text{Cd}^{2+}$  for mannoproteins (65) and  $\text{Co}^{2+}$  interaction with the protein component of both the upper and lower layers of the cell wall (41).

Present in the cell wall are an abundance of ligands capable of complexing with heavy metal and other cations. Each of the major cell wall components contributes specific binding ligands. Depending on the nature of these ligands they can selectively bind certain metals, though in general the cell exhibits non-selective accumulation of metal ions.

Metal ions can be separated into three categories: class A, class B and borderline metal ions (66). Class A metal ions are referred to as hard acids, whilst class B metal ions are called soft acids. The former are found at the left hand side of the periodic table and include ions of the alkali metals and alkaline earths. In contrast class B metal ions form a small block of roughly triangular shape with  $\text{Cu}^{2+}$  at its apex ( $\text{Cu}^{2+}$  is a borderline metal) and  $\text{Ir}^{3+}$  and  $\text{Bi}^{3+}$  at its base. The heavy metals are categorized under borderline metal ions (66). When plotted graphically according to their class A or ionic index ( $Z^2/r$ ) vs their class B or covalent index ( $X^2_{mr}$ ), the borderline ions show a greater affinity towards class B characteristics, with the class B character increasing in the order:



An exception is  $\text{Zn}^{2+}$  which exhibits considerable class A character (66).

A marked difference occurs between the types of binding sites preferred by class A and class B metal ions (52, 66). Class A ions favour oxygen binding sites viz. carboxylate, carbonyl, alcohol, phosphate or phosphodiester groups, whilst class B ions prefer nitrogen and or sulphur centres, eg. sulphhydryl (-SH), disulphide (-S-S-), thioester (-SR), amino (- $\text{NH}_2$ ), or heterocyclic nitrogen (imidazole of histidine or nucleotide bases) (52, 66). Variations of binding affinity within the heavy metal group occurs. For example  $\text{Cu}^{2+}$  prefers the nitrogens of histidine, whilst  $\text{Zn}^{2+}$  binds in preference to the sulphhydryl groups of cysteine.

Yeast biomass contains several of these binding ligands on its cell walls, eg. the carboxylate, hydroxyl, phosphate and amino groups of the carbohydrate and protein components (43, 67). The outer layer plays a more significant role in metal binding than the inner layer, suggesting the importance of the mannoprotein fraction in comparison to the glucan and chitin components (67).

### 1.3.2. YEAST VACUOLES

Vacuoles in plants and fungi occupy between 25 - 95% of the cell and have essential and dynamic physiological roles (68). Several hydrolytic enzymes are contained therein and in addition, this organelle is involved in pH homeostasis and the compartmentalization of metabolites and ions.

Many different cellular constituents including amino acids (69 - 71), polysaccharides and polyphosphates (72, 73) are stored within the fungal vacuole. The storage and homeostasis of cellular metal ions are also controlled by this organelle. Cells are obligated to regulate their cytosolic ion

concentration for several reasons. Some of the ions are potentially harmful, eg.  $\text{Co}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Hg}^{2+}$  and  $\text{Fe}^{3+}$  and cannot be allowed to accumulated within the cytosol but must be transferred elsewhere. Even the physiological cations may become toxic in excessive concentrations. Their levels must be precisely controlled if they are to play a role in the regulation of cellular processes (73, 74). Vacuole stored metal ions include such physiologically useful cations as  $\text{Zn}^{2+}$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$  (74 - 77) as well as the non-physiological cations eg.  $\text{Co}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Au}^{3+}$ ,  $\text{Cd}^{2+}$  and  $\text{Fe}^{3+}$  (78 - 81) (for review 73).

Studies on the internal distribution of metal ions in yeast cells after accumulation indicate a selective accumulation of metal ions by the vacuole. Whilst the proportion of  $\text{Na}^+$  and  $\text{K}^+$  in the cytosol exceeds that of the vacuole, 21.2% of accumulated  $\text{Cd}^{2+}$  is found in the vacuole compared to 0.8% in the cytosol. Likewise, 31.1% of  $\text{Ag}^{3+}$  is compartmentalized within the vacuole compared to 7.8% which remains soluble in the cytosol (82). Intracellular ferritin, zinc and other heavy metals are also found concentrated within the vacuole. Mutants with defective vacuoles are more sensitive to heavy metal poisoning and antibiotics. Even  $\text{Ca}^{2+}$ , a physiological ion, may be inhibitory to the growth of strains with defective vacuoles (75).

Like the storage of nutrients and metabolites, the accumulation of metal cations within the vacuoles requires a transport system. Vacuolar uptake of several cellular constituents is carried out by  $\text{H}^+$ /ion transport systems, *ie.* ion uptake into the vacuole is driven by a proton efflux from the organelle. In *vitro* studies with purified vacuole vesicles have shown that  $\text{H}^+$ /ion antiports are the principle mechanism of vacuolar uptake of arginine, the divalent cations  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$  and several other amino acids (15, 22, 78, 80, 83, 84).

The proton gradient is generated by an  $\text{H}^+$ -ATPase, the V-ATPase enzyme, comprised of eight subunits (85). Similar to the plasma membrane system, the energy capacity of the vacuolar  $\text{H}^+$  gradient can be increased by the corresponding  $\text{K}^+$  gradient. The efflux of vacuolar  $\text{K}^+$  initiates the accumulation of  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  within the vacuole (38, 39, 79, 86) .

Substantial evidence (73) indicates that once the accumulated metal ions and amino acids have entered the vacuole they interact with polyphosphate ions. Polyphosphates are macromolecular anions and are unique to the vacuole. Within the vacuole they serve a dual function. Due to their anionic nature they coordinate with basic amino acids and inorganic cations, thereby acting as storage

macromolecules. Secondly, because they lower the concentration of free inorganic phosphate, amino acid and inorganic cations, they reduce the osmotic pressure within the cell. Hence both the cytosol and vacuole remain isotonic (73).

### 1.3.3. YEAST MITOCHONDRIA

Although the mitochondria do not appear to be a major site of metal ion accumulation,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$  and other monovalent ions have been reported to interact with the mitochondrial enzymatic system, whilst  $\text{Ca}^{2+}$  uptake and release has been documented in yeast mitochondria (87 - 89).

Yeast mitochondria have diverse  $\text{Na}^+$  and  $\text{K}^+$  transport systems. A  $\text{K}^+/\text{H}^+$  ATPase antiport system, similar to that of the plasma membrane and tonoplast has been identified in the mitochondrial membrane. Ion transport is non-specific,  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Rb}^+$ ,  $\text{NH}_4^+$  and  $\text{Li}^+$  all enhance ion transport (87). Mitochondrial ATPases also have an absolute requirement for monovalent cations (89). Should the same non-specificity apply to divalent cations this would imply that the mitochondria could participate in intercellular metal cation sequestration.

## **PART 2: BIOREMEDIATION**

### **2.1. WATER AVAILABILITY AND DRINKING WATER CRITERIA IN SOUTH AFRICA**

The need to take precautions with drinking water to protect public health was recognized as many as 4000 years ago. Today the quality of drinking water is still of primary concern (90). South Africa is not only faced with the problem of maintaining the quality of its drinking water, but has to protect its dwindling supplies. It has been calculated that the supply and demand curves for potable water available in South Africa will cross in the year 2020 and beyond that, demand will exceed supply (90).

How is drinking water described? Criteria have been established, not as regulatory requirements, but merely to serve as guidelines. No legally enforceable drinking water standards exist in South Africa (90). In short the drinking water quality criteria represent the maximum level of contaminants present therein that the water can be consumed with adequate safety (90).

The task of establishing criteria is not an easy one. The criteria can only be as reliable as the body of knowledge existing for a given constituent (91). Due to technological advances more information has become available which has prompted the need to focus on a new range of determinants, previously regarded as insignificant. Important determinants include microbiological factors, aesthetic considerations, organic chemical material, radionuclide and physical and inorganic determinants. Included within the latter group of determinants are the natural elements which were long regarded as having primarily aesthetic or industrial significance and therefore being of only marginal health importance. However, there are inorganic constituents of more obvious public health significance, eg. the toxic metals. Industrial and mining activities provide numerous opportunities for toxic metal release into the environment and although the waters produced as by-products from these activities have been subject to pollution control guidelines, the water quality continues to deteriorate.

## 2.2. METALS AS POLLUTANTS IN WATER

The toxicity of metals in natural waters is widely assumed to result from a combination of their solubility, bioavailability and mode and kinetics of uptake in the target organism (1, 66). Microbes in aquatic systems are key components in biochemical cycling of elements and serve as the basis for all food chains and food webs. Micro- and macrobiota sensitive to metal pollutants will be affected at various physiological levels thereby disturbing the equilibrium of the food chains.

Elements exhibiting the most toxic effects on micro- and macrobiota in aqueous systems include in particular the heavy metals. The heavy metals are comprised of 40 elements with a density greater than 5. Many of these metals are essential for growth of both prokaryotic and eukaryotic organisms, but also have a comprehensively toxic effect on cells. Others do not fulfill any physiological functions, but cause metal toxicity within the cell. The mechanisms of metal ion toxicity can be divided into 3 categories: 1) inhibition of the essential biological functional groups of biomolecules, 2) displacement of the essential metal ions in biomolecules and 3) modification of the active conformation of biomolecules (66, 92, 93, 94). Heavy metal contamination of the lowest strata of the food web will have a cumulative effect throughout the food chain. Microbes and fish are more sensitive to the effect of metal interaction than man, and hence the suggested criteria for river and dam water differs from the levels stated for drinking water.

The metal ions can originate from a variety of sources including the nuclear power, defense and fuel reprocessing industries, surface finishing and electroplating processes, mining operations, smelting in the metal processing industry and textile industries (90, 95). In addition to contaminating streams, the ground water at many of the sites is affected. By limiting the loss of metal ions during processing, or by removing and concentrating the ions, not only would the impact on the environment be vastly reduced, but the industrial processes would become economically more viable.

The three categories of heavy metal cations: class A, class B and borderline ions have the toxicity sequence:

Class B > Borderline > Class A (66)

In aquatic systems class B and borderline ions are able to form water-soluble organometallic cations capable of crossing biological membranes. Toxicity of the metal ions is largely influenced by

speciation, pH, temperature, inorganic ionic concentration, organics and hydrous metal oxides (66, 94, 96).

### 2.3. BIOREMEDIATION OF METAL-CONTAINING WATER

Bioremediation of polluted water is not a new concept (6), yet it offers an alternative to the existing, conventional metal removal technologies of metal ion exchange, evaporative recovery, electrochemical treatment and chemical reduction. Both approaches have their respective advantages and disadvantages. The disadvantages of bioremediation are compensated for by the high metal binding capacity and selectivity of microorganisms for certain metals and most importantly the low operational costs.

Bioremediation of waste water involves biotechnology-based processes whereby metal ions are removed from the waste water. Microorganisms play an active role in the solubilization, accumulation, transport and deposition of metals in the environment (3).

The mode of action whereby microorganisms remove metal ions from water includes absorption, adsorption and bioaccumulation. Collectively absorption and adsorption constitute biosorption. Due to the physico-chemical nature of biosorption, waste ions are removed from aqueous solutions rapidly and efficiently, thereby enhancing the biomass potential in waste water bioremediation. In addition non-viable biomass can be used, thereby avoiding such problems as the provision of nutrients, contaminations and toxicity to the biomass (97, 98).

The metal accumulative properties of microbial biomass have prompted research into the removal of radionuclides from industrial wastes. Radionuclides, eg. Tc, Zr, U are formed during fission and activation reactions in nuclear reactors and are released as part of the fission products or enter the waste water due to technical breakdowns, eg corrosion, (99 - 103). Due to the longevity of radionuclides, eg.  $^{93}\text{Zr}$  :  $1.5 \times 10^6$  (100) and Tc-99 :  $2.1 \times 10^5$  years (99), the waste will remain active in the natural environment for long periods of time.

Biotechnological processes, for removal of toxic metals and radionuclides from waste has gained impetus and credibility over the years and include a number of microbial interactions: bioleaching (3, 104, 105), biotransformation (104, 106) and bioaccumulation (4, 5, 104, 105).

## 1. GENERAL INTRODUCTION

The use of microbes in leaching metals from ores has been intensively studied. The leaching may involve direct enzymatic oxidation of the substrate, or indirect leaching and biotransformation, eg. *Thiobacillus ferrooxidans* (7). Related to bioleaching are microbial transformations based on the mobilization and immobilization of the toxic metals. Aerobic, anaerobic, mesophilic, autotrophic and heterotrophic groups are involved in the microbial activities, which include solubilization and leaching of the metals, oxidation, reduction and precipitation of metal ions (106). Although bioleaching and biotransformation can mobilize metals from deposits, thereby reducing their environmental impact, subsequent accumulation and removal of the metals ions is necessary.

Bioaccumulation systems can employ a single or a mixture of microorganisms or higher plants. Algal and cyanobacterial blooms have reduced the levels of  $\text{Cd}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Fe}^{2+}$  and  $\text{Co}^{2+}$  in laboratory solutions (107 - 116),  $\text{Cd}^{2+}$  in water leached from rice paddies (117),  $\text{Cr}^{3+}$  from tannery effluent (118) and  $\text{Zn}^{2+}$  from river water (112). In addition microalgae (100, 99) and cyanobacteria (100) are capable of accumulating Zr and Tc from nuclear reactor waste.

Removal and subsequent sequestration of metal ions by bacteria has also been successful. Yttrium (107) and  $\text{Cr}^{6+}$  (92) have been removed from nuclear waste, whilst  $\text{Cu}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Se}^{2+}$ ,  $\text{Ag}^{3+}$ ,  $\text{As}^{2+}$  and  $\text{Hg}^{2+}$  have been sequestered from spiked aqueous solutions (119 - 125) and industrial waste (95, 126 - 132).

Plants, although not as frequently used as a bioremediation tool for the removal of metal ions from aqueous solutions, play an important role in metal ecology. A relationship exists between the soil and the vegetation covering it. Plant species colonizing metal contaminated soil or areas containing a high metal content are often stunted with unusual growth features (8, 133, 134). Metal ions removed from the soil are most commonly concentrated in the storage tissue, eg. roots (133, 134).

Fungi and yeasts can accumulate significant amounts of heavy metals and radionuclides. Although this feature is common to other microbial and plant cells, fungi may be better suited for this purpose than other microbial groups because of their high tolerance towards metals and other adverse conditions, eg. high cell wall binding capacity and high intracellular uptake by viable cells, low pH or osmotically unstable conditions (102, 135). Additional advantageous attributes of fungal biomass include the range of morphological types available, which include unicellular and filamentous forms. Large amounts of fungal biomass are derived as waste products from industrial processes and

fermentations, eg. *R. arrhizus*, a filamentous fungus, which appears to be a highly successful biosorbent for metal and radionuclide removal (10 - 12, 49, 102, 135, 136). Similarly *S. cerevisiae*, a byproduct from industrial fermentation industries has been employed to monitor and treat aquatic pollutants. *S. cerevisiae* can accumulate numerous metal, eg.  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Au}^{3+}$ ,  $\text{Ag}^{3+}$  and  $\text{Pb}^{2+}$  from both synthetic solutions and polluted waste water (4, 86, 102, 105, 108).

## 2.4. INDUSTRIAL APPLICATIONS

### 2.4.1. INDUSTRIAL EFFLUENTS

Since the onset of industrialization, the deposition of heavy metals into the natural water streams has altered the cyclic fluctuations of metal and trace elements previously found therein. The toxicity of these metals in natural waters results from a combination of their solubility, bioavailability and the effect on the organism at a cellular level.

The major sources of metal pollution into aquatic ecosystems include domestic waste water effluents, coal burning power plants, non-ferrous metal smelters, iron and steel plants, mine and metal finishing industries and the dumping of sewage sludge. In 1988 it was estimated that 25% of industrial effluents in Europe were discharged into lakes and rivers (137). However, due to the rapid onset of industrialization and poor control of the water quality guidelines this can be considered a conservative estimate.

#### 2.4.1.1. ELECTROPLATING EFFLUENT

The industrial waste effluents of interest in this study were those generated by electroplating processes. With the exception of a few large concerns, the majority of electroplating industries operate as small entities, discharging their waste into the underground disposal system. In comparison to several other types of industries, eg. the mining industry, the volumes of waste discharge are relatively low. The annual water consumption by the electroplating industry in South Africa is approximately 9 million  $\text{m}^3$  of which 80% is discharged as effluent. The primary cause for concern regarding this effluent remains the extremely high levels of metals present.

Depending on the type of electroplating industry, the species of metal present in the discharge varies.

## 1. GENERAL INTRODUCTION

Predominant sources of contamination during the finishing operations include the discharge of untreated solutions obtained from: 1) alkali cleaning to remove oil and grease, 2) acid descaling and pickling of various basis metals, 3) metal plating solutions from cyanide complexes of Zn, Cd, Cu, Ag and Au, 4) metal plating solutions based on the acid complexes of Ni, Cu and Cr, 5) tin plating based on acid or alkaline complexes, 6) sulfuric acid anodizing of aluminum, 7) chromium compound based conversion coating on Al, Cd, Zn and Cu alloys, 8) accidental overflows of solutions or possible leaks as well as the periodic discharge of exhausted process solutions.

### 2.4.2. BIOMASS SYSTEMS FOR INDUSTRIAL BIOREMEDIATION

In view of the low toxicity, cost effectiveness and comparable results to those of activated charcoal (10, 97, 139) and ion exchange resins, microbial biomass systems appear to be an attractive alternative for the remediation of industrial waste water.

The choice of biomass varies, ranging from easily obtained biomass types, to specially isolated or even genetically engineered strains. Raw biomass which has been processed or modified to improve its metal sorption characteristics may also be utilized. Although the biosorbent biomass can be selected from algal, fungal and bacterial strains, the most convenient source of raw material is that obtained as by-products from large scale fermentations or pharmaceutical processes (138). The biomass can either be applied as viable or non-viable biomass. Advantages of non-viable biomass include low maintenance costs, enhanced metal sorption levels and absence of maintaining the cultures. Moreover, living cells are prone to the toxic effects of effluents which may result in cell death, thereby nullifying any of the advantages of using live cells (97, 139).

*Bacillus spp.* either immobilized (140) or contained within a packed-bed reactor (141) was exposed to spiked metal solutions. The immobilized cells exhibited good metal loading characteristics, viz. 70 mg Cr<sup>3+</sup>/g cell weight and 64 mg Cu<sup>2+</sup>/g cell weight. Alkali treatment of the immobilized pellets improved the metal retention capabilities, viz. 118 mg Cr<sup>3+</sup>/g cell weight and 116 mg Cu<sup>2+</sup>/g cell weight respectively (140). Uranium removal by *Bacillus subtilis* cells in a packed-bed reactor has exhibited the potential to become an effective, yet simple system whereby metal removal could commence (141).

Numerous applications exist for microbial biomass in the treatment of industrial and mining waste.

## 1. GENERAL INTRODUCTION

Pelleted and immobilized yeast, fungal and waste bacterial and algal biomass have been used in the removal and recovery of heavy metals and radionuclides. Waste biomass of *Streptomyces noursei* exhibited the following affinity series for metals:  $\text{Ag}^{3+} > \text{Pb}^{2+} > \text{Cr}^{3+} > \text{Cu}^{2+} > \text{Zn}^{2+} > \text{Cd}^{2+} > \text{Co}^{2+} > \text{Ni}^{2+}$ . The affinity sequence and amount of metal removed differed depending on the biomass, a fact which facilitates specific removal and subsequent sequestration by the microbial biomass (139 -141). Application of this characteristic to mining effluent containing economically viable metals would provide a cheap alternative to gold and silver recovery.

Two systems incorporating microbial biomass as bioremediation agents which have been particularly successful are firstly a granular metal-accumulating, non-viable biomass product developed by an American company. The biomass, referred to as metal recovery agent (MRA), is capable of accumulating large quantities of metal cations and is more than 99% efficient in cation removal from dilute metal solutions. Its accumulative capacity extends to a wide range of metal cations, inclusive of gold, silver, palladium, platinum, lead, copper, cadmium and zinc (142).

MRA is prepared by exposure of the microbial biomass to caustic conditions and removes metal ions from solution in a pH range between pH 2 - 11, with optimum metal accumulation between pH 4 - 8. Although regarded as a good metal removal agent which has been successfully employed in pilot scale trials, limitations with respect to its metal accumulating abilities do occur. Similar to other metal-microbial interactions, metal speciation does influence MRA binding capacities. For example, MRA is only capable of accumulating reduced levels of gold from gold-cyanide complexes, compared to other salts of gold (142).

The second system which has been successfully employed in industrial bioremediation is one developed by the U.S. Bureau of Mines (143, 144). The Bureau developed BIO-FIX beads (an acronym for biomass-foam immobilized extractant) from a variety of raw materials, eg. blue-green algae (*Spirulina sp.*), marine algae (*Ulva sp.*), yeast (*S. cerevisiae*) and sphagnum peat moss. These systems were subsequently utilized in hydrometallurgical processing.

The BIO-FIX beads displayed superior physical and chemical stability, withstanding several adsorption-desorption cycles. Sphagnum-containing BIO-FIX beads demonstrated the greatest metal sorption capacity. The beads were tested on acid mine drainage metals using stirred tanks, fixed bed columns, fluidized bed columns and a low maintenance passive system. Metal sorption was rapid,

with > 50% of equilibrium sorption occurring within 5 minutes, and in excess of 90% sorption achieved after 20 minutes (143, 144).

A number of other systems for heavy metal removal from industrial solutions have been reported, but these have been performed on a smaller scale.

## 2.5. RESEARCH AIMS

The aims of the present study were twofold, to identify and utilize a biological system of metal removal from waste water. Commercially produced bakers yeast, *S. cerevisiae* was used as a source of biomass due to the low cost factor and abundant supply. In order to implement a successful bioremediation system for the treatment of metal contaminated waste water, the mechanisms of metal biosorption and uptake by the yeast cells need to be understood.

The accumulative capacity of yeast for a wide range of metals has been known for a considerable time (see section 1.2.2). The initial aims of this research were thus to elucidate the mechanisms of metal accumulation by *S. cerevisiae*. The uptake and localization patterns of metals in intact yeast cells was studied and following on from this work, a more detailed analysis was focussed on metal accumulation by isolated yeast cell walls and cell wall components as well as isolated vacuoles. The three metals used throughout the biochemical studies, viz.  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$  differ in many respects, but are representative of the heavy metal group.  $\text{Cu}^{2+}$ , a physiological cation and the least toxic of the metals, is often required by cells as a metalloenzyme. The other two cations  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$  have a greater toxicity, eg.  $\text{Cd}^{2+}$  serves no function within the cell, and causes cell damage and ultimately cell death. The interaction of the cells and cellular components with these metals of varying toxicities was monitored.

A detailed knowledge of the mechanistic and biochemical aspects of heavy metal processing by viable and non-viable yeast cells enabled the development of subsequent biotechnological processes. Bioaccumulation of metals from electroplating effluent by viable yeast cells were studied and since the biosorptive properties of non-viable yeast cells exhibit similar or greater affinities for heavy metals, additional metal uptake studies were conducted with non-viable yeast biomass preparations.

Three immobilization techniques (viz. embedding and crosslinking) were employed to produce potentially viable industrial yeast products, viz PVA Na-alginate, PVA Na-orthophosphate and

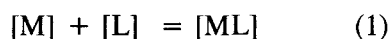
## *1. GENERAL INTRODUCTION*

PEI:GA pellets, which could be implemented in bioremediation processes. These preparations were tested for their metal biosorptive and mechanical properties, with the biomass most suited as a biosorbent being selected for further studies. The analytical results and performance of the biomass as a biosorbent were compared using batch reactors, continuous-flow fixed bed biomass columns and continuous-flow stirred bioreactors. All of the later experimental work was conducted using industrial waste obtained from electroplating plants. This waste contains a wide range of metals in levels exceeding the stipulated criteria for aquatic and drinking water. The environmental threat brought about by the direct discharge of this untreated waste into the underground sewage system initiated the above-mentioned research.

## 2. INTERACTION WITH, AND ACCUMULATION OF METAL IONS BY YEAST CELL WALLS AND CELL WALL COMPONENTS

### 2.1. INTRODUCTION

The binding of metals to cell walls is an equilibrium reaction, with the interaction between the divalent metal cations and the anionic ligands of the cell wall expressed as:



Where M represents the free metal cations in solution, L the ligands and ML the metal-ligand interaction. The square brackets denote the concentration in appropriate units for aqueous solutions (40).

Rothstein and Hayes (40) subsequently developed a mass law equation whereby K, the dissociation constant could be determined. Their determination of K was dependent on the three activity coefficients:  $f_M$ ,  $f_L$  and  $f_{ML}$ . The value for  $f_M$  can be determined from the ionic strength, but the activity coefficient for the two solid phases are unknown:

$$K = [M][L]/[ML] \times f_M f_L / f_{ML} \quad (2)$$

Others (66, 145) chose to express the formation of a metal-ligand complex differently, and express K as the equilibrium constant  $K_{ML}$ :

$$K_{ML} = [ML]/[M][L] \quad (3)$$

As an equilibrium (145) or dissociation (40) constant, K represents the ratio between the forward and reverse reaction rates of metal-ligand complexation. The larger the magnitude of the equilibrium constant  $K_{ML}$ , the more stable the complex ML is in solution (66). Both of the groups (40, 145) assumed the independent nature of K, whereby the formation of a complex at one site does not alter the affinity of other sites for metal cations. Although the value of  $K_{ML}$  is almost independent of the nature of the ligand, it depends strongly on the particular metal cation involved. Cations with a high  $K_{ML}$  value will occur mostly as a complex, eg.  $Cu^{2+}$ , whilst those with low equilibrium values eg.  $Na^+$ ,  $K^+$ , will remain as free cations. Metal cations can be classified with the following decreasing values for the equilibrium constant:

$Fe^{3+} > Pb^{2+} > Cu^{2+} > Ni^{2+} > Co^{3+} > Zn^{2+} > Co^{2+} > Cd^{2+} > Fe^{2+} > Mn^{2+} > Mg^{2+}, Ca^{2+} > Sr^{2+} > Ba^{2+} > Na^+, K^+$  (145).

## 2. CELL WALLS AND CELL WALL COMPONENTS

Metal-ligand formations are however, not always simplistic. The afore-mentioned classification is based on thermodynamic factors independent of kinetic considerations. In a few cases, the ligand preference may be controlled kinetically, one such example being the interaction of  $Pt^{2+}$  with simple ligands, proteins and nucleotides (52, 66). In addition, ligands may be mono-, bi- or multidentate, enabling them to attach to the metal ions using one, two or more donor atoms. If a bidentate ligand co-ordinates with a metal ion through both donor atoms a ring structure is formed. pH is also an important factor in regulating the access of metal ions to binding sites in biological molecules (52, 66, 145). Protons will directly compete with metal ions for binding sites, with metal ions frequently having to displace protons in biological systems (66). Thus equation (1) can be rewritten as a proton displacement reaction:



For example, when an amino acid is the ligand, a heavy metal cation will bind to the carboxylate group in a mildly acidic solution. Binding to the amino nitrogen will not occur until the pH is raised to deprotonate this group.

Initial research work (40) confirmed the presence of multiple binding sites on the cell surface of yeast, although only a small fraction of the cellular structure is involved in the binding of exogenous cations. Due to the composition of the yeast cell wall (section 1.3.1.2.) and the three dimensional arrangement of the ligands, a range of binding sites are available for metal complexation (43, 50, 52, 66, 67, 146).

The amorphous orientation and cross linkages between the cell wall polymers may limit the availability of the ligands for metal binding, through masking. For example, selective removal of mannan causes an increase in copper binding, possibly by exposing metal binding sites on the protein fraction (67). However, enzymatic degradation of the yeast cell wall indicates that the relationship between wall proteins and metal ions, although important, is not the sole metal binding interaction of the wall. The outer mannoprotein layer appears to dominate metal binding. Removal of this layer with its phosphodiester links decreases the cation binding capacity of isolated cell walls by 50% (44).

Each of the respective cell wall components contribute metal ligands for metal binding. The ligands contributed by the carbohydrate moieties are inclusive of a range of groups *viz.* hydroxyl, carboxyl, amino or phosphate groups, which can either interact with class A or B metals thereby facilitating ionic or covalent interactions. In comparison, protein-metal interactions favour covalent

interactions, due to the presence of amino, carbonyl groups and the nitrogen/sulphur centres. Chemical modification of the ligands of isolated cell walls confirmed the importance of amino, carboxyl and hydroxyl groups for metal binding. Brády *et al.* (43), tested the ability of cell walls to bind  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Co}^{2+}$  by chemically modifying the respective cell wall components. From this work it was concluded that most, if not all, of the cell wall components play a role in heavy metal accumulation.

The aim of this experimental section was to establish the importance of the carbohydrate fractions of isolated yeast cell walls and cell wall components in metal binding. The metal binding affinities of the polysaccharide fractions for three cations, viz.  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$  were compared. Qualitative analysis using infrared spectroscopy was undertaken to determine any change in configuration of carbohydrate groups due to metal interaction.

## 2.2. MATERIALS AND METHODS

### 2.2.1 PREPARATION OF ISOLATED CELL WALLS

Commercial preparations of *S. cerevisiae* (production strain, approximately 90% cell viability) were obtained from Anchor Yeast Inc. The cells had been grown aerobically in molasses wort, adjusted with phosphoric acid and harvested in the stationary phase. Wet, pressed bakers yeast was stored at 4°C prior to use. Before being used, the yeast was washed three times (3000 g x 10 min) using ultra-pure water (Milli-Q water) and harvested. Preparations of the isolated cell walls were provided courtesy of B. Prior and P. van Zyl (Dept Microbiology and Biochemistry, University of Free State, South Africa). Yeast cells were disrupted using a Braun homogenizer in the presence of glass beads (0.5 mm diameter). To prevent heat build-up and the effects of autolytic enzymes, the disruptions were completed discontinuously at 4 - 10°C using ice and liquid nitrogen to maintain the temperature. This technique achieved cell disruption of 95%. Lysed cells could be seen as dark "ghosts" when viewed under phase contrast microscopy. Removal of the cell debris was achieved through centrifugation. Yeast cell walls were freeze-dried and this fraction was then suspended in 5 mM 1,4 piperazinediethanesulphonic acid (PIPES) (PIPES, Sigma Co) buffer and centrifuged at 4°C (3000 g x 10 min). PIPES buffer (pH 6.5) was used throughout the biochemical studies due to its negligible metal accumulating properties (Sigma buffer catalogue). The

## 2. CELL WALLS AND CELL WALL COMPONENTS

supernatant was discarded and the pellet freeze-dried and stored in a desiccator at room temperature until required.

### 2.2.2. CELL WALL COMPONENT EXTRACTION

#### 2.2.2.1. CHITIN AND CHITOSAN EXTRACTION

Chitin and chitosan were successfully extracted from a 100 mg sample of yeast cell wall material following the method of Muzarelli (63). The cell walls were demineralized with 5% HCl for 5 hrs, using 11.8:1 (w/w) ratio of HCl to dry weight of yeast cell walls. After washing the walls to neutrality using Milli-Q water, the solution was centrifuged (3000 g x 10 min) and the pellet subsequently deproteinated with 2% NaOH (65°C for 2 hrs under nitrogen) using 30:1 (w/w) ratio of NaOH solution: dry weight yeast cell walls. The material was once again washed to neutrality using ultra-pure water, centrifuged and the pellet dialyzed twice against Milli-Q water (4°C, 4 hrs) before being freeze-dried.

#### 2.2.2.2. MANNAN ISOLATION

Mannan was extracted according to the method of Northcote and Horne (57). Cell wall material (150 mg) was digested with 3% NaOH (w/w) for 6 hrs at 100°C. The digested material was centrifuged (3000 g x 10 min) and the supernatant retained and acidified to pH 6.0 using 2 M-acetic acid. The mannan in this solution was precipitated using 4 volumes of ethanol. The supernatant was discarded, the precipitate redissolved in water and reprecipitated using ethanol. The white solid obtained was washed using diethylether to remove lipids, dialyzed twice against Milli-Q water and freeze-dried.

#### 2.2.2.3. GLUCAN ISOLATION

The alkali-insoluble pellet from the mannan extraction was digested using 3% NaOH (w/w) (100°C, 6 hrs) and subsequently by 0.5 M acetic acid (75°C, 6 hrs) before being washed in 2 ml of ethanol and ether respectively (54). The remaining white precipitate was dialyzed twice against Milli-Q water (4°C, 4 hrs) prior to being dried in the normal fashion.

### 2.2.3. PROTEIN AND CARBOHYDRATE DETERMINATION

Prior to analysis, cell wall components were hydrolyzed using HCl: 1) chitin was dissolved in 4 M HCl at 1 mg/ml concentration. Digestion proceeded for 4 hrs at 100°C (147), 2) mannan and glucan (1 mg/ml) fractions were hydrolyzed for 6 hrs at 100°C with 2 M HCl.

The Folin-Coicalteau assay was used for protein determination (Appendix 1). Cell wall components were analysed in triplicate using 0.1 ml aliquots of the prepared samples. All absorbance readings were made at 500 nm against a blank. The Nelsons test for reducing sugars was utilized to determine the amount of carbohydrate present in each of the cell wall acid digest samples. All determinations were done in triplicate against a blank at 540 nm (Appendix 2).

### 2.2.4. QUANTITATIVE METAL BINDING STUDIES

The dialysis apparatus used was similar to that used by Marrack and Smith (148). Two identical perspex units, each containing five separate 1.2 ml chambers were clamped together to form five 2.4 ml chambers bisected by a semi-permeable dialysis membrane (Spectrapor dialysis tubing, 6-8 kDa cut off). Two small perspex balls were placed in each of the chambers to agitate the solution during shaking.

Comparative metal binding studies of the isolated yeast cell walls and cell wall components, using  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$  cations were conducted in the dialysis chamber at room temperature (22°C) in a shaking water bath. Initial range finding experiments were conducted to determine the required metal concentration and duration of contact for optimal metal accumulation.

One milliliter of the metal solution (4 mM  $\text{CuCl}_2$  and  $\text{CdCl}_2$  and 2 mM  $\text{CoCl}_2$ ) was pipetted into one side of the chamber, whilst 1 mg of yeast cell wall material, suspended in 5 mM PIPES/TMAH (Tetramethylammonium hydroxide, Sigma Chemical Co) buffer (pH 6.5) was pipetted into the other side. After three hours of gentle shaking in a water bath, equilibrium was reached for  $\text{Cd}^{2+}$  and  $\text{Co}^{2+}$ , whilst 4 hrs was required to achieve equilibrium for  $\text{Cu}^{2+}$ . 0.1 ml aliquots were removed from the respective metal ion compartments, diluted to 1 ml and the amount of free metal determined using atomic absorption spectroscopy. On conclusion of the experiment, the empty chambers were filled with Milli-Q water and the apparatus shaken in the water bath for a period equivalent to that of the

## 2. CELL WALLS AND CELL WALL COMPONENTS

experiment. The amount of bound metal could be expressed as:  $\text{Bound}_{[M]} = \text{Total}_{[M]} - \text{Free}_{[M]}$ , where  $\text{Free}_{[M]}$  constitutes both the amount of metal left in solution as well as that associated with the chamber walls and dialysis membranes after incubation with water as described above. In addition, the affinities ( $K_A$ ) of the cell wall and cell wall components for the respective metals could be determined from the slopes of the plots representing the ratio of free/bound metal versus the amount of complexed metal.

### 2.2.5. QUALITATIVE ASSESSMENT OF METAL BOUND TO CELL WALL AND CELL WALL COMPONENTS

In understanding the function of biomolecules, the most critical step is often the determination of its structure. Using infrared spectroscopy, the wall structure can be quantitatively determined and the fingerprints unique to each of the biomolecules obtained. In the case of cell wall biomolecules, exposure of native cell wall material to metal cations will result in ligand-metal complexation occurring, thereby modifying this infrared fingerprint region.

The infrared spectra of yeast cell wall material were recorded prior to, and after metal biosorption, in an attempt to acquire information on the nature of the interactions between the metals and cell wall material. Using a Perkin-Elmer model 180 infrared spectrophotometer, infrared analysis of the native and metal-complexed cell walls and cell wall components were undertaken. The spectra of the respective samples were determined as alkali pellets. One milligram of an oven-dried sample and 100 mg of anhydrous KBr were mixed, ground to fine powder and pressed into solid state disks (96). To obtain information regarding the nature of the chemical interactions between the divalent cations and yeast cell wall material, the shift in the spectra in the  $1800 - 800 \text{ cm}^{-1}$  region (fingerprint region) was measured (11, 12, 149, 150).

## 2.3. RESULTS

### 2.3.1. QUANTITATIVE ANALYSIS

The accumulation of  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$  by intact cell wall and cell wall isolates can be seen in Figure 2.1. The cell wall isolates accumulated greater quantities of each of the three cations than the intact cell wall. This suggests that each of the wall components possess numerous binding sites,

but due to their orientation within the intact cell wall, these ligands are masked or involved in cross-linkages between wall polymers and thus unavailable as binding sites. The general order of binding of the three metals was  $\text{Cu}^{2+} > \text{Cd}^{2+} > \text{Co}^{2+}$ , the exception being mannan, which accumulated slightly more  $\text{Co}^{2+}$  than  $\text{Cd}^{2+}$ .

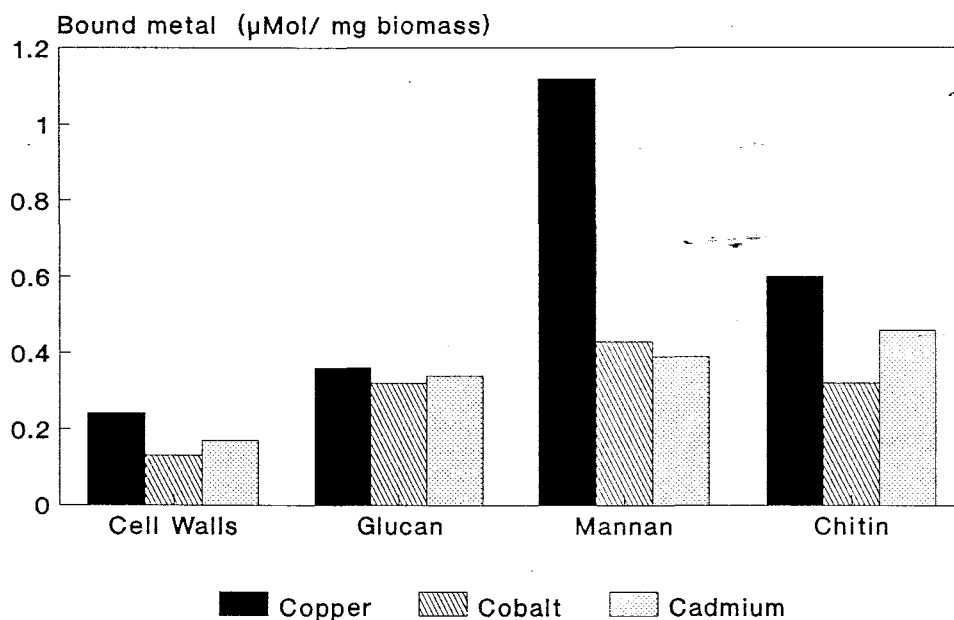


Figure 2.1. Comparison of heavy metal cation binding by *S. cerevisiae* isolated cell walls and extracted cell wall macromolecular components.

No direct relationship appears to exist between the protein content of the extract and the ability to bind metal ions (Table 2.1).

## 2. CELL WALLS AND CELL WALL COMPONENTS

Table 2.1. Parameters of metal binding by isolated cell wall and cell wall components of yeasts. The mean values are obtained from five replicates.

Sample	Prot/CHO ratio	Cu <sup>2+</sup> binding μmol.mg <sup>-1</sup> dry wt extract	Co <sup>2+</sup> binding μmol.mg <sup>-1</sup> dry wt extract	Cd <sup>2+</sup> binding μmol.mg <sup>-1</sup> dry wt extract
Yeast Cell Walls	0.21 <sup>a</sup>	0.2 +/- 0.17	0.13 +/- 0.05	0.17 +/- 0.12
Mannan	0.32	1.12 +/- 0.11	0.43 +/- 0.10	0.39 +/- 0.09
Glucan	0.02	0.36 +/- 0.17	0.32 +/- 0.21	0.34 +/- 0.11
Chitin	0.27	0.60 +/- 0.20	0.32 +/- 0.27	0.46 +/- 0.04

a: Theoretical ratio calculated from Northcote and Horne (57)

A high protein content within the extracted cell wall component fractions signified contamination of the sample with protein. In comparison to the intact cell wall, the protein : carbohydrate ratios of the mannan and chitin fractions were high. During the isolation of these compounds, the amount of carbohydrate present was decreased whilst the amount of residual protein remained relatively high, and is subsequently reflected by these ratios. Mannans are closely linked to the protein moiety, thus the high protein : carbohydrate ratio may be indicative of incomplete extraction. Although metal accumulation is not proportional to the protein content, the latter influenced the amount of metal bound by the extracted fractions. For example, the accumulation of copper by the extracted cell wall fractions decreased with a decline in the protein concentration, suggesting some relationship between protein content and Cu<sup>2+</sup> binding capacity of the cell wall. This phenomenon was limited to Cu<sup>2+</sup> accumulation, while Co<sup>2+</sup> and Cd<sup>2+</sup> binding, although influenced by the presence of protein, appeared to be independent of protein concentration.

The order of metal accumulation capacity of mannan > chitin > glucan > intact cell walls as seen in Figure 2.1. is also reflected by the affinity constants (Table 2.2.) obtained from inverted Scatchard plots. Once again metal ions appear to show a greater affinity for isolated cell wall components than for the intact cell wall.

Table 2.2. Affinity constants ( $K_A$ ) (mol/l) of the intact and extracted cell wall components for  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$ .

Sample	$\text{Cu}^{2+}$ ( $K_A$ )	$\text{Co}^{2+}$ ( $K_A$ )	$\text{Cd}^{2+}$ ( $K_A$ )
Yeast Cell Walls	0.04	0.03	0.04
Mannan	0.55	0.16	0.07
Glucan	0.06	0.13	0.06
Chitin	0.11	0.08	0.09

### 2.3.2. QUALITATIVE ANALYSIS

Infrared analysis of the cell walls and components yielded distinct patterns in the fingerprint region ( $1800 - 800 \text{ cm}^{-1}$ ) (Figure 2.2.). The characteristic bonds of the isolated virgin *S. cerevisiae* cell walls included: 1) the secondary amide peak between  $1680 - 1630 \text{ cm}^{-1}$  (amide I: C=O stretching) and that between  $1570 - 1515 \text{ cm}^{-1}$  (amide II: N-H bending), which are considered to represent the protein fraction of the cell wall, 2) the sharp peak which dominates the  $1385 - 1365 \text{ cm}^{-1}$  region (C-H saturation) and 3) the region between  $1200 - 1000 \text{ cm}^{-1}$  which represents the carbohydrate fraction of the cell wall (C-O stretching, viz.  $1150 - 1040 \text{ cm}^{-1}$ ; C-OH stretching,  $1150 - 1070 \text{ cm}^{-1}$ ; C=O stretching).

The reduction in the intensity of the peaks between  $1680 - 1630 \text{ cm}^{-1}$  and  $1580 - 1490 \text{ cm}^{-1}$  wavelength bands of the isolated cell wall components is indicative of deproteination which should occur during component isolation. Whilst the intensity of the carbohydrate peak of the isolated components did not change, the shape of the peak assumed the characteristics of the specific component. Traces of similarity between the native wall structure and isolated mannan fraction could be distinguished. Due to the dominance of mannan and its peripheral location within the wall, this is to be expected.

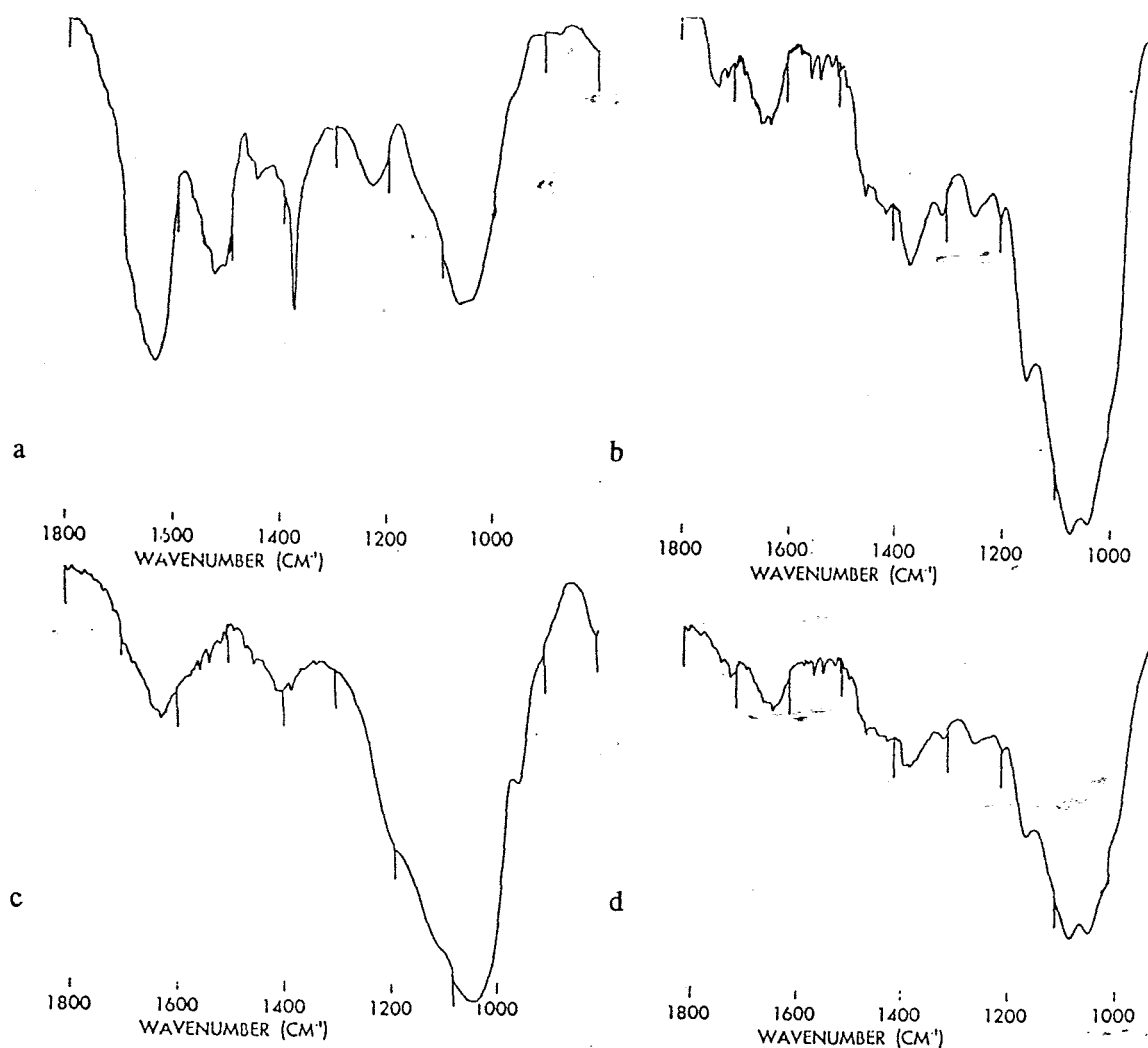


Figure 2.2. Infrared analysis ( $1800 - 800 \text{ cm}^{-1}$ ) of *S. cerevisiae* cell walls and extracted components: (a) isolated yeast cell walls, (b) chitin/chitosan, (c) mannan and (d) glucan preparations.

Metal exposure to isolated, intact cell walls (Figure 2.3.) primarily affected the secondary amide band between  $1570 - 1575 \text{ cm}^{-1}$ . The three heavy metal cations appeared to bind to the amide region of the wall in a similar manner, and metal interaction with the cell wall resulted in a change of the peak frequencies of the spectra. Following metal biosorption, the shape of the amide peak changes from a single to multiple peaked band. However, no discernable shifts could be observed in the carbohydrate region of the spectra of the intact cell walls, suggesting a lack of metal binding or alternatively inadequate sensitivity of the technique to monitor metal complexation by this component.

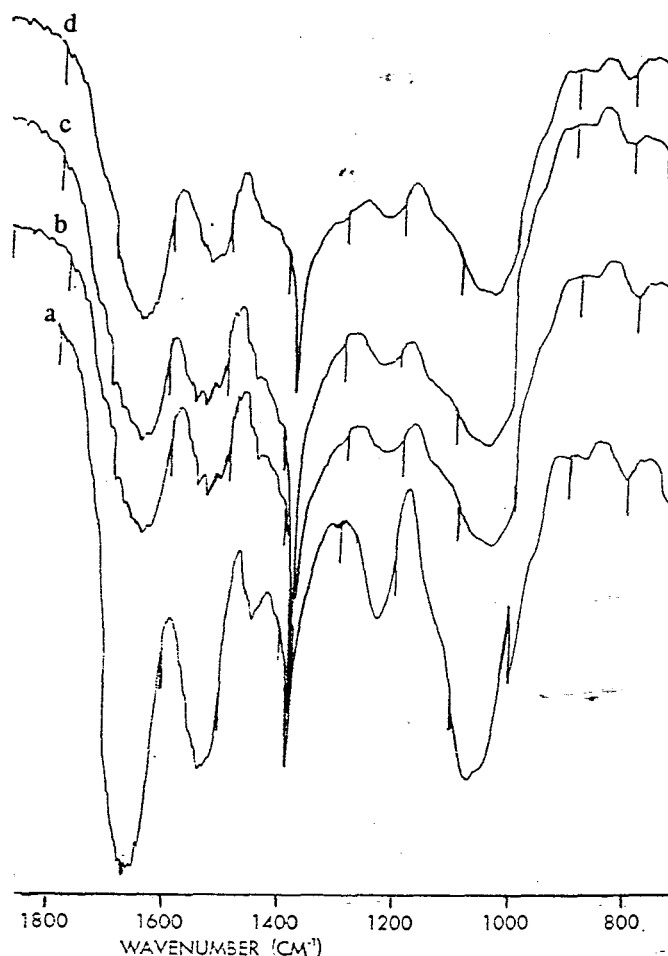


Figure 2.3. Infrared spectra ( $1800 - 800 \text{ cm}^{-1}$ ) of isolated *S. cerevisiae* cell walls before and after metal biosorption. (a) Untreated cell walls, (b)  $\text{Cd}^{2+}$ , (c)  $\text{Co}^{2+}$  and (d)  $\text{Cu}^{2+}$  exposed cell walls.

The involvement of mannan, glucan and chitin polymers in metal biosorption was confirmed by the infrared spectra of the isolated components (Figures 2.4 - 2.6). Once again the respective metal ions appeared to interact with each of the components in a similar fashion.  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$  interacted with the C-OH and C=O groups of mannans (Fig 2.4). Although these may only be low affinity interactions, they were sufficient to alter the shape of the  $1200 - 1000 \text{ cm}^{-1}$  band. In addition a separate peak was formed at  $950 \text{ cm}^{-1}$  in the metal equilibrated spectra. This peak may have resulted from the interaction with residual phosphate groups of the mannoprotein fraction, as phosphate groups have been identified in the  $970 - 910 \text{ cm}^{-1}$  wavebands. A similar peak was

identified in the spectra of thorium-equilibrated *R. arrhizus* cell walls (12), but was ascribed to thorium-chitin interactions. A second peak due to metal interaction was formed at  $1500\text{ cm}^{-1}$  which is characteristic of  $-\text{NH}_3^+$  groups or may be due to a shift in the amide II groups.

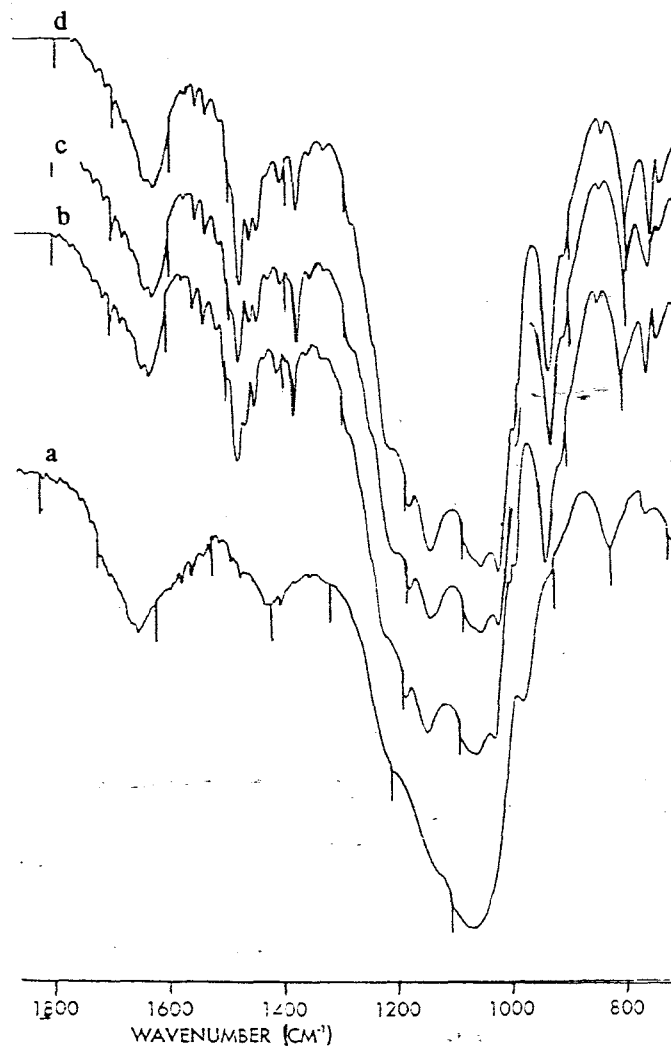


Figure 2.4. Infrared spectra ( $1800 - 800\text{ cm}^{-1}$ ) of mannan extracted from *S. cerevisiae* cell walls before and after metal biosorption (a) Untreated mannan, (b)  $\text{Cd}^{2+}$ , (c)  $\text{Co}^{2+}$  and (d)  $\text{Cu}^{2+}$  interaction with mannan.

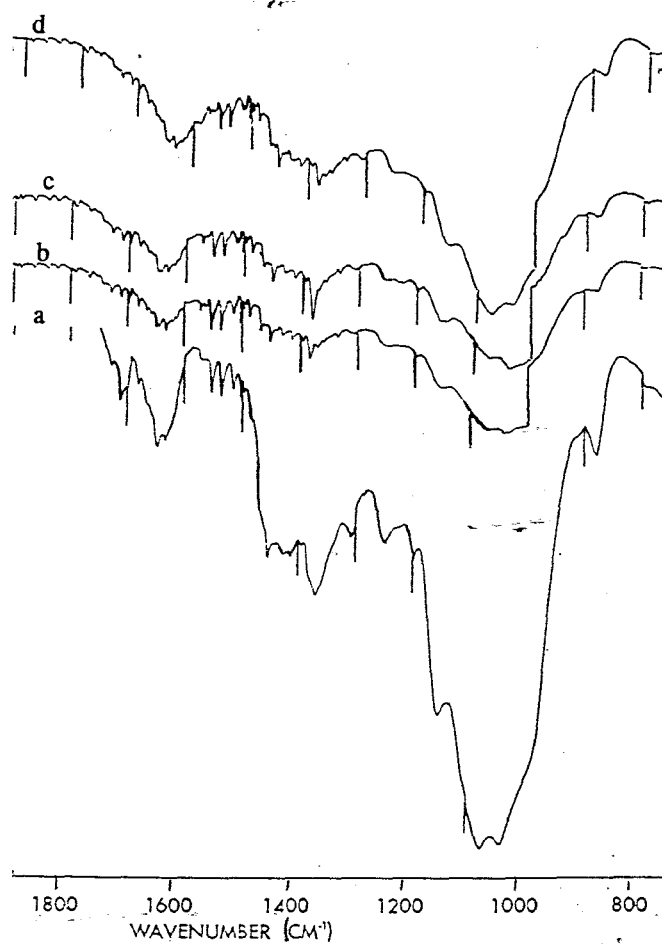


Figure 2.5. Infrared spectra ( $1800 - 800 \text{ cm}^{-1}$ ) of glucan extracted from *S. cerevisiae* cell walls before and after metal exposure; (a) Untreated glucan, (b)  $\text{Cd}^{2+}$ , (c)  $\text{Co}^{2+}$  and (d)  $\text{Cu}^{2+}$  interaction with glucan.

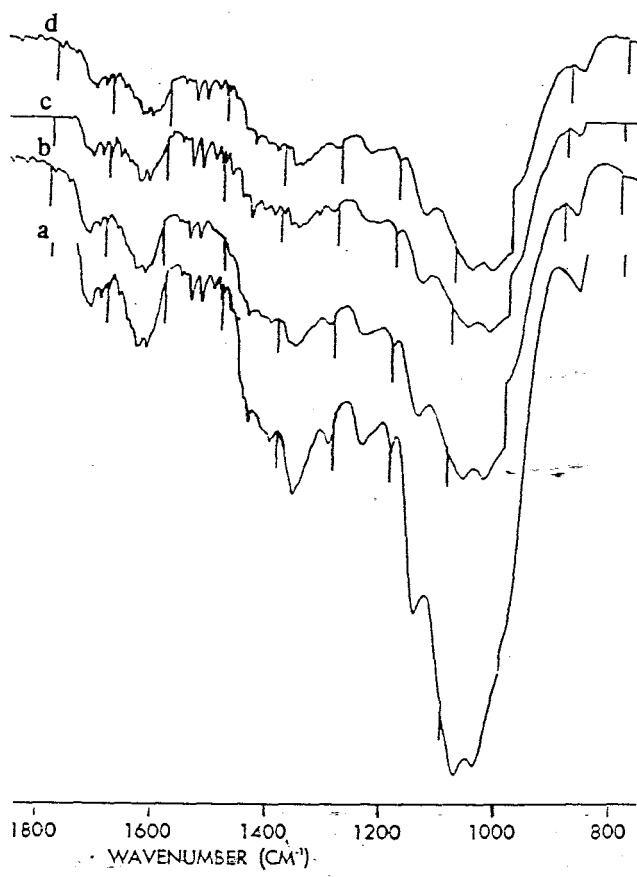


Figure 2.6. Infrared spectra (1800 - 800  $\text{cm}^{-1}$ ) of chitin isolated from *S. cerevisiae* cell walls before and after metal biosorption, (a) Untreated, (b)  $\text{Cd}^{2+}$ , (c)  $\text{Co}^{2+}$  and (d)  $\text{Cu}^{2+}$  interaction with chitin.

## 2.4. DISCUSSION

Alkali and acid treatment to intact cell walls of *S. cerevisiae* resulted in the liberation of the primary carbohydrate components. The purity of the isolated fractions was established through determining their protein : carbohydrate ratios. A high protein : carbohydrate ratio reflected incomplete extraction, whilst a pure extract was characterized by a low protein content. In addition the characteristic fingerprint spectra of each of the components was provided by infrared spectroscopy.

The primary metal-chelating component of the cell walls appeared to be represented by the mannan fraction. Though a more efficient metal-binding component than glucan and chitin, the affinity of mannan for metals depends strongly on the metal involved. The affinity of the three test metals for mannan decreased in the order  $\text{Cu}^{2+} \gg \text{Co}^{2+} > \text{Cd}^{2+}$ . The high  $K_A$  of the mannan for  $\text{Cu}^{2+}$  reflects a number of ligands or species of ligands available on the mannan component for metal interaction. Expressed in terms of the equation  $\text{M} + \text{L} = \text{ML}$ , this implies that the formation of ML is favoured with the metal-ligand forming a stable complex, whilst minimal  $\text{Cu}^{2+}$  dissociation occurs. This has been supported by other studies which have shown that  $\text{Cu}^{2+}$  binding displaces protons associated with the mannans, resulting in the subsequent acidification of the external environment (145). The affinities of the other cell wall isolates used in this study for the respective metal ions varied, depending on both the isolate and the metal species in question. However, the intact cell wall exhibited similar overall affinities for the three metal species, thereby confirming the participation of the glucan and chitin components in metal binding.

Due to the association of mannan with a protein fraction in the intact cell wall, and the presence of phosphate containing side chains, the ligands available on this component for metal biosorption include the hydroxyl and phosphate groups of the carbohydrate moiety, together with the wide range of groups provided by the protein fraction. In other studies bioaccumulation of uranium has been related to the phosphate groups of mannan, whilst experimental evidence has linked cadmium binding predominantly to the protein and not the carbohydrate moiety of mannoproteins (65). Hydroxyl-heavy metal interactions, although of a low affinity (66) have been confirmed by the present study.

The accumulation of heavy metal cations by the isolated cell wall components reflected their spatial arrangement in the cell walls. In comparison to mannoprotein which forms the outer layer of the cell wall, the primary constituents of the inner layer of the wall, glucan and chitin though capable

of forming metal complexes were less involved with metal binding. The affinity of glucan for heavy metals was low, yet due to its dominance in the cell wall (approximately a third), it contributes towards the metal binding characteristics of the wall. Chitin has however, been documented as a chelating agent for copper (63), thorium (12) and uranium (11, 149) in the cell walls of *R. arrhizus*. The mechanism hypothesized for chitin metal binding suggests that the amino groups of the cell wall chitin act initially as metal concentration sites. Subsequent nucleation of the metal occurs, eg. uranium which is complexed as uranyl hydroxide (149). A similar phenomenon has been reported regarding the complexation of gold with carboxyl groups (151). This characteristic enables the cell wall to bind metal in excess of the number of available binding sites.

While important, the carbohydrate moiety is not the sole metal accumulator in the cell wall. Infra-red spectra characterized the predominant cell wall-metal interactions to occur between the secondary amide groups of the cell wall and the metal ions. Other studies (43) have also implicated the protein component as an important heavy metal chelator. Protease digestion of mannoproteins greatly decreases the mannan accumulation by mannan (65). This does not exclude the carbohydrate components of the cell wall from complexing with metal ions and the involvement of mannan, glucan and chitin in metal binding was also confirmed in this study by infra-red spectra. The respective heavy metal ions appeared to interact with each of the cell wall isolates in a similar fashion, viz. predominantly through the C-OH and C=O groups, though the residual phosphate groups of the mannan component also played a role in metal binding.

### 2.5. CONCLUSIONS

The experimental results presented in this chapter suggest the trend of cell wall components binding to heavy metals in the order: mannan > chitin > glucan. Binding of heavy metals does not appear to be a function of the polysaccharide backbone alone, but rather due to the complexity of the nature of each polymer. Attached amine and phosphate groups in addition to the protein fractions increase the binding capacity of these polysaccharide components. The affinity of the cell wall components for different metals depends on the species of the metal and the nature of the ligands available for metal complexation. The binding of  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$  to the cell walls of yeast appear to be properties of all the major cell wall components.

### 3. UPTAKE OF HEAVY METALS BY VACUOLES ISOLATED FROM *S. cerevisiae*

#### 3.1. INTRODUCTION

In addition to cell wall accumulation, yeast cells also accumulate metals internally. Studies on the distribution of metal ions in yeast cells after uptake into the cell indicate a selective accumulation of metal ions by the vacuole (73). Evidence suggests that active accumulation of metals by the vacuole occurs (152) whereby the transport of divalent cations across the tonoplast is effected by a  $H^+$ -ATPase enzymatic pump which generates an electrochemical proton gradient (15, 80, 84, 153). The electrochemical potential not only maintains internal vacuolar modification, but also operates a  $H^+$ /ion antiport transport system across the tonoplast.

Several  $H^+$ /antiport ATPase systems are located in the tonoplast. Kinetic studies have indicated the presence of seven independent  $H^+$ /amino acid antiport systems driven by protonmotive forces and which are responsible for the active accumulation of ten amino acids by the vacuole (154). The arginine/ $H^+$  antiport system which regulates the arginine influx into the vacuole coupled to  $K^+$  efflux from the vacuole, is accepted as the model system. This arginine pool is transferable with both lysine and histidine (70, 71, 154). The efflux of vacuolar  $H^+$  or  $K^+$  also initiates the accumulation of  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $K^+$  and  $Zn^{2+}$  by the vacuole (75, 77, 79, 80), although precise clarity regarding the uptake properties of certain ions including  $Fe^{2+}$ ,  $Co^{2+}$  and  $Ni^{2+}$  appears to be lacking (73). A specific  $Ca^{2+}/H^+$  antiport system regulates  $Ca^{2+}$  homeostasis within the cell (74, 80, 84).

Evidence of the involvement of ATPase activity in the transport system was obtained through subjecting isolated vacuoles to ATPase uncouplers and inhibitors (77, 84, 86). Oligomycin and sodium vanadate, inhibitors of the mitochondrial and plasma membrane ATPase, as well as the ionophores Valinomycin and Nystatin fail to inhibit vacuolar  $H^+$  - ATPase activity and subsequent amino acid uptake into the vacuole (69, 155), yet the vacuolar transport activity is sensitive to the  $H^+$  - ATPase inhibitor N,N - dicyclohexylcarbodiimide (DCCD) and is also inhibited by  $Cu^{2+}$  and  $Zn^{2+}$  cations. Not only does the vacuolar membrane ATPase differ from the mitochondrial and plasma membrane enzymes regarding its mode of action and sensitivities to certain chemical agents, but also with regard its pH optimum.

Divalent cation accumulation by the vacuole of *S. cerevisiae* has been predominantly studied using  $Mg^{2+}$ ,  $Ca^{2+}$  and  $Zn^{2+}$ . The aim of this study was therefore to determine whether or not the same accumulation criteria applied to the uptake of  $Cu^{2+}$ ,  $Co^{2+}$  and  $Cd^{2+}$  by isolated yeast vacuoles. Through the addition of an ATPase uncoupling agent 2,4 dinitrophenol (DNP), the role of the vacuolar  $H^+$  - ATPase antiport system in the uptake of these cations could be determined.

## 3.2. MATERIALS AND METHODS

### 3.2.1. PRETREATMENT OF YEAST

The washing procedure of yeast cells adhered to the protocol described previously (section 2.2.1.). Washed cells were pretreated according to an adapted methodology of Rose *et al.* (156), and Wiemken (157). In order to predispose the yeast cells to the action of the lytic enzyme, they were exposed to a thiol component, 2-mercaptoethanol (Fluka Chemie, AG). The thiol component acts on the disulphide bonds of the cell wall proteins.

Washed yeast cells were suspended in 10 mM sodium citrate buffer containing 0.6 M sorbitol (SOB, pH 6.8) in the ratio 1 g cells (wet wt) : 5 ml buffer. The solution was supplemented with 0.05 ml mercaptoethanol for every gram of the washed yeast cells. The suspension was incubated for 20 min at 28°C, prior to centrifugation (3000 g x 10 min). The centrifuged cells were washed in buffer to remove any residual mercaptoethanol.

### 3.2.2. SPHEROPLAST FORMATION

Enzymatically treated yeast cells are referred to as spheroplasts in preference to protoplasts, due to the residual material which remains adhered to the plasma membrane. Throughout spheroplast formation the yeast cells remained suspended in the SOB buffer (pH 6.8) to maintain their osmotic stability. Lysing enzyme from *Cytophagia sp.* (Sigma Chemical Co) containing yeast glucanase, protease and cell lytic components was used for spheroplast formation.

The washed, pretreated cells were resuspended in SOB buffer (pH 6.8) and incubated with the enzyme (1.0 mg enzyme : 1.5 g wet wt yeast cells) at 30°C for 2 hrs with shaking. Spheroplast formation was monitored using phase contrast microscopy and spectroscopy. A few drops of the

spheroplast solution were heavily diluted in respective buffer (SOB, pH 6.8) and water solutions. These suspensions were allowed to stand with occasional shaking for 5 min. The optical density of the solutions were read at 600 nm, and a comparison was made between the initial and final absorption readings at this wavelength. Spheroplast lysis occurs in water, causing the OD (optical density) to drop, whilst the buffered sorbitol prevents lysis and the OD remains virtually unchanged.

### 3.2.3. VACUOLE ISOLATION

Vacuoles were obtained through metabolic lysis of the spheroplasts following the method of Indge (158). One volume of the SOB buffer (pH 6.8) was added to 50 volumes of 10 mM Imidazole - HCl buffer containing mannitol (10% w/v) and 5 mM EDTA (ethylenediaminetetraacetic acid) (pH 6.5). After equilibration at room temperature for 10 min a 10% (w/v) glucose solution (2.5 ml glucose/ml spheroplasts) was added to initiate the reaction. The reaction mixture was incubated at 30°C for 30 min with gentle shaking.

The released vacuoles were contained in a pellet collected after centrifugation (2000 g x 4 min). To obtain the vacuolar fraction the pellet was suspended in Imidazole - HCl buffer containing only 10 mM mannitol (0.1 ml vacuole suspension/ ml buffer). The gelatinous material containing the cell debris readily sedimented out through gravitational forces, leaving a vacuole-rich supernatant which could easily be removed.

### 3.2.4. DETERMINATION OF VACUOLE INTEGRITY

Vacuole integrity was determined using neutral red dye (159). Vacuoles were placed in freshly prepared neutral red dye (made up to 0.01% in 0.8 M phosphate buffer, pH 7.2) for one hour, rinsed and viewed under the microscope. Due to the ion trap mechanism of accumulation by the vacuoles (73, 159) intact vacuoles stain red to brick red in colour. Their integrity in suspension could thus be determined using phase contrast microscopy.

### 3.2.5. METAL UPTAKE

Metal uptake by vacuoles was determined by incubating the vacuolar fraction with metal salts in a 1:1 ratio (v/v). 1 ml of respective vacuole solutions (25 mg wet weight/ ml buffer) was suspended

in buffered solutions of the chloride salts of  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Co}^{2+}$  (Merck Chemical Co) to yield final concentrations of the metals ranging from 2.5 - 30  $\mu\text{M}$ . Metal uptake was determined over time, with sampling occurring at 0, 5, 10, 20, 30, 60, 90 and 120 minutes respectively. Throughout the experiment the vacuolar fractions were kept on ice.

Sample fractions were subsequently filtered through membrane filters (Millipore filters, 25 mm diameter, 0.45  $\mu\text{m}$  pore size) according to the method of Norris and Kelly (4). The filters containing the vacuolar fraction were washed using 1 ml Imidazole - HCl buffer containing 5 mM EDTA to remove metal ions bound to the surface of the vacuole. (To determine the integrity of the filtered vacuoles, the results from the EDTA washed filters were compared to those washed with Imidazole - HCl buffer not containing EDTA, and unwashed filters.) The filters containing the vacuole-metal fraction were placed in 10 ml of 2 M HCl and digested (100°C, 2 hrs) to release the accumulated metal ions. Control samples, excluding vacuoles were run to determine the amount of metal bound by the filter.

Levels of accumulated and free metal ions were detected using atomic absorption spectroscopy (Varian AA 1275 Spectrophotometer) at the relevant wavelengths for each of the respective metals.

### 3.2.6. ATPase INHIBITION

Prior to addition of the metal solutions to the vacuole fraction, 0.2 ml of a 2 mM solution of the ATPase uncoupling agent, 2,4 dinitrophenol (DNP, Sigma Chemical Co) was added. Incubation of the vacuole-DNP fraction on ice for 10 min enabled the DNP to interact with the vacuolar ATPase. One ml of the appropriate metal solution was added resulting in the final concentration of the DNP in solution being 0.182 mM. The remainder of the protocol was as outlined in section 3.2.5.

## 3.3. RESULTS

### 3.3.1. SPHEROPLAST AND VACUOLE INTEGRITY

Microscope analysis indicated successful spheroplast formation. These observations were substantiated by those obtained from optical density studies. A substantial change in optical density of cells suspended in water reflected the instability of the spheroplasts, while spheroplasts suspended

in SOB (pH 6.8) buffer showed a minimal change in optical density. Spheroplasts suspended in Milli-Q water underwent lysis due to the lack of a buffering agent. In comparison, the SOB (pH 6.8) buffer maintained the osmotic environment, minimizing lysis.

Due to the permeability of the tonoplast to the molecular (carbonium) form of the neutral red dye, the latter was chosen in preference to others in establishing vacuole integrity. The dye is taken up by the vacuole where in the presence of an acidic environment between 88 - 99% of the neutral red molecules are converted to a monovalent cation (159). The tonoplast is impermeable to these cations and the latter are trapped within the vacuoles, resulting in a deep red colour (152, 159). The interior of the vacuoles stained with neutral red dye appeared to contain darkly stained granular material. Very little, or no other cellular material was observed, indicating vacuolar integrity.

### 3.3.2. HEAVY METAL UPTAKE

Isolated yeast vacuoles accumulated heavy metal cations from solutions of differing concentrations of metal ions. The vacuoles had however, a limited accumulation capacity, with their maximum uptake depending on the species of metal present. Vacuole suspensions accumulated relative amounts of metal in the order of  $\text{Cu}^{2+} > \text{Co}^{2+} > \text{Cd}^{2+}$  (Figure 3.1 - 3.3).

Vacuolar sequestration of the three heavy metal species exhibited similar trends. The heavy metal cations were removed from the external environment and concentrated within the vacuole. The majority of the metal ions were taken up within the first 30 min of exposure to the test solutions (Figure 3.1 - 3.3). Sequestration of the metal ions within the vacuole appeared to be independent of the external metal ion concentration. A minimal difference was observed between the amounts of metal accumulated by the vacuole from 5, 10 and 30  $\mu\text{M}$  solutions. The initial rapid phase was followed by a secondary phase during which the rate of metal sequestration was slower, resulting in the levels of the metals in the vacuole only increasing slightly, eg.  $\text{Cu}^{2+}$  and  $\text{Co}^{2+}$ , or alternatively levelling off, eg.  $\text{Cd}^{2+}$ .

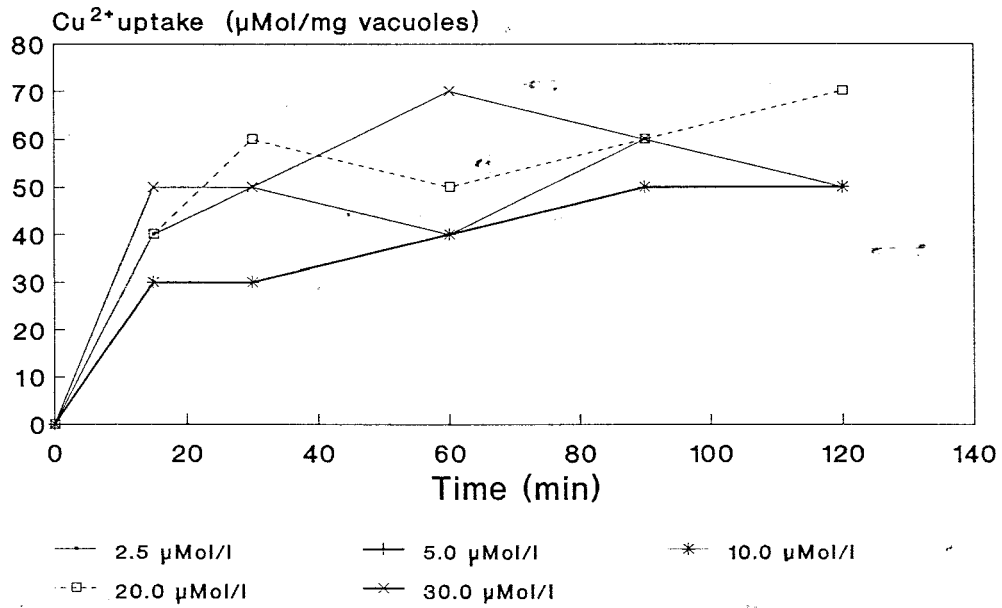


Figure 3.1. Accumulation of Cu<sup>2+</sup> by isolated yeast vacuoles over time from Cu<sup>2+</sup>-containing solutions. The amount of metal accumulated is ascertained through digestion of the metal-treated vacuoles. Note: the 2.5 µMol/l and 10.0 µMol/l curves overlap.

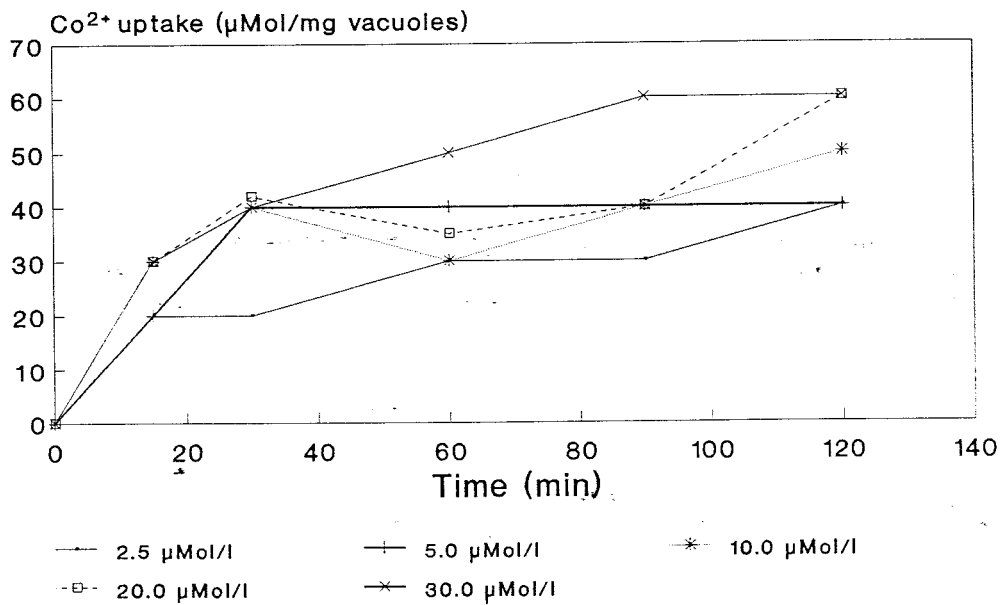


Figure 3.2. Accumulation of Co<sup>2+</sup> by isolated yeast vacuoles over time from Co<sup>2+</sup>-containing solutions.

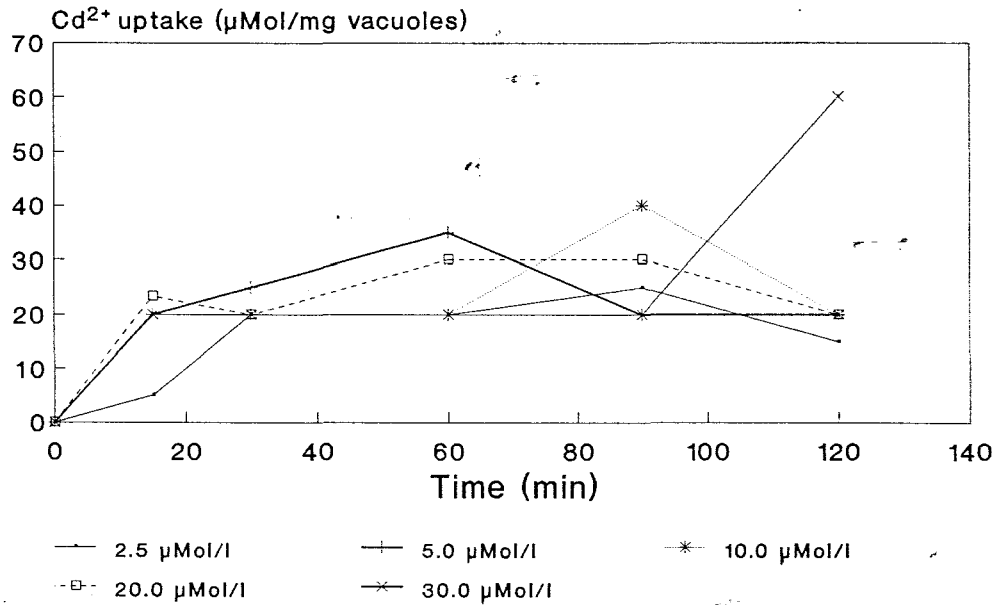


Figure 3.3. Accumulation of  $\text{Cd}^{2+}$  by isolated yeast vacuoles over time from  $\text{Cd}^{2+}$ -containing solutions.

The ATPase uncoupler, DNP, did not exhibit a strong inhibitory effect on the uptake of  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$  into the vacuoles (Figures 3.4 - 3.6).

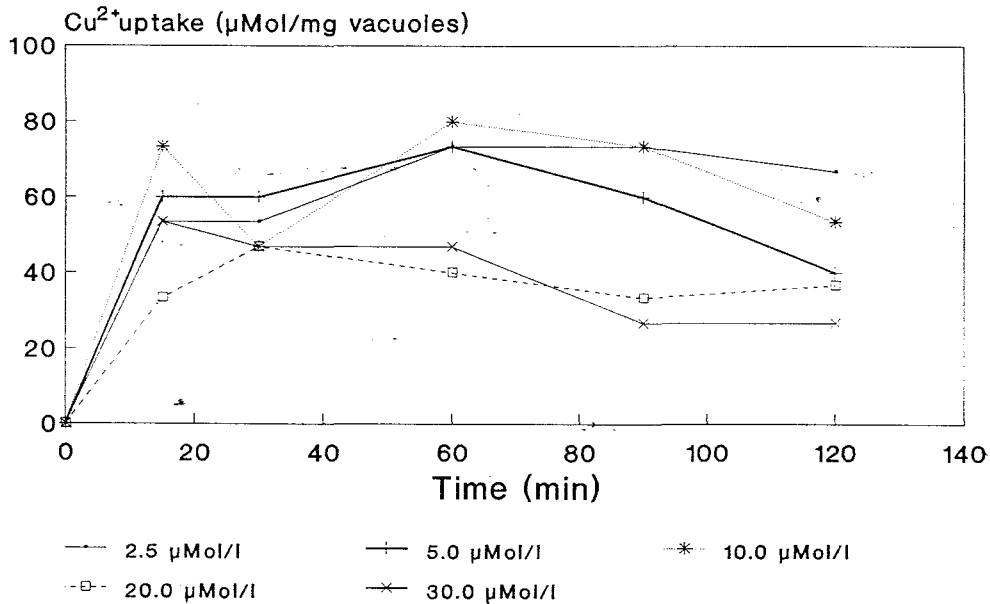


Figure 3.4. Effect of DNP-pretreatment on vacuolar uptake of  $\text{Cu}^{2+}$ . Experimental conditions remained identical to those during uptake by untreated vacuoles.

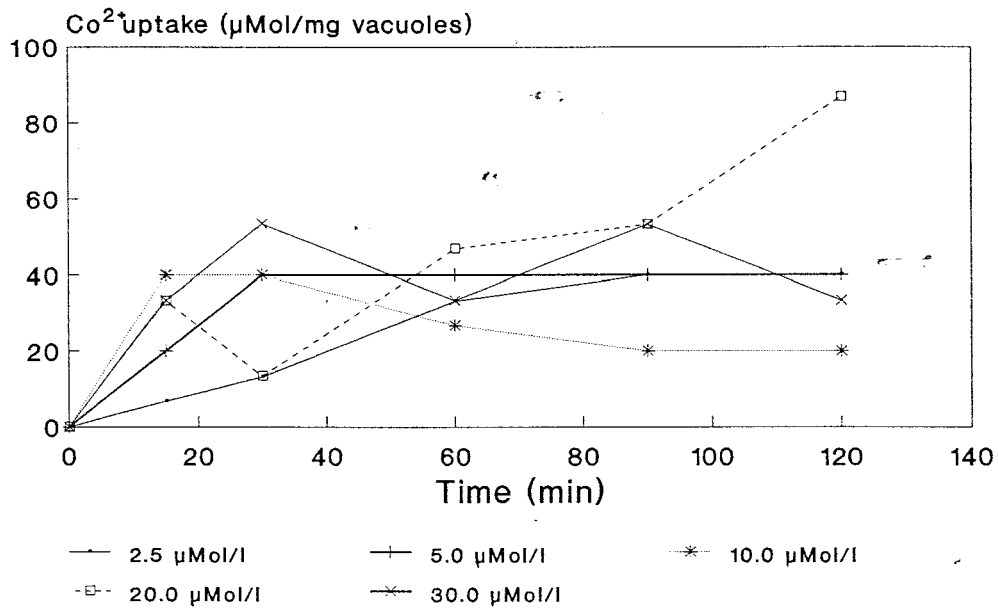


Figure 3.5. Effect of DNP-pretreatment on vacuolar uptake of Co<sup>2+</sup>. Experimental conditions remained identical to those during uptake by untreated vacuoles.

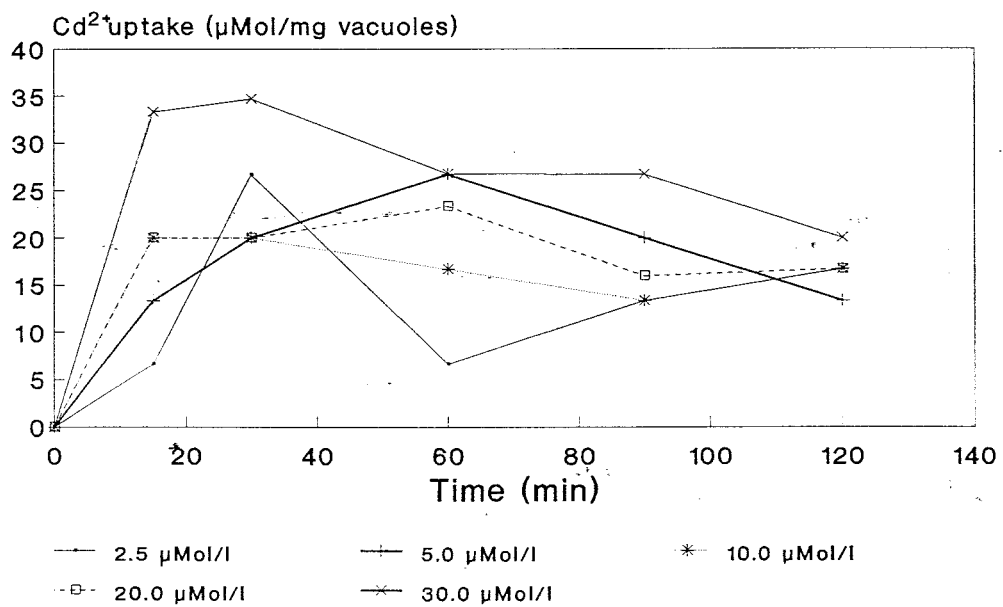


Figure 3.6. Effect of DNP-treatment on vacuolar uptake of Cd<sup>2+</sup>. Experimental conditions remained identical to those during the uptake by untreated vacuoles.

The accumulation of heavy metal cations during the first 30 min (the initial rapid phase) was comparable to that of untreated vacuoles, irrespective of the metal species or their ambient concentrations. With time the effectiveness of the DNP as an ATPase uncoupler was slightly enhanced. After 120 min the levels of the respective metal cations accumulated within the DNP-pretreated vacuoles were less than those of their untreated counterparts.

### 3.4. DISCUSSION

Vacuolar uptake of heavy metal cations (Fig 3.1 - 3.3) exhibit characteristics similar to those of cellular uptake. Both accumulation mechanisms have an initial, rapid phase of uptake. Whereas this phase is followed by a defined, slower secondary uptake phase into intact yeast cells, the amount of metal accumulated by the vacuole levels off, or increases only slightly. Although the vacuole has been reported to possess remarkable elasticity (160), its saturation of metal accumulation may in fact be due to spatial limitations. The sequestration of metal cations by the vacuole in the order of  $\text{Cu}^{2+} > \text{Co}^{2+} > \text{Cd}^{2+}$  as reported in this study may be related to size, with a corresponding decrease in atomic radii of  $\text{Cd}^{2+} > \text{Co}^{2+} > \text{Cu}^{2+}$ .

The mechanisms whereby metals are transported across the tonoplast are complex, with the regulation of these transport systems not being well understood. Both metabolic and non-metabolic transfer systems remain feasible options. Based on data obtained from the present study, the uptake of the three metals by untreated vacuoles could be metabolism dependent. The initial, rapid metal uptake, followed by the decline in accumulation suggests the utilization of a metabolizable energy source during the first phase of uptake. Depletion of this energy source, or alternatively the inhibition of the ATPase system would result in the subsequent decline in the amount of metal accumulated.

Several independent transport and translocation systems have been located and identified in the vacuolar membrane. The existence of two  $\text{H}^+$ -translocase systems for chloride (161) and seven for  $\text{H}^+$ /amino acid antiport mechanisms have been identified (154). The transport systems of the vacuolar membranes differ from those of the plasma membranes by having lower substrate affinities (154, 160). Due to the low substrate affinity it is proposed that substitution of the amino acids by heavy metal ions occurs in the ATPase antiport transport system, enabling the uptake of these metals into the vacuolar lumen. Evidence for this transport mechanism was provided by Okorokov (162) who suggested that  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  activate the ATPase system, thereby initiating

their uptake into the vacuolar interior.

A feasible hypothesis for metal accumulation by vacuoles, would be for metal uptake via an energy dependent  $H^+$ /antiport system. The initial rapid accumulation of the metal ions could be ascribed to ATPase activity in the presence of an energy source. Several reasons could account for the subsequent decline in the rate of metal accumulation: 1) in the absence of a renewable energy source, utilization of the available ATP during the initial uptake would place limitations on the amount of metal accumulated, 2) the presence of high levels of heavy metal cations could act as ATPase inhibitors preventing further accumulation and 3) the spatial limitations of the vacuole may also prevent further metal accumulation.

Researchers have also implicated alternative routes of metal accumulation in yeast. Ohsumi *et al.* (71, 84), reported the permeabilization of the plasma membrane by relatively low amounts of  $Cu^{2+}$ , whilst  $Cd^{2+}$  has also been known to permeabilize membranes. Using fluorescing agents, results were obtained during this study (data presented in chapter 4) which implicate the permeabilization of yeast membranes by certain heavy metal ions. Based on this evidence the possibility of tonoplast permeabilization by heavy metals cannot be excluded. This permeabilization would enable the slow movement of metal ions into the vacuole lumen, thus explaining the gradual increase in the vacuolar metal content after the initial rapid uptake phase.

The results obtained from DNP-treated vacuoles suggest the inability of the metabolic uncoupler DNP to inhibit the accumulation of heavy metal ions by the yeast vacuole. DNP, inhibits the uptake of citrate into vacuoles isolated from the plant *Hevea latex* (152), with the transport of citrate across the vacuolar tonoplast postulated to be similar to that of metals. However, the inability of the DNP to inhibit metal accumulation by yeast vacuoles suggests a different mechanism of metal uptake across the yeast tonoplast, or alternatively the ineffectiveness of DNP as an ATPase uncoupler in yeast vacuolar membranes.

### 3.5 CONCLUSIONS

From this study, and evidence provided in the literature (160), it appears that more than one mechanism for solute uptake into the vacuole exists. It is proposed that the primary route of heavy metal uptake into the vacuole is via the ATPase-dependent  $H^+$ /antiport system. Dependent on ATP

### 3. ISOLATED VACUOLES

as an energy source, this system transfers both essential and non-essential components into the vacuole. A possible secondary accumulation system for heavy metals is based on membrane permeabilization by the cations. The contribution towards the total vacuolar metal accumulation by the latter system is thought to be minor. The possibility exists that membrane permeabilization is triggered by very high levels of heavy metals in the cytosol, or in the absence of a functioning antiport system. Inhibition of the ATPase antiport mechanism can be effected in the presence of metabolic or ATPase-inhibitors and uncouplers, though vacuolar ATPase inhibition appears to be limited to selective agents, eg. DCCD. It can be concluded that DNP, a metabolic uncoupler, is ineffective as an uncoupling agent for vacuolar ATPase, and subsequently unable to prevent metal accumulation by the vacuole.

Having examined metal accumulation by components of yeast cells (cell walls and vacuoles) further studies were conducted on metal accumulation by whole cells.

## 4. ACCUMULATION AND INTERNALIZATION OF HEAVY METALS BY VIABLE YEAST CELLS

### 4.1. INTRODUCTION

A unique attribute of yeast and other viable microbial cells is their ability to maintain an electrochemical balance between the intra- and extracellular environment. To accomplish this, physiological elements are internalized via facilitated transport. In addition to the uptake of essential ions, non-essential and toxic metal cations, eg. heavy metals, can also be accumulated by the cells. Heavy metals are well recognized for their negative effect on the environment and microbial life forms. The severity of the effect on the microorganisms depends largely on the species of heavy metal. The metals used in the present study,  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$  represent a broad spectrum of heavy metals in terms of their chemical and physical properties, toxicity and behaviour.  $\text{Cu}^{2+}$ , the smallest of the three cations is a physiological cation and in low doses is the least toxic, whilst  $\text{Cd}^{2+}$  is regarded as one of the "big three" toxic metals together with  $\text{Hg}^{2+}$  and  $\text{Pb}^{2+}$  (138).

The affinity displayed by microbial cells for heavy metals is not equimolar. An affinity series of accumulation of  $\text{Mg}^{2+} > \text{Cu}^{2+} > \text{Zn}^{2+} > \text{Mn}^{2+} > \text{Ni}^{2+} > \text{Ca}^{2+} > \text{Sr}^{2+}$  has been demonstrated by *Saccharomyces carlsbergensis* cells (22). Regardless of the affinity for heavy metals in the presence of high extracellular concentrations of these ions, metal uptake into yeast cells will occur. As previously noted (chapter 1), metal accumulation by viable yeast cells involves a biphasic uptake mechanism. The initial rapid phase is metabolism-independent, consisting of metal-cell wall interactions. A slower, metabolism-dependent second stage is responsible for the uptake and internalization of the heavy metal cations within the yeast cell interior (4).

The cellular response to metals varies. A relationship between heavy metal transport into yeast cells and toxicity is often observed, with sensitive strains accumulating more metal than resistant strains (163). Regardless of the sensitivity of the yeast strain, the initial response to internalized metal ions is compartmentalization (163, 164). Internalized cations are distributed between the soluble cytosol and the insoluble fractions (organelles) of the cell. Deposition primarily occurs in the vacuole (69 - 71, 73), whilst the majority of heavy metal cations show a lower affinity for cytosolic deposition (82).

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Exposure of yeast cells to high levels of toxic heavy metals can confer metal tolerance and resistance upon these cells (163 - 166). Resistance and tolerance mechanisms include: 1) a reduction in metal uptake and the impermeability of the cell plasma membrane. Alternatively the implementation of energy-driven efflux pumps occurs, which keep toxic metal levels low in the interior of the cell. Such mechanisms have been described for  $\text{As}^{2+}$  and  $\text{Cd}^{2+}$  (167). 2) Sequestration of metals in or around the cell. Metal ions can either bind to cell surfaces or precipitate as insoluble complexes, eg. as metal sulfides or oxides at the cell surfaces (92, 164, 167). 3) Chemical modification or biotransformations to less toxic forms of the elements. Oxidation, eg.  $\text{AsO}_3^{2-}$  to  $\text{AsO}_4^{3-}$  or reduction, viz.  $\text{Hg}^{2+}$  to  $\text{Hg}^0$  which occurs intracellularly via enzymatic processes converting these metals to less toxic forms. Methylation and demethylation also constitute mechanisms of resistance (163, 164, 167). 4) One of the most common metal-induced responses in many microorganisms, inclusive of yeast, is the synthesis of intracellular metal binding proteins which not only play a role in detoxification, but also regulate the storage and cytosolic metal ion concentrations (163). A class of proteins which are capable of chelating relatively large quantities of metal are the metallothioneins (MT). These proteins which are rich in cysteine residues bind a wide range of metal cations eg.  $\text{Co}^{2+}$ ,  $\text{Ag}^{3+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  (165). Up to 60% of cellular copper has been associated with MT (168), and a percentage of  $\text{Cd}^{2+}$  in Cd-resistant strains has been found bound to soluble MT fractions (35, 169). 5) In addition to forming complexes with chelating agents such as phosphoglycoproteins (170), ferritin (81), siderophores (81) and ferridoxins,  $\text{Mg}^{2+}$ ,  $\text{Sr}^{2+}$  and  $\text{Ca}^{2+}$  are sequestered in cytoplasmic granules in phosphate-rich cells (16). Polyphosphates, similar to those found in vacuoles have been detected in these granules.

The alternative to metal resistance and tolerance in heavy metal exposed yeast cells, is the loss of cell viability. Although a certain degree of metal resistance can be induced in yeast cells when grown in the presence of elevated levels of extracellular metals (165, 167), eg.  $\text{Cu}^{2+}$  (171),  $\text{Cd}^{2+}$  (169) and  $\text{Co}^{2+}$  (169), most yeast cells are sensitive to such levels of toxic metals. The loss of cell viability can manifest itself in several ways. High levels of intracellular  $\text{Cd}^{2+}$  inhibit nucleic acid and protein synthesis (92) and have also been reported in the permeabilization of the cell plasma membrane as has  $\text{Cu}^{2+}$  (4, 56).

The aim of this section of the study was to monitor the effect of heavy metals on viable yeast cells over time with respect to the sites of deposition of the internalized metal cations and the subsequent cellular response they elicited. In addition, the effect of heavy metals on the integrity of the cell

plasma membrane was determined.

## 4.2. METHODS AND MATERIALS

### 4.2.1. SCANNING ELECTRON MICROSCOPY (SEM) OF Cu-LOADED CELLS

#### 4.2.1.1. METAL ACCUMULATION

Bakers yeast was suspended in ultra-pure Milli-Q water, washed three times (3000 g x 10 min) and subsequently suspended in 5 mM PIPES/TMAH buffer (pH 6.5) in a 1 : 2.5 wet weight (g) : volume ratio (ml).

Duplicate 20 ml volumes were removed as test and control fractions. To the test fractions a 0.5 mM  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  stock solution was added to obtain a final concentration of 0.357 mM. The yeast was exposed to the metal for 90 minutes at 25°C with shaking. The control fractions were not exposed to any metals ions. On completion of incubation, the yeast was spun down (3000 g x 10 min) and the pellet air-dried for 5 min prior to being mixed with warm 6% (w/v) agar and allowed to set.

#### 4.2.1.2. CRYO-SEM PREPARATION

Small rectangular blocks ( $2\text{mm}^3$ ) were cut from the agar. These were cooled to  $-200^\circ\text{C}$  in liquid nitrogen before being transferred to the cryo-chamber of the SEM (JOEL JSM 840 Scanning electron microscope). At no stage of the transferral was the sample allowed to come into contact with air.

The sample was sectioned to expose the cell morphology. To enhance the image obtained the sections were gold coated and subsequently viewed at an acceleration voltage of 20kV. Both backscattered (BEI) and secondary (SEI) electron images of the sliced cells were examined.

#### 4.2.2. DISTRIBUTION STUDIES OF ACCUMULATED HEAVY METAL CATIONS BY TRANSMISSION ELECTRON MICROSCOPY (TEM)

##### 4.2.2.1. METAL ACCUMULATION

Washed yeast cells were suspended in 5 mM PIPES/TMAH buffer (pH 6.5) in a 1:2.5 g:ml ratio. 20 ml volumes of yeast suspensions were exposed to respective Cu, Co or Cd-solutions to yield a final metal concentration in solution of 1M. As for section 4.2.1.1, the control fraction was not exposed to the heavy metal solutions. Metal incubation took place for 1, 2, 5, 12 and 24 hrs respectively with shaking at 25°C. Air-dried cells were mixed with warm 6% (w/v) agar and allowed to set.

##### 4.2.2.2. EMBEDDING PROTOCOL FOR MICROSCOPIC ANALYSIS

2mm<sup>3</sup> blocks were cut from the yeast-agar molds and placed into labelled specimen tubes containing cold 2.5% glutaraldehyde in 0.1 M phosphate buffer. Primary fixation of the sample proceeded overnight, after which the glutaraldehyde was decanted and the blocks washed with 0.1 M phosphate buffer. The washing procedure was repeated.

No secondary fixation protocol was observed due to the elemental conflicts caused by the secondary fixative (OsO<sub>4</sub>) in the energy dispersive X-ray spectra of Cu and other ions. Dehydration of the samples was achieved using an alcohol gradient of 30 - 100%. The samples were exposed to each concentration of alcohol for 5 min, with two final washes using absolute ethanol. After dehydration, infiltration and embedding of the samples using resin could commence. The first stage of infiltration required two 15 min washes using propylene oxide.

The embedding process was gradual. On decanting off the second propylene-oxide wash, the tubes were refilled with a 75 : 25 propylene oxide : resin (Araldite) mixture. This was replaced by a 50 : 50 mixture after 60 min, and the process repeated until the samples were transferred into tubes containing 100% pure resin for 12 hrs to enable embedding to proceed overnight. On completion of infiltration and embedding the samples were polymerized at 60°C for 36 hrs.

#### 4.2.2.3 SECTIONING FOR TRANSMISSION ELECTRON MICROSCOPY (TEM) AND X-RAY DIFFRACTION ANALYSIS

Prior to sectioning, the polymerized blocks were trimmed to the correct shape; a trapezium. All sectioning and trimming was conducted on a RMC MT-7 Ultramicrotome, using glass knives.

##### 4.2.2.3.1. ULTRATHIN SECTIONING AND STAINING OF SAMPLES FOR TEM

Ultrathin sections (100 nm) were cut from the trimmed block. On cutting, the 100 nm sections formed silver-grey ribbons which were collected by floating them onto 300 mesh copper grids. The sections were air-dried prior to staining.

TEM sections were stained in aqueous uranyl acetate for 30 min, and subsequently in lead citrate for 5 min. The grids containing the stained sections were examined under a JOEL JSM 100 CK TEM at an acceleration voltage of 80kV.

##### 4.2.2.3.2. X-RAY DIFFRACTION PREPARATION AND ANALYSIS

Due to the higher acceleration voltage used for X-ray analysis, thicker sections were required. 250 nm thick sections which formed blue-green ribbons when floated on water, were cut. Due to various elemental conflicts not all the samples could be collected onto identical grids. The Cu-containing samples were floated onto gold grids, Co-samples onto formvar coated wide slot copper grids and Cd-samples onto either wide slot formvar coated copper grids or alternatively nickel grids.

Once again interference in heavy metal detection prevented the staining of the samples. The presence of intracellular elements was confirmed through examining the carbon coated sections using a Phillips EM 420 TEM at an acceleration voltage of 120kV. The sites of metal ion deposition, localization and evidence of ion exchange were confirmed from EDAX spectra obtained from an EDAX PV 9100 probe attached to the TEM. The limits of the probe were set between 1 - 20 KeV thereby excluding the detection of the essential elements: H, C, N, O and foreign ions. Analysis of the cells or specific cellular components (eg. cell walls, vacuoles and cytosolic inclusions) was accomplished by focussing the electron beam on the area of interest.

### 4.2.3. INFLUENCE OF METALS ON MEMBRANE INTEGRITY

Based on the method of Slavik (172), the membrane permeability of yeast cells was determined using fluorescence techniques. Non-fluorescent fluorescein diacetate (FDA, Sigma Chemical Co) when incubated with viable yeast cells, is internalized and deacetylated to yield fluorescent fluorescein. An advantage of this conversion is the fact that fluorescein shows very small permeability across the cell plasma membrane, thus in experiments with intact yeast cell plasma membranes, very little fluorescent dye is lost back to the medium. The amount of fluorescein in the media are an indication of the loss of plasma membrane integrity.

Yeast cells were washed twice (3000 g x 10 min), aerated and suspended in buffer (50 mM PIPES/TMAH, pH 6.5). Prior to the addition of metals, 5 ml of suspended cells were added to each tube and incubated with 0.1 ml fluorescein diacetate (FDA, Sigma Chemical Co) and 4.9 ml Milli-Q water at 25°C for 30 min. Incubated cells were centrifuged, washed and the pellet resuspended in 9.5 ml of PIPES/TMAH buffer (pH 6.5). 0.5 ml of the appropriate metal solution ( $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  or  $\text{CdCl}_2 \cdot \text{H}_2\text{O}$ ) was added to the respective tubes (the blank contained 0.5 ml Milli-Q water). This suspension was incubated for a further 30 min at 25°C, spun down (3000 g x 10 min) and the supernatant measured for fluorescence. Fluorescence determinations were accomplished using a Perkin-Elmer Fluorescence Spectrophotometer 203. Fluorescence intensity was recorded at 520 nm after excitation at 435 and 490 nm respectively.

## 4.3. RESULTS

### 4.3.1. HEAVY METAL INDUCED MORPHOLOGICAL CHANGES

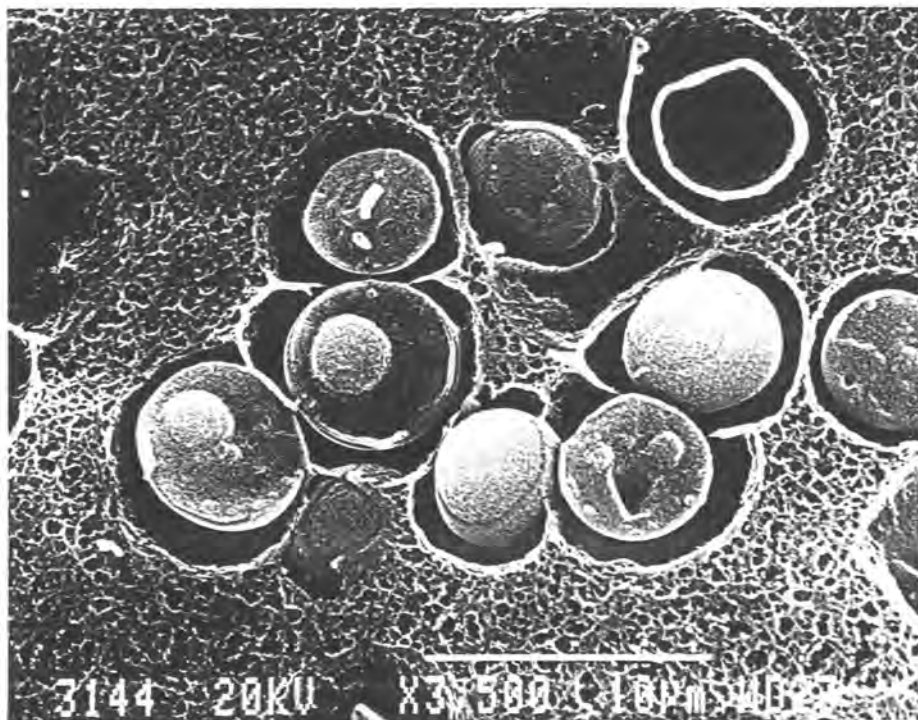
The morphological consequences of  $\text{Cu}^{2+}$  accumulation on yeast intracellular structure is shown in Fig. 4.1. and 4.2. The normal ultrastructure of *S. cerevisiae* in the absence of  $\text{Cu}^{2+}$  contains well defined vacuoles. On uptake of  $\text{Cu}^{2+}$  the cytosol appeared to become dehydrated (Fig 4.2) whilst vacuolar enlargement occurred (Fig 4.3 - 4.5). Also apparent from this study was the effect of heavy metal ion accumulation on the integrity of the cellular tissue. Extensive exposure of yeast cells to metal ions increased the extent of disruption of cellular tissue during sectioning.

Further consequences of  $\text{Cu}^{2+}$  exposure on the morphology of yeast cells is apparent in Fig 4.3.

#### 4. ACCUMULATION BY VIABLE CELLS

$\text{Cu}^{2+}$  accumulation and precipitation after 5, 12 and 24 hrs are represented as dark granules or inclusions throughout the cytosol and in the vacuole. Little evidence of bound or precipitated  $\text{Cu}^{2+}$  was observed on the cell wall, though due to the nature of metal sorption and the reversible association between metal ions and the yeast cell walls, the absence of cell surface metal binding may have been an artifact of the embedding procedure.  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$  exhibited similar patterns of cellular deposition (Fig 4.4 - 4.5) with  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$ -rich inclusions located throughout the cytoplasm. However, vacuolar enlargement was not as pronounced as for the  $\text{Cu}^{2+}$  exposed cells.

Sequestered metal ions were either associated with the insoluble fraction (cell wall or vacuole) or the soluble (cytosolic) fractions of yeast cells. The presence of electron dense metal depositions within the cytosol suggested saturation of the soluble fraction, with the subsequent precipitation of metal ions. In addition to these granular depositions, metal association with smaller vacuoles within the cytoplasm was observed.



a)



Figure 4.1. Electron micrographs of native *S. cerevisiae* cells. a) scanning electron microscopy micrograph (SEM) (magnification 3 500x) and b) transmission electron micrograph (TEM) (10 000x).

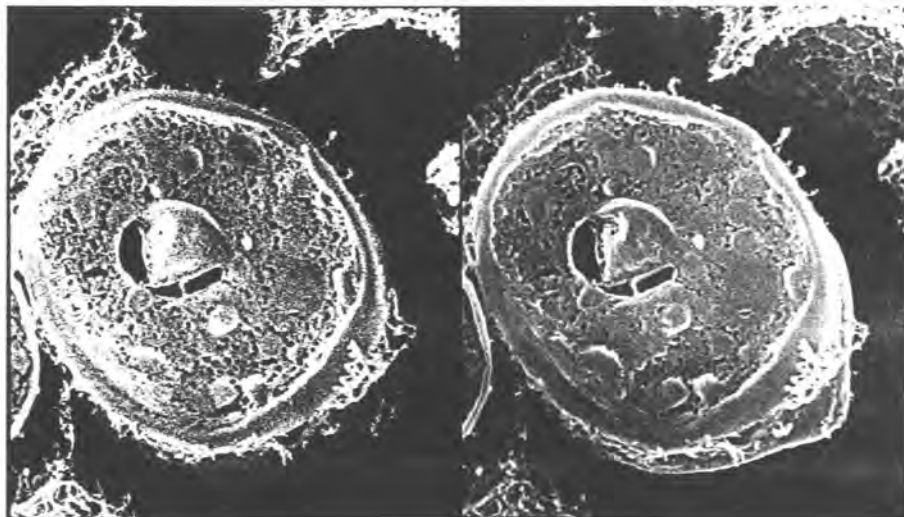


Figure 4.2 SEM micrographs (BEI and SEI images) of  $\text{Cu}^{2+}$  exposed *S. cerevisiae* cells (8 000x).

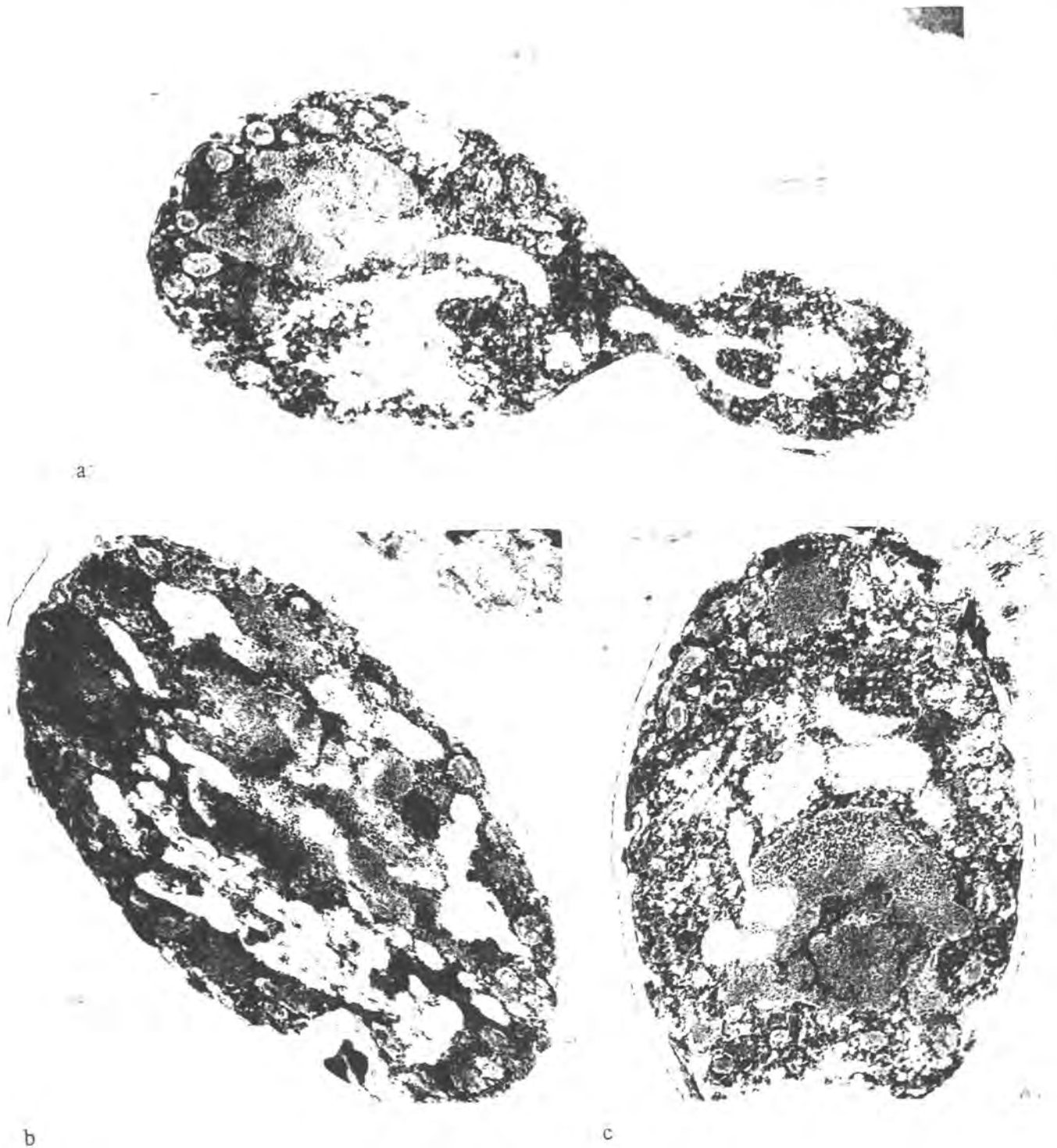


Figure 4.3. TEM micrographs (10 000x) of *S. cerevisiae* cells after a) 5, b) 12 and c) 24 hrs exposure to  $\text{Cu}^{2+}$  ions. The granulated electron dense areas in both the cytosol and the enlarged vacuole are  $\text{Cu}^{2+}$  rich areas.

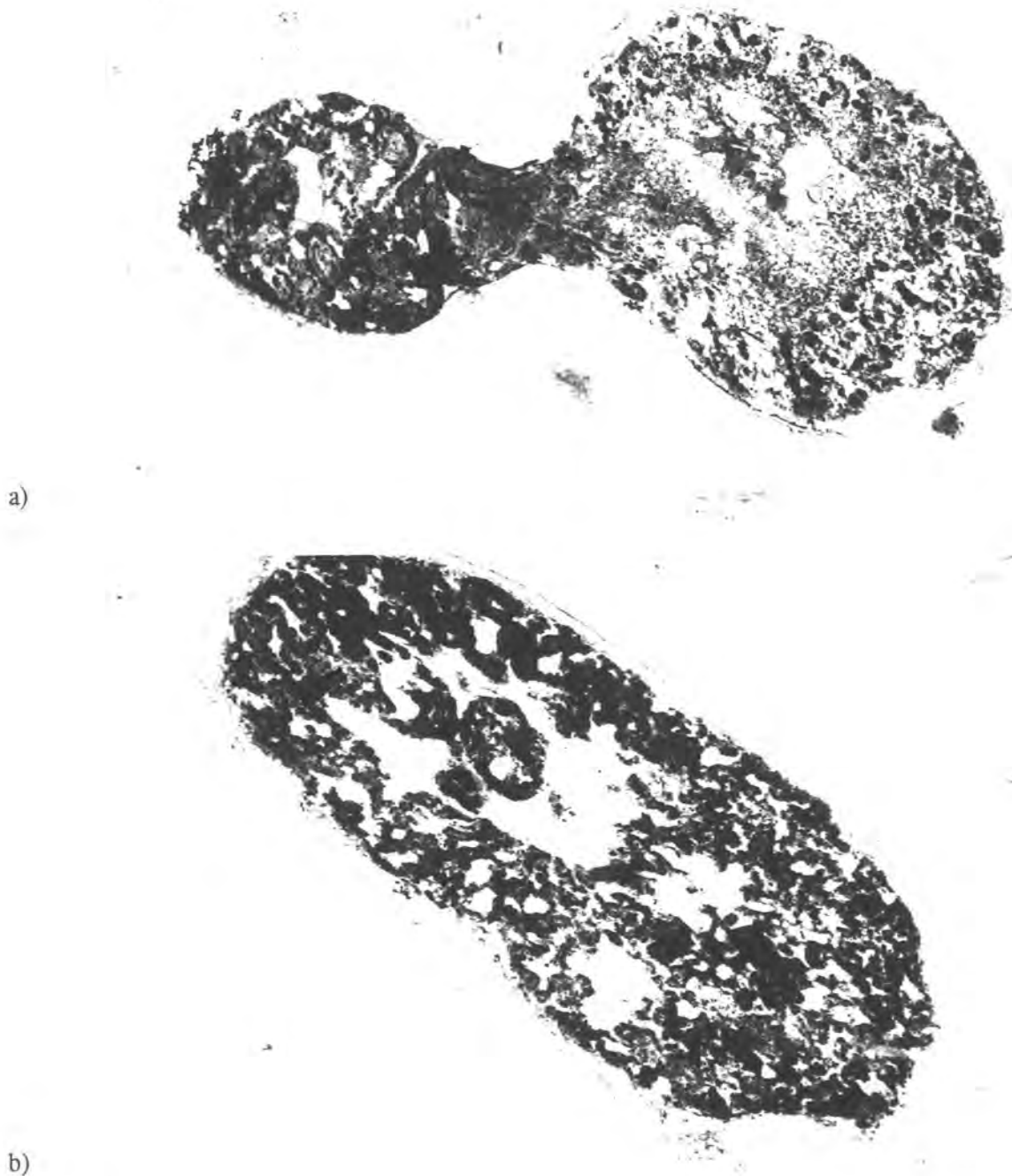


Figure 4.4. TEM micrographs (14 000x) of yeast cells after a) 5 and b) 12 hrs exposure to  $\text{Co}^{2+}$  ions. Vacuolar enlargement is apparent in the presence of  $\text{Co}^{2+}$

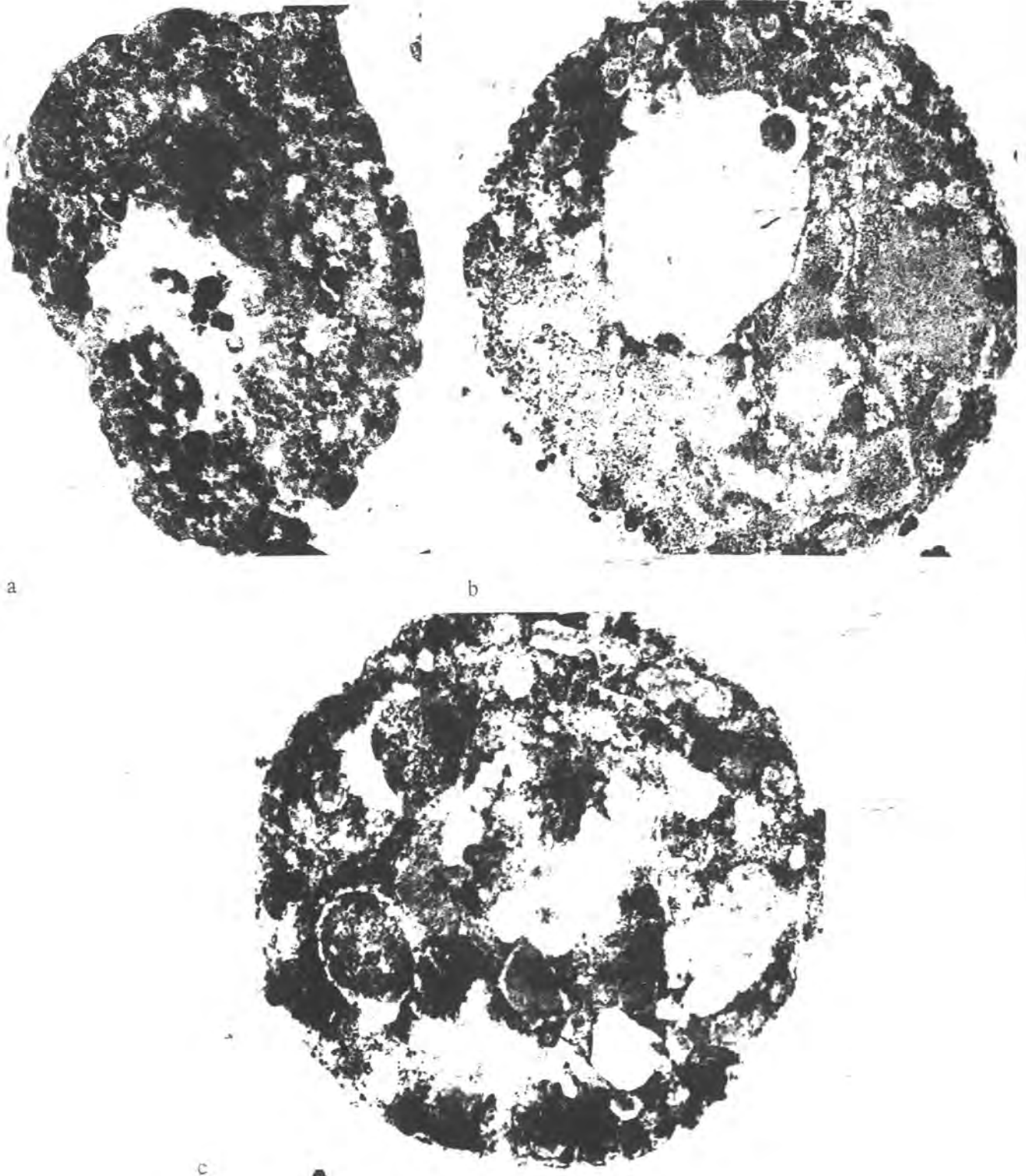


Figure 4.5. TEM micrographs (14 000x) of *S. cerevisiae* after: a) 5, b) 12 and c) 24 hrs exposure to Cd<sup>2+</sup> ions. Deposition of Cd<sup>2+</sup> rich inclusions in the cytosol and vacuole occur.

## 4.3.2. CELLULAR DISTRIBUTION OF METAL IONS

A prerequisite for the detection of elements on the EDAX spectra was a minimum composition of 1% of the area under analysis. Restrictive criteria, eg. biological variables, prevented quantitative analysis of the metal ion concentration within yeast cells, resulting in qualitative interpretation of the results. Elements present in native yeast cells, viz. Na, Mg, Si, P, S, K, Cl and Ca (Figure 4.6) are not all inherent to the yeast cells, but some originate from the external sources, eg. Si, Cl, P and S, which are provided by metal solutions, buffers and embedding resin. The physiological ions: Na, K, Cl and Ca, maintain the ionic equilibrium within the cell, whilst the presence of P, often as a conspicuous phosphorous peak in vacuolar bodies can be ascribed to polyphosphate molecules (173).

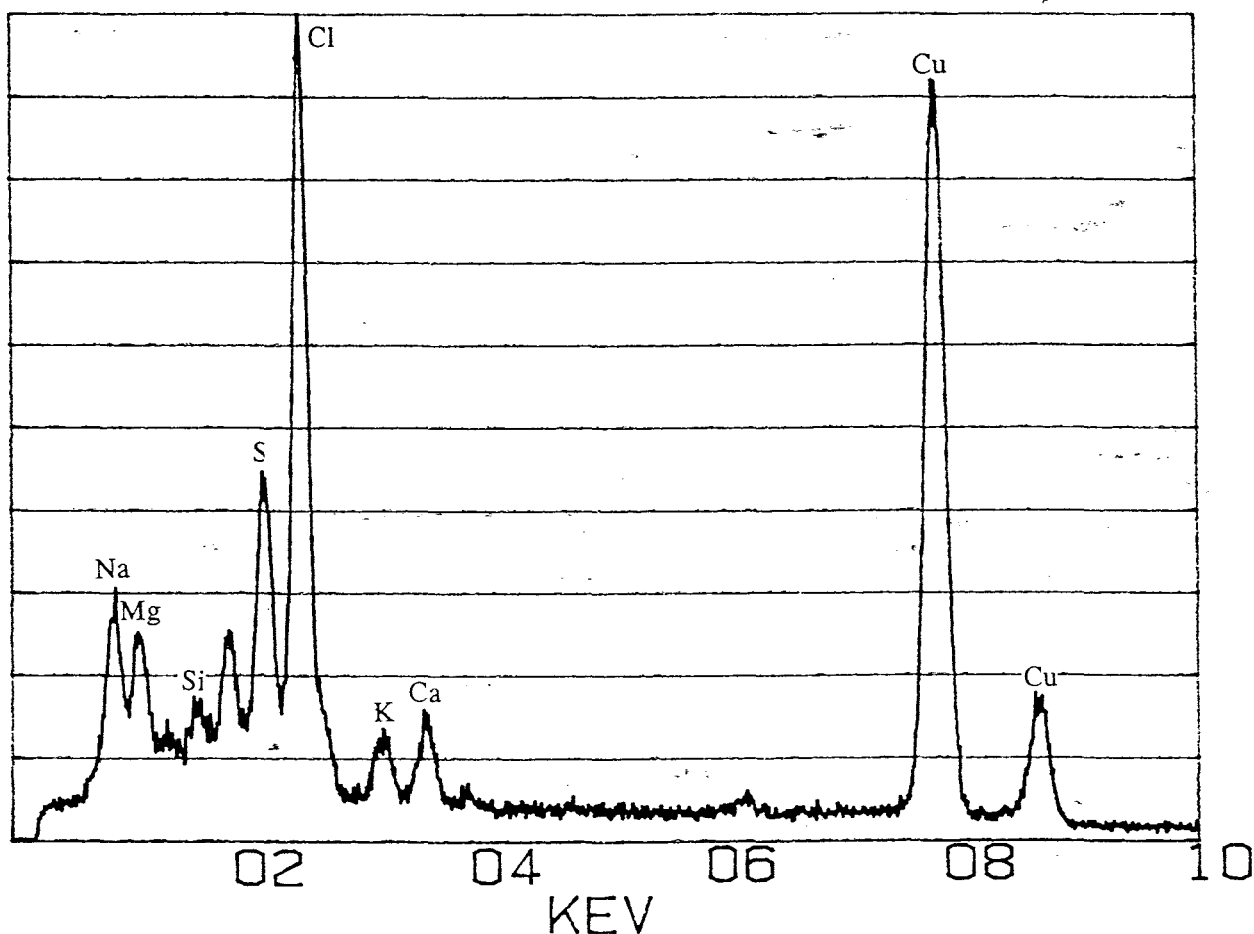


Figure 4.6. A typical EDAX ion profile spectra of native *S. cerevisiae* biomass. The Cu peaks originate from the 300 mesh grids used.

#### 4. ACCUMULATION BY VIABLE CELLS

Not all cells accumulated heavy metal cations, even though high external concentrations of metals were maintained to encourage internalization. Internalization of heavy metal cations occurred as a function of time. After 1 hr of metal exposure (Table 4.1) not all intact cells presented a positive identification spectra of the respective heavy metals, regardless of the presence of cytosolic electron dense inclusions, eg. the  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$  exposed cells. An increase in the number of inclusions over time (or those located in different cells) resulted in positive metal identification in the respective cells. Exposure to 1M solutions containing  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$  in excess of 5 hrs resulted in the majority of cells having accumulated heavy metals. Metal ion assessment of the intact cells, cytosol, cell walls and vacuoles were undertaken. The electron dense cytosolic inclusions noted in TEM micrographs (Fig 4.3 - 4.5) were analysed and proven to contain heavy metal ions (Figure 4.7). Though little visual evidence existed to implicate metal interaction with cell walls, positive spectral M-lines of the respective metal ions were noted in spectra of treated cells.

Table 4.1 Ion profiles of yeast cells exposed to respective 1M  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$  solutions for 1 hr. This data, obtained from EDAX spectra represent typical results.

CONTROL			$\text{Cu}^{2+}$ -CELLS			$\text{Co}^{2+}$ -CELLS			$\text{Cd}^{2+}$ -CELLS		
IC <sup>a</sup>	Cyt <sup>b</sup>	Vac <sup>c</sup>	IC	Cyt	Vac	IC	Cyt	Vac	IC	Cyt	Vac
Na	P	Na	Si	Si	Si	Na	P	Na	P	Na	Na
Mg	S	Mg	P	P	P	Mg	S	Mg	S	P	Mg
Si	Cl	Si	Cl	S	S	P	Cl	P	Cl	S	Si
P	K	P	Cu	Cl	Cl	S	Ca	S	K	Cl	P
S	Ca	S		Cu	Cu	Cl		Cl	Ca	Cd	S
Cl		Cl				K		K			Cl
K						Ca		Ca			Cd
Ca								Co			

a: Intact cell

b: Cytosol

c: Vacuole

The amount and rate of  $\text{Cu}^{2+}$  accumulation exceeded that of  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$ . Sufficient  $\text{Cu}^{2+}$  was taken into the yeast cells to provide positive detection after 1 hr. Whilst  $\text{Cd}^{2+}$  and  $\text{Co}^{2+}$  depositions

#### 4. ACCUMULATION BY VIABLE CELLS

within the cells could be located after 1 hr, this accumulation represented less than 1% of the total cell deposition thereby failing to provide positive cellular detection. Based on random analysis of sites within yeast cells, it appeared that the vacuoles were the initial site for intracellular metal ion deposition. These deposits became enlarged with increasing time of exposure to the metal containing solutions. Subsequent accumulation and deposition of metal ions in the cytosol may arise as a result of vacuolar saturation.

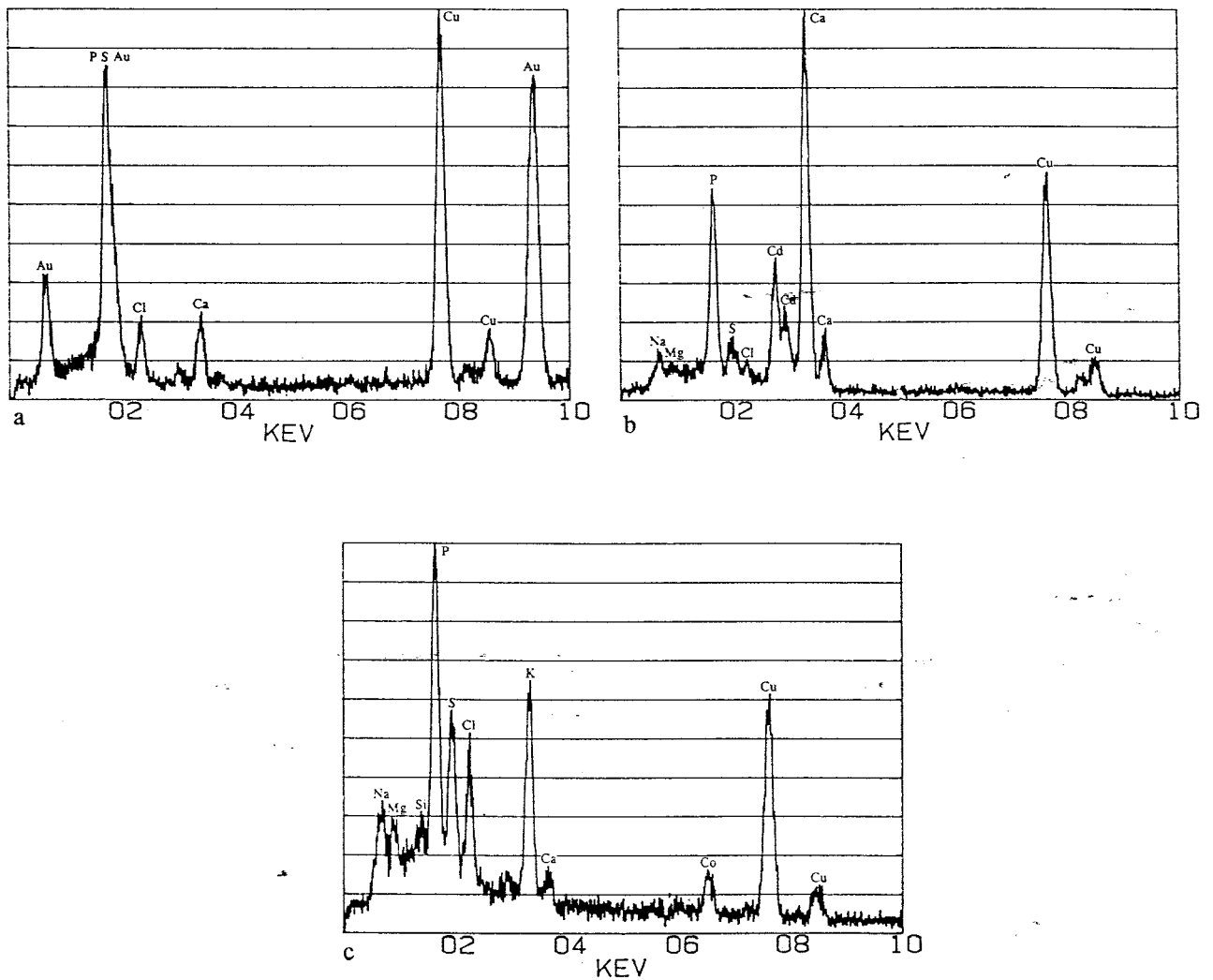


Figure 4.7 EDAX spectra of the electron dense inclusions in a)  $\text{Cu}^{2+}$ , b)  $\text{Cd}^{2+}$  and c)  $\text{Co}^{2+}$  loaded cells after 1 hr. Conspicuous P peaks are observed in each of the spectra. The Cu peaks present in (b) and (c) and the Au peaks in (a) are due to grid interference.

#### 4. ACCUMULATION BY VIABLE CELLS

The cellular ion profiles were monitored over time in the presence of high concentrations of the respective heavy metal species (Figure 4.8 - 4.10).

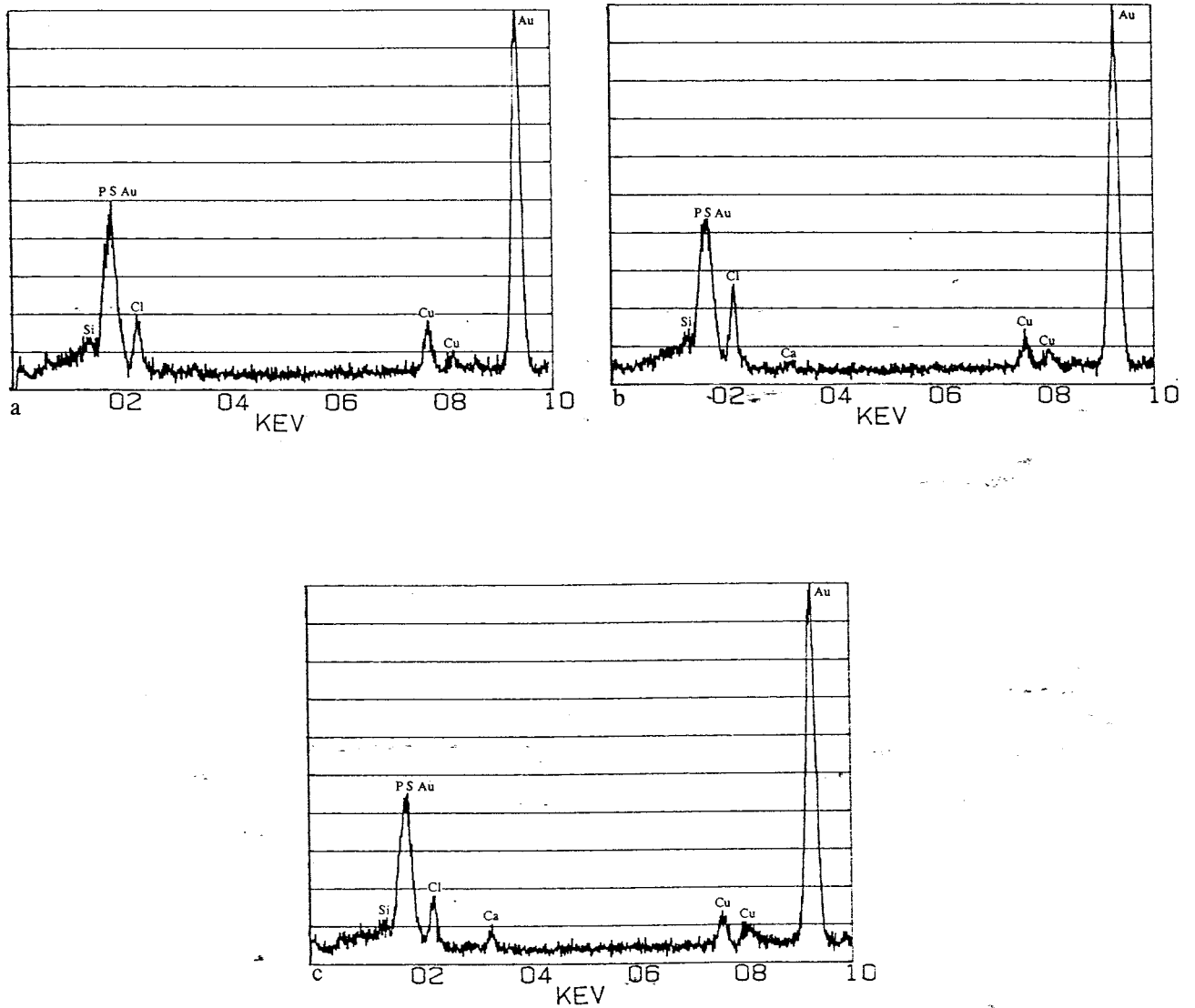


Fig 4.8 Intracellular ion composition of yeast cells exposed to a  $\text{Cu}^{2+}$  containing solution after a) 1, b) 12 and c) 24 hrs of metal exposure. Au peaks represent grid interference. The absence of physiological ions emphasized the ongoing ion exchange.

#### 4. ACCUMULATION BY VIABLE CELLS

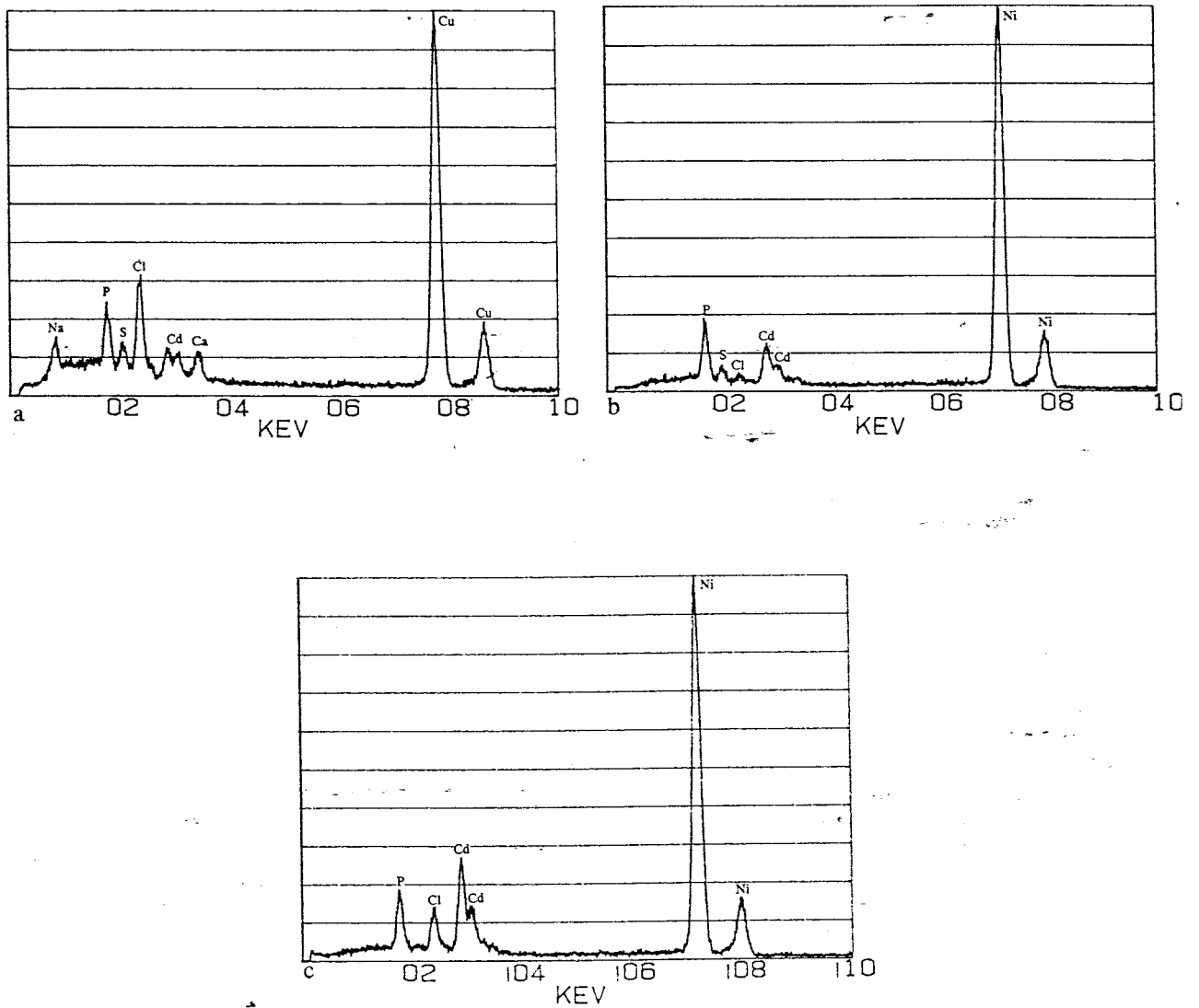


Fig 4.9. Intracellular ion composition of yeast cells exposed to a Cd<sup>2+</sup>-containing solution after a) 1, b) 12 and c) 24 hrs. Any K<sup>+</sup> present in the sample would be masked by Cd<sup>2+</sup> peaks. Cu and Ni grids were used. The disappearance of the physiological cations implies ion exchange.

4. ACCUMULATION BY VIABLE CELLS

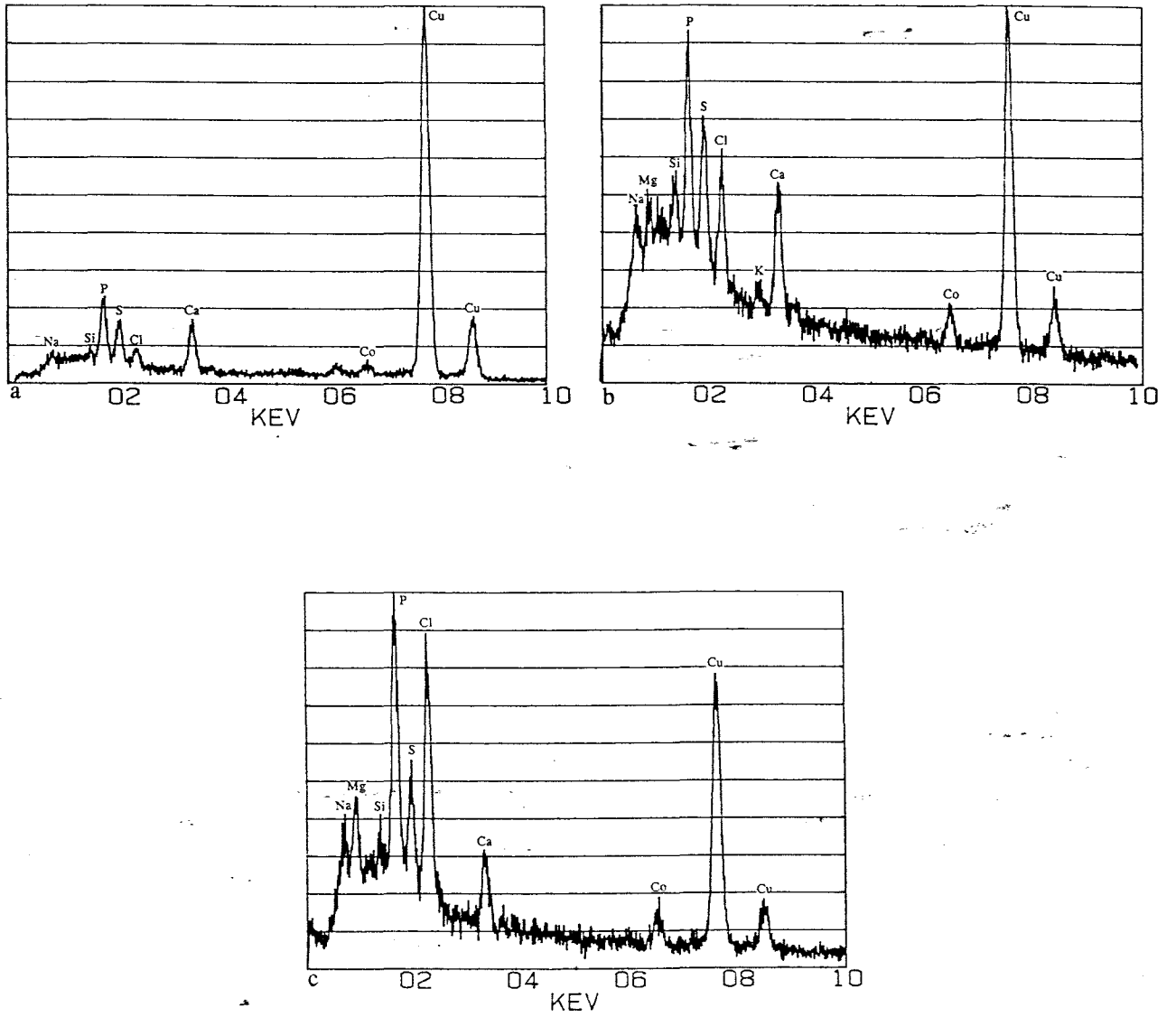


Fig 4.10. Intracellular ion composition of yeast cells exposed to a  $\text{Co}^{2+}$ -containing solution after a) 1, b) 12 and c) 24 hrs. Cu peaks originate from the grids used in the experiment.

#### 4. ACCUMULATION BY VIABLE CELLS

These spectra suggested a varied cellular response dictated by the heavy metal ions. The response elicited by  $\text{Cu}^{2+}$  (Fig 4.8) was similar to that of  $\text{Cd}^{2+}$  (Fig 4.9), whilst in the presence of  $\text{Co}^{2+}$  the cell responded differently (Fig 4.10). Though  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  elicited similar cellular responses, the pattern of accumulation of these two elements by the cells differed. At the onset of exposure of the yeast cells to  $\text{Cu}^{2+}$  initial, rapid accumulation of the metal by the cells appeared to occur with the intensity of the  $\text{Cu}^{2+}$  peaks in the EDAX spectra not increasing after 1 hr (Fig 4.8). In contrast,  $\text{Cd}^{2+}$  accumulation occurred gradually, with the intensities of these peaks becoming more prominent with time (Fig 4.9).  $\text{Co}^{2+}$  accumulation also occurred gradually, though saturation appeared to occur at an earlier stage than for  $\text{Cd}^{2+}$  (Fig 4.10).

Over time, exposure to, and accumulation of  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  by yeast cells resulted in the decline of intracellular metals. Not all the cellular cations were affected to the same extent, the decline in the amount of  $\text{Na}^+$ ,  $\text{Mg}^{2+}$  and  $\text{K}^+$  occurred in preference to that of  $\text{Ca}^{2+}$ , suggesting selective ion exchange or alternatively high levels of intracellular  $\text{Ca}^{2+}$ . The onset of the decline of these cations occurred rapidly. After 1 hr, analysis of  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  loaded cells failed to detect any physiological cations remaining within the cells. In contrast,  $\text{Co}^{2+}$  interaction with yeast cells failed to trigger an ion-exchange based decline in physiological cations, and after 24 hrs, detectable amounts of  $\text{K}^+$ ,  $\text{Na}^+$  and  $\text{Mg}^{2+}$  remained within the cells. A similar phenomena was noted by Okorokov (153) who found that  $\text{Co}^{2+}$  translocation into yeast cells did not result in any  $\text{K}^+$  efflux from the cells.

#### 4.3.3. THE EFFECT OF METALS ON MEMBRANE INTEGRITY

Membrane integrity of metal exposed cells was established using a diacyl derivative of fluorescein, FDA. FDA only fluoresces after uptake into the cells and subsequent decomposition to fluorescein by cellular hydrolases. The decomposition of FDA within the cells occurs within a few minutes and the resultant fluorescein shows very small permeability across the cell plasma membrane and is thus contained within the cell (172). The presence of fluorescein outside the cell thus indicates loss of plasma membrane integrity.

Extracellular fluorescence of metal treated cells (Fig 4.11) implicated  $\text{Cd}^{2+}$  as a predominant membrane disrupting agent. The effect of  $\text{Cu}^{2+}$  and  $\text{Co}^{2+}$  on membrane permeability appeared to be similar, and was less severe than that of  $\text{Cd}^{2+}$ . (Final fluorescence values were determined taking

#### 4. ACCUMULATION BY VIABLE CELLS

the values obtained from the control cells (no metal ions present) into account) The  $\text{Cu}^{2+}$  and  $\text{Co}^{2+}$  induced fluorescence intensities of the supernatants comprised 56% and 60% respectively of that induced by  $\text{Cd}^{2+}$ . Corresponding quantitative metal determination revealed that marginally less  $\text{Cd}^{2+}$  was accumulated by yeast cells than  $\text{Co}^{2+}$  or  $\text{Cu}^{2+}$ , ( $2.76 \mu\text{mol Cd}^{2+}/\text{g yeast}$ , compared to  $2.94 \mu\text{mol Cu}^{2+}/\text{g yeast}$  and  $2.96 \mu\text{mol Co}^{2+}/\text{g yeast}$ ).

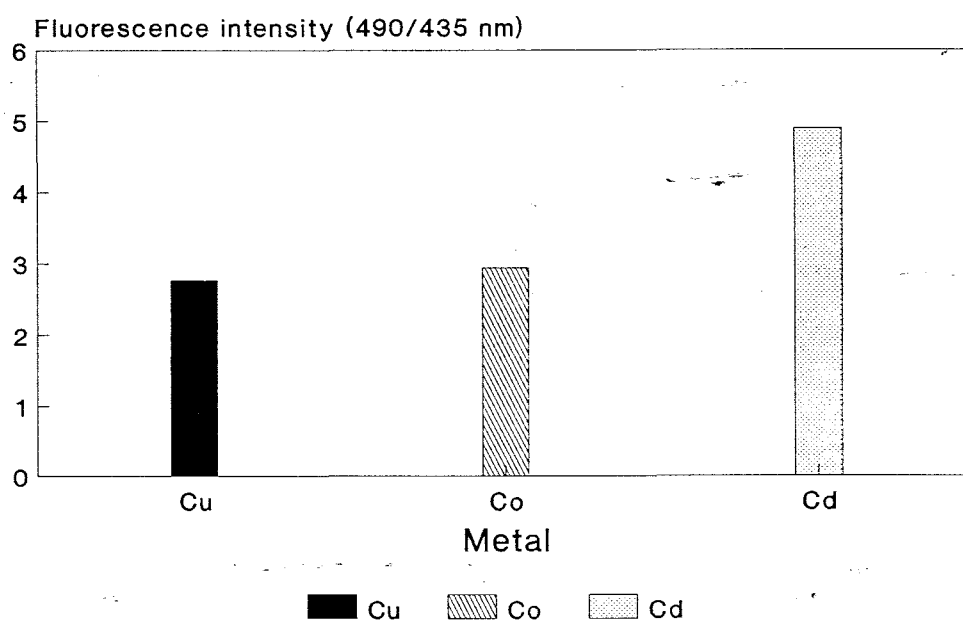


Fig 4.11. Fluorescence determination of the supernatant of  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Co}^{2+}$  exposed cells as an indicator of the effect of metals on plasma membrane integrity of yeast cells.

#### 4.4. DISCUSSION

While numerous other authors have provided evidence regarding internalization and compartmentalization of heavy metal cations, the benefits of using X-ray diffractive analysis (EDAX

#### 4. ACCUMULATION BY VIABLE CELLS

spectra) for this purpose include the ease with which metal ion deposition and the subsequent cellular response can be monitored over time.

Distribution of metal cations within yeast cells occurs between the soluble and insoluble fractions. Asymmetrical distribution of metal ions occurs with metal ions generally having a greater preference for the insoluble fraction than the soluble fraction. Work done on *S. cerevisiae* and *K. marxianus* (82) proved accumulation of  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Ag}^{3+}$  by the insoluble fraction to be the main mechanism of tolerance and accumulation, with 21%  $\text{Cd}^{2+}$ , 46%  $\text{Cu}^{2+}$  and 31%  $\text{Ag}^{3+}$  associated with the vacuole. Similar affinities for vacuolar deposition were exhibited by other ions. Perkins and Gadd (94) found that 35% of  $\text{Li}^{+}$  was accumulated in the vacuoles of *S. cerevisiae* cells. These organelles have also been shown to be the major Fe storage compartment in the yeast cell and the only compartment capable of greatly increasing its Fe content when subjected to high external levels of Fe (81).

The role played by the vacuole in  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Co}^{2+}$  sequestration was emphasized during the present study. Vacuolar ion deposition occurred at an early stage, often prior to the detection in the cytosol (Table 4.1). After 1 hr the amounts of  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$  deposited within the vacuole exceeded 1% of its composition, yet this was inadequate for cellular detection. In contrast, rapid uptake and distribution of  $\text{Cu}^{2+}$  occurred with adequate amounts of  $\text{Cu}^{2+}$  being accumulated to enable cellular detection after 1 hr.

White and Gadd (167) described an increase in vacuolar  $\text{Co}^{2+}$  accumulation in the presence of elevated levels of external  $\text{Co}^{2+}$ . In comparison vacuolar accumulation of  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  under identical conditions was far less marked, instead a significant increase in accumulation by the soluble fraction was noted. Similar to the darkened deposits observed during the present study (Fig 4.5),  $\text{Cd}^{2+}$  was noted as dark deposits in the cytosol of bacterial cells on uptake in the presence of elevated external concentrations of this metal (174). The  $\text{Cd}^{2+}$  was shown to interact with phosphorous groups within the cytosol. Roomans (cited by 16), when using energy dispersive X-ray analysis, showed that  $\text{Mg}^{2+}$ ,  $\text{Sr}^{2+}$  and  $\text{Ca}^{2+}$  are also sequestered as phosphorous-rich cytoplasmic granules. Similarly EDAX spectra of the electron dense cytoplasmic inclusions in  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Co}^{2+}$  treated cells in the present study yielded conspicuous phosphorus peaks. Presumably phosphates are contained within these granules, though this does not necessarily contradict with the view of vacuolar polyphosphate concentration. However, the presence of cytosolic polyphosphates as an

#### 4. ACCUMULATION BY VIABLE CELLS

adaptive mechanism to excessive levels of heavy metals cannot be excluded (173).

An observation drawn from the present study was the inability of some cells to accumulate detectable levels of metal ions. Similar phenomena were noted by others during  $\text{Cd}^{2+}$  accumulation by *S. cerevisiae* (173) and  $\text{UO}_2^{2+}$  uptake by *Streptomyces sp.* (cited by 173), which suggest the metabolic driven accumulation of metal ions. In the absence of operational metal uptake pathways, non-viable cells would not accumulate any metal ions.

Two distinct cellular responses were detected in response to metal accumulation. Uptake of  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$  triggered the loss of the physiological cations:  $\text{K}^+$ ,  $\text{Na}^+$  and  $\text{Mg}^{2+}$  in a distinct ion-exchange process, whereas in the presence of  $\text{Co}^{2+}$  no release of intracellular ions (*ie.* ion-exchange) occurred.  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$ -induced cellular cation loss have been reported on previous occasions (36, 39, 175 - 179).  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Mg}^{2+}$  ions are distributed between the vacuole and the cytosol of yeast cells, with  $\text{Na}^+$  and  $\text{K}^+$  ions predominantly concentrated within the cytosol (73). The rapid loss of cellular  $\text{K}^+$  during this and other studies on  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  uptake (39, 105, 178, 180) may be enhanced by the effect of these metals on the permeability of the plasma cell membrane.  $\text{Co}^{2+}$  uptake is however not necessarily coupled to  $\text{K}^+$  loss (4, 175). Similar to the present study, Okorokov (153) and Norris and Kelly (4) observed no loss of  $\text{K}^+$  during  $\text{Co}^{2+}$  accumulation. No simple relationship exists between  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  uptake and the amounts of  $\text{K}^+$  released. Norris and Kelly (4) approximated the release of 4  $\text{K}^+$  ions for each  $\text{Cd}^{2+}$  ion accumulated, whereas Kessels, *et al* (36), observed the loss of 22  $\text{K}^+$  ions per  $\text{Cd}^{2+}$  ion accumulated. In addition, the release of  $\text{K}^+$  appears to be an integral component of intracellular  $\text{Cu}^{2+}$  accumulation. After  $\text{Cu}^{2+}$  treatment, approximately 30% of the total  $\text{K}^+$  remained within the cells (39). In addition  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  have been found to cause the loss of  $\text{Mg}^{2+}$  from yeast cells (4, 5).  $\text{Ca}^{2+}$  release from cells is least affected by  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Co}^{2+}$  (175).  $\text{Ca}^{2+}$  stores remained undepleted in  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Cd}^{2+}$  exposed cells in the present study (Fig 4.8 - 4.10).

$\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$ -induced cellular ion leakage from yeast cells can also occur as a result of selective permeabilization of the plasma cell membrane by the metals. In addition to  $\text{K}^+$ ,  $\text{Na}^+$  and  $\text{Mg}^{2+}$ , other studies have shown that low molecular weight substances inclusive of inorganic phosphate, 260 nm absorbing materials and ribose containing molecules are released from the cell (39, 177). The release of cellular ions is indicative of the loss of cell viability due to heavy metal toxicity. Cadmium toxicity is reported to be caused by structural lesions in the plasma membrane (35, 178, 180), thereby

#### 4. ACCUMULATION BY VIABLE CELLS

explaining the elevated fluorescence level noted in the supernatant of  $\text{Cd}^{2+}$  treated cells in the present study.

#### 4.5. CONCLUSIONS

In conclusion, the present study has investigated the uptake of  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Co}^{2+}$  into yeast cells, their subsequent cellular deposition and the response elicited by viable yeast cells. The accumulation of the above-mentioned metals is metabolism-dependent with intracellular deposition primarily occurring in the vacuoles, although cytosolic deposition does occur. Accumulation of heavy metals within the cytosol often occur as granular deposits. The uptake of heavy metal cations by viable yeast cells has been shown to elicit certain cellular responses.  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  accumulation results in the loss of the physiological cations,  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Mg}^{2+}$ . In contrast,  $\text{Co}^{2+}$  has no such effect. Not all the physiological cations are affected, eg.  $\text{Ca}^{2+}$  stores within the yeast cell remain undepleted. Loss of these intracellular cations most likely occurs via an ion-exchange mechanism as all three heavy metals induce plasma cell membrane permeabilization.

Further investigations in this study were aimed at using yeast cells as a biomass for heavy metal accumulation from waste water.

## 5. BIOACCUMULATION OF HEAVY METALS FROM WASTE WATER BY VIABLE *S. cerevisiae* CELLS

### 5.1. INTRODUCTION

The bioaccumulation of heavy metals has received a great deal of attention in recent years, not only as a scientific novelty, but also for its application potential in industry. It is necessary to differentiate between the process of bioaccumulation and biosorption. Although Gadd and White (98) refer to the latter as the uptake of metals by viable or non-viable biomass via various mechanisms, biosorption remains the physico-chemical property of non-viable biomass to retain and concentrate metallic elements from solution via non-energy dependent processes. In comparison, the bioaccumulation of metal by viable microorganisms occurs by a number of processes, such as intracellular uptake by transport, adsorption to cell walls and entrapment in extracellular capsules, precipitation and biotransformation reactions. Both of these processes can be used in the bioremediation of metal containing water.

One of the most ubiquitous biomass types available on a large scale is *S. cerevisiae*. Depending on the manner of propagation, the yeast strains are either referred to as "bakers yeast" (aerobic propagation) or "brewers yeast" (anaerobic propagation). Although this biomass has previously been referred to as a mediocre biosorbent (138) its ready availability and low cost in certain countries make it a favourable choice of biosorbent in these cases. Furthermore, yeast cells retain their ability to accumulate a broad range of heavy metals to varying degrees, under a wide range of external conditions (181). For example, *S. cerevisiae* has been shown to accumulate Ag (4.7 mg/g), Cd (11 - 40 mg/g), Co (4.7 mg/g) (181), Cu (6 - 40 mg/g) (181, 182), U (55 - 140 mg/g) (182), Th (70 mg/g) and Zn (14 - 40 mg/g) (138, 182). Earlier investigations in this study also showed that *S. cerevisiae* was capable of accumulating Cd, Cu and Co.

Obviously the bioavailability and subsequent amounts of the respective metals accumulated are not fixed, but remain as variables, moderated by various factors, such as the chemical and physical properties of the metals (165), cellular physiology (15) and the ambient conditions (181, 183, 184). Included in the range of chemical parameters which influence the uptake of metals are ionic radius, ionic charge, preference for coordination with certain ligands, solubility and subsequent availability

of the metal ions (165).

Metal accumulation is susceptible to both incubation temperature and ambient pH (15, 181, 184). The extent to which temperature affects the uptake depends on the species of metal in question. Brady *et al.* (181), reported minimal temperature related effects on the bioaccumulation of  $\text{Cu}^{2+}$  over a temperature range of 5 - 40°C, although maximum accumulation occurred between 25 - 30°C. Accumulation of uranium by *S. cerevisiae* is highest at 40°C. In comparison, the susceptibility of *S. cerevisiae* to the toxic effects of  $\text{Cd}^{2+}$  is greatly enhanced by elevating the incubation temperature from 25°C to 37°C. An elevated incubation temperature also enhances the susceptibility of yeast to  $\text{Hg}^{2+}$ , although the effect is less pronounced (184). Accumulation processes that depend on cellular metabolism such as the second phase of the uptake procedure are those most likely to be affected by variances in temperature. Active uptake is most likely to be inhibited at low temperatures, whereas elevated temperatures increase the fluidity of the lipids in membranes and subsequently affect the membrane integrity.

Transport of cations into yeast cells is influenced by both the intracellular and extracellular pH (15). The alteration of ambient pH over a wide range does not measurably alter the intracellular pH suggesting that the peripherally located binding sites are influenced to a greater extent by the variability of the extracellular pH than the constancy of the intracellular pH (40, 135). Ambient pH is a major factor affecting the amount of metal accumulated owing to the competition between metal cations and  $\text{H}^+$  ions for uptake. In general, low external pH reduces both the surface binding and the intracellular influx of ions (135). The biological availability of metals is also determined by the charge. Shifts in pH affect metal bioaccumulation by changing the metal speciation. A low pH increases metal solubility and mobility whilst at neutral or high pH, insoluble oxides and hydroxides tend to form. The optimal pH for metal accumulation remains species specific, eg. maximum  $\text{Cu}^{2+}$  accumulation by yeast occurs between pH 5.0 - 9.0, with rapid decreases in accumulation at either extreme of the pH range (181). Uptake of  $\text{Ni}^{2+}$  increases with increasing pH up to pH 5.0, while the maximal uptake rate for  $\text{Ca}^{2+}$  occurs at pH 7.0 and is reduced by 85% at pH 4.5 (15). Jones and Gadd (145) found that the uptake of divalent ions by *S. cerevisiae* is significantly reduced below pH 5.0.

The metabolism-dependent uptake of metal ions can be influenced by the presence of competing metal cations, or even inhibited by glucose analogues or lack of glucose. The uptake of  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$

and  $Zn^{2+}$  is low in starved *S. carlsbergensis* cells, yet the addition of, or pretreatment with glucose results in a 5 - 20 fold increase in the amount of metal accumulated (25). Contrasting results presented by Brady *et al.* (181), report no enhanced accumulation of  $Cu^{2+}$ ,  $Cd^{2+}$  and  $Co^{2+}$  by *S. cerevisiae* cells in the presence of glucose. The difference could however, reflect the growth phase of the cells. Non-energy dependent accumulation may occur in stationary phase cells, whilst developing cells in log phase may require an additional energy source.

In yeast an energy-dependent influx of heavy metal cations has been demonstrated with an affinity series of  $Mg^{2+} > Co^{2+} > Zn^{2+} > Mn^{2+} > Ni^{2+} > Ca^{2+} > Sr^{2+}$  (25). The selectivity of heavy metal accumulation can be further influenced by the presence of competing monovalent or divalent metal cations. Monovalent cations eg.  $K^+$  may antagonize the uptake of divalent cations such as  $Ca^{2+}$  or  $Mg^{2+}$  and *visa versa* (15). Mutual interactions occur between divalent cations. In the presence of specific metal cations, uptake of other divalent cations may be enhanced. For example, the uptake of  $Zn^{2+}$  is increased specifically by  $Cu^{2+}$ , whereas the uptake of  $Mn^{2+}$  remains unaffected (16). Similarly  $Co^{2+}$  enhances  $Zn^{2+}$  uptake, with the reciprocal also being true (25). In comparison  $Ca^{2+}$  inhibits  $Zn^{2+}$ ,  $Co^{2+}$  (25) and  $Cd^{2+}$  uptake (4). The presence of  $Mg^{2+}$  results in a minimal reduction of  $Cd^{2+}$  accumulation. The inhibition of metal cation influx by other metal has been reported to be related to the ionic radii (4), though this is based on the assumption that a general mechanism for uptake of divalent cations exists (16, 25). In general, a mixture of heavy metals can produce three possible types of behaviour: synergism, antagonism or non-interaction, where the effects produced are either greater than, less than or the same as the individual effects of the constituents in the mixture (185).

A relationship exists between metal uptake and the ambient metal concentration.  $Cu^{2+}$  accumulation by yeast has proven to be dependent on the ratio of external free metal concentration to the available biomass (181). Similar effects have been noted for the accumulation of  $UO_2^{2+}$ , where accumulation was dependent on uranyl ion concentration. The movement of  $Hg^{2+}$  into yeast cells is also a function of the external  $Hg^{2+}$  concentration (186).

A central aim of this study has been to forge a link between academic interest and industrial applicability. While earlier chapters in this report have focussed on more academic aspects of metal accumulation such as mechanisms of metal uptake and sites of deposition, the object of the study reported in this chapter was to examine the effectiveness of *S. cerevisiae* biomass to accumulate

metals from waste water. Although metal uptake in viable yeast cells have been studied on previous occasions, this study investigates the bioaccumulation of metal ions from multi-metal solutions in which the aqueous phase differed from those used previously. Instead of using a spiked metal containing solution, waste water was obtained from industrial electroplating plants. This waste water was obtained from three different plants (effluents A, B and C), each differing in metal species and content. The capacity of native bakers yeast cells for metal ion removal from these solutions was determined and the effect of glucose and external pH on this uptake monitored.

## 5.2. METHODS AND MATERIALS

### 5.2.1. METAL EQUILIBRATION

Similar to the method of Tsezos (187), Kuyucak and Volesky (150) and others, the yeast biomass was brought into direct contact with the effluent in batch reactors. Washed viable yeast cells were mixed with the respective effluents (A, B or C) at known concentrations (0.1 g (wet wt)/ml). Equilibration of the yeast-effluent solutions proceeded for 25 hrs at 25°C with gentle shaking. At 1, 5, and subsequent 5 hourly intervals, 20 ml fractions were removed, centrifuged (3000 g x 10 min) and the supernatant analysed to determine the residual metal concentration (Varian 1275 Atomic Absorption Spectrophotometer). In the absence of yeast, loss of metal due to metal-glass interactions was ascertained from control fractions. The external pH was monitored throughout the duration of the experiment.

### 5.2.2. OPTIMIZATION STUDIES

#### 5.2.2.1. BIOMASS RATIO DETERMINATION

Due to the number of contaminating metal elements therein and the severity of the Cd problem (extremely high levels and subsequent toxicity) all further optimization studies were performed using effluent A. Additional trials were conducted to determine the optimum yeast : metal ratio for metal accumulation. Yeast was suspended in effluent in 0.1, 0.25, 0.5, and 1.0 g/ml (wet wt) ratios, shaken for 15 hrs (completion of yeast metal equilibration) at 25°C, and the efficiency of metal ion removal determined. The 0.5 g/ml ratio of yeast : waste water attained adequate metal removal for industrial purposes and was used in all subsequent experiments for this section.

#### 5.2.2.2. GLUCOSE SUPPLEMENTATION

The effect of an energy source on the efficiency of metal removal was ascertained. The importance of glucose as an energy source was determined by comparing the metal removal by glucose-treated cells to that of untreated cells. In addition, the effect of pretreating the cells with glucose was compared to direct supplementation of glucose to the yeast-effluent solutions.

Suspended yeast cells (0.5 g/ml H<sub>2</sub>O) were pretreated with glucose following the method of Perkins and Gadd (42). Glucose was added to the yeast suspension to a final concentration of 50 mM, 30 min prior to effluent addition. After 30 min the suspension was centrifuged and the pelleted yeast cells washed. The pelleted yeast cells were resuspended in Effluent A (0.5 g/ml) and the metal removal procedure commenced.

The effect of direct glucose addition over time was monitored by the supplementation of the yeast-effluent solution with glucose at 0, 5 and 10 hourly intervals and metal accumulation determination after 15 hrs. Glucose was added to obtain a final concentration in solution of 50 mM.

#### 5.2.3. BIOACCUMULATION FROM WASTE WATER USING BATCH REACTIONS

Multiple metal containing effluents (A, B and C) collected from electroplating factories were treated using batch reaction systems. The percentage metal removed from each of the effluents during 15 hr continuous batch systems were compared to those of 3 x 5 hr batch reaction systems run in series. The latter involved suspending yeast in effluent (0.5 g/ml) for 5 hrs at 25°C with gentle shaking. After 5 hrs the suspensions were spun down (4000 g x 10 min) and the supernatant added to fresh yeast. On completion of incubation (after 15 hrs) both continuous and series batch systems were spun down and the percentage metal removed, determined.

Identical experiments were conducted using glucose pretreated cells. Optimal pH conditions were also introduced into some of the reactions. The pH of effluents A, B and C varied, viz. pH 6.0, 3.6 and 2.2 respectively. Using NaOH the pH of B was altered to pH 5.0 (precipitation occurred at higher pH values) and that of C from pH 2.2 to pH 6.0. As for previous experiments, the pH was monitored for the duration of incubation.

## 5. HEAVY METAL BIOACCUMULATION

Comparative bioaccumulative analysis was undertaken using a spiked metal solution containing multiple metals in solution. Metal uptake from a stock solution containing 50 mg/l of Zn, Cr, Cu, Cd and Ni was compared to that from industrial effluent. Experimental parameters remained identical to those used previously.

### 5.3. RESULTS

The electroplating effluents used during this study were obtained from three different metal plating factories. All contained metal ions in excess of the stipulated criteria for both drinking and river water (Table 5.1).

Table 5.1. Heavy metal profiles for electroplating effluents A, B and C. Metal levels of the effluents are compared to suggested median levels for drinking and dam/river water. The levels are expressed as mg/l. Total Cr (*ie.* Cr<sup>3+</sup> and Cr<sup>6+</sup>) was measured

	Metal Concentration (mg/l)				
	Drinking water	Dam/river water	A	B	C
Cd	0.01	0.003	16.0	<0.05	<0.05
Cr	0.05	0.05	<0.05	71	4
Cu	1.00	0.005	0.1	0.5	30
Ni	0.05	0.05	0.1	<0.05	15.6
Zn	5.00	0.1	260	460	0.3

With the exception of Zn<sup>2+</sup> removal from effluent A and C, the majority of metal ions were taken up during the initial stages of equilibration (Fig 5.1 - 5.3). The rate of accumulation ( $\mu\text{g/g/hr}$ ) of metals decreased over time with the onset of biomass saturation. Complete biomass saturation was assumed to have occurred after 15 hrs. No adjustments were made to the pH of the yeast-effluent solutions prior to equilibrium, yet in the presence of yeast cells the external pH was regulated and preferentially maintained between pH 4.0 - 5.0. The greatest change in the external pH of effluent A and B occurred within the first hour (Fig 5.1 and 5.2). After 25 hrs pH values within the same range were recorded for effluents A and B, *viz.* pH 5.01 and pH 5.33 respectively. The initial pH

## 5. HEAVY METAL BIOACCUMULATION

of the yeast-effluent solution for C was lower than that of A and B (*viz.* pH 2.01). During equilibration an active cellular response to this acidic environment was maintained, resulting in an increase in the alkalinity of the solution to a final pH of 3.81. In the absence of metal ions the pH of the solutions of yeast cells suspended in Milli-Q water gradually increased over time.

Pretreatment of the yeast cells with glucose enhanced heavy metal ion uptake from effluent A over a 15 hr period (low amounts of  $\text{Cu}^{2+}$  and  $\text{Ni}^{2+}$  were present in this effluent, hence the graphic representation was limited to  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$  accumulation) (Fig 5.4). In contrast to pretreatment, direct addition of glucose to the yeast-effluent A suspension did not stimulate metal removal, when compared to the control (no glucose addition). Glucose was supplemented directly to respective yeast-effluent solutions at 0, 5 and 10 hrs (metal accumulation measured after 15 hrs). The lack of response to the treatment suggests an inability of the cells to utilize glucose in the presence of metal ions.

The effect of time, glucose pretreatment and pH modification on the metal removed from the three effluents can be compared in Fig 5.5 - 5.7. Under these conditions, increased metal accumulation was recorded from multiple shorter incubation periods in series. Metal accumulation is an energy dependent process. Glucose pretreatment had a greater effect on bioaccumulation than an increase in the external pH. The combined treatment (*ie.* glucose pretreatment and pH modification) was marginally less effective regarding metal uptake than glucose treatments. (Note: pH treatment was not required for effluent A due to an influent pH 6.0) Though a trend can be established regarding the metal bioaccumulation, due to the variation in the initial concentrations of metals present in the respective effluents, it is difficult for a quantitative comparison to be drawn regarding the amount of metal accumulated.

5. HEAVY METAL BIOACCUMULATION

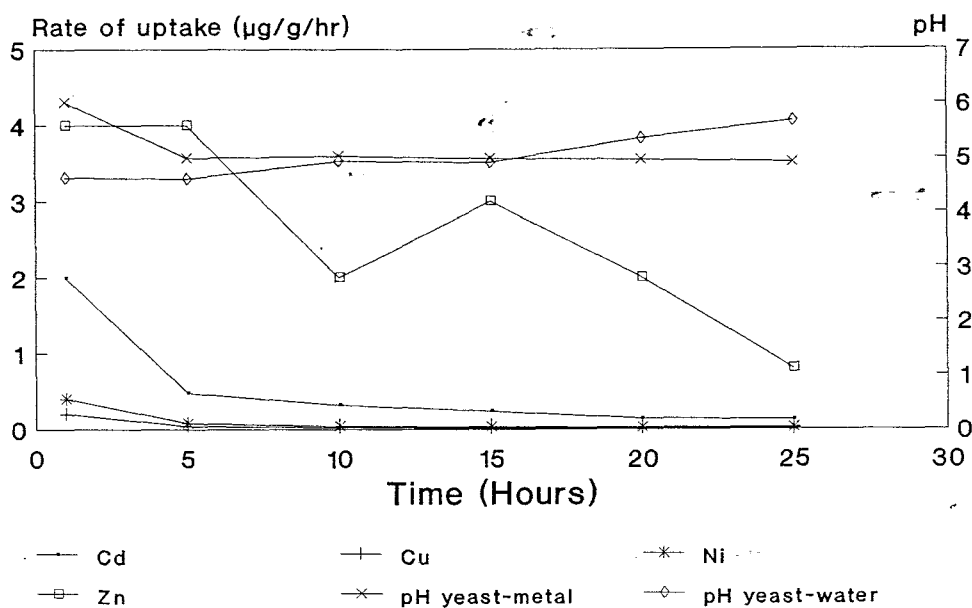


Figure 5.1. The rate of bioaccumulation of metal ions from effluent A over time ( $\mu\text{g/g}$  biomass/hr) by viable yeast cells. The change in pH of yeast-metal solutions can be compared to that of a yeast-water solution over the same period of time.

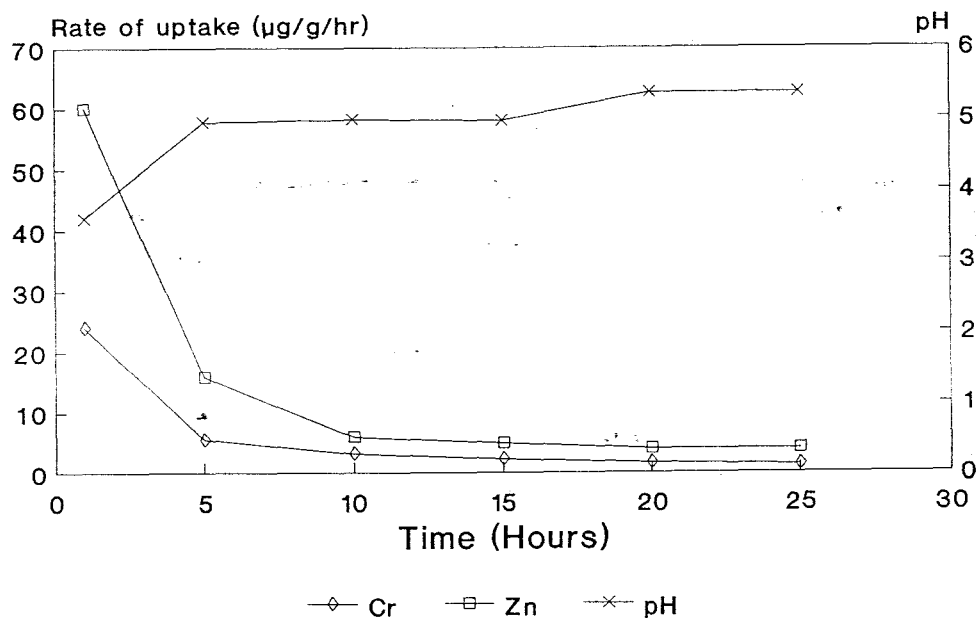


Figure 5.2. The rate of bioaccumulation of metal ions from effluent B over time ( $\mu\text{g/g}$  biomass/hr) by viable yeast cells. The effect of time and metal bioaccumulation on the external pH was monitored.

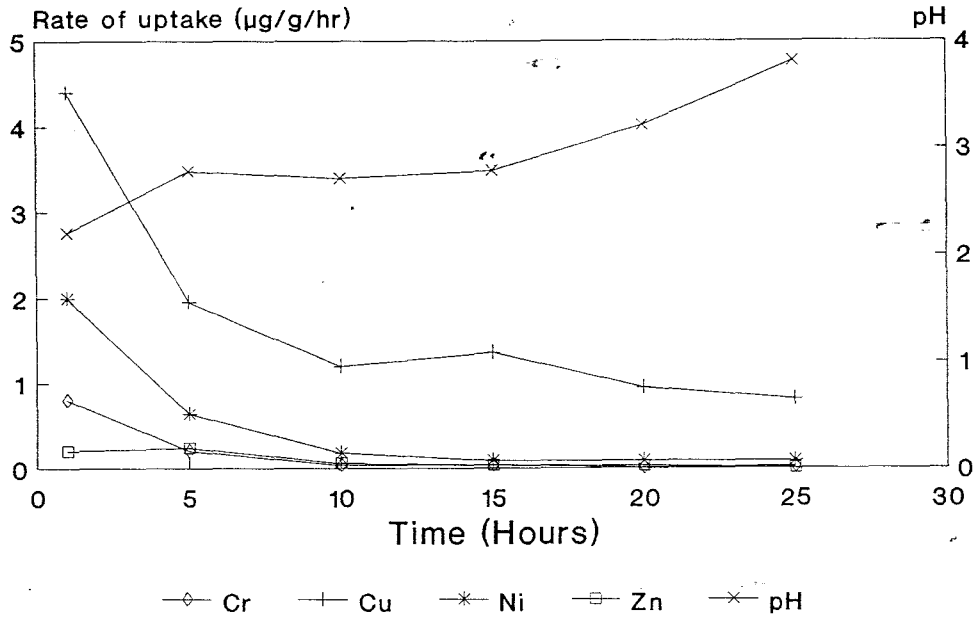


Figure 5.3. The rate of accumulation of metal ions from effluent C over time ( $\mu\text{g/g biomass/hr}$ ) by viable yeast cells. The effect of time and metal bioaccumulation on the external pH was monitored.

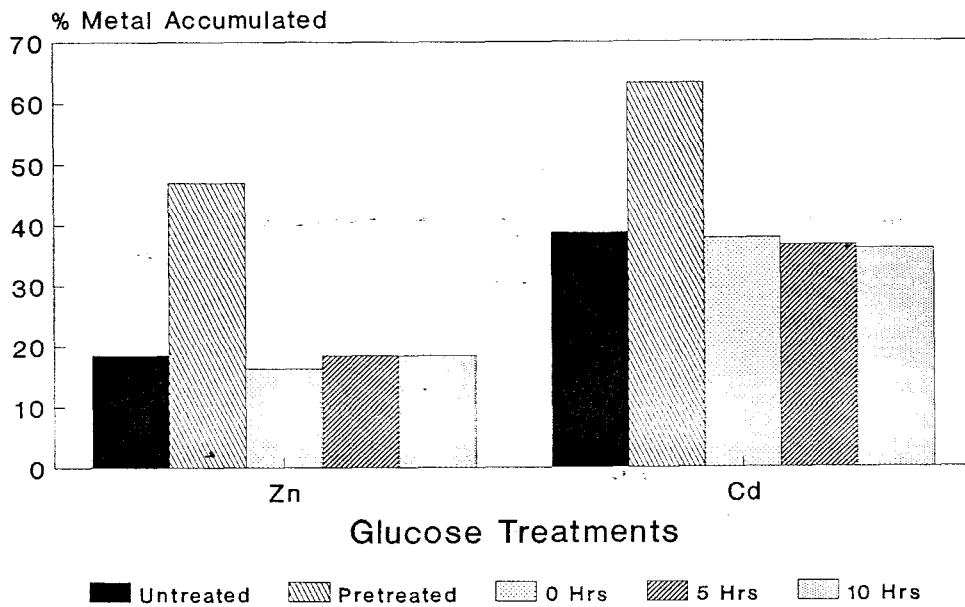


Figure 5.4. The effect of a metabolizable energy source on the uptake of  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  from effluent A by viable yeast cells. Glucose treatment involved either a 30 min pretreatment period or direct addition of glucose at 0, 5 and 10 hrs respectively.

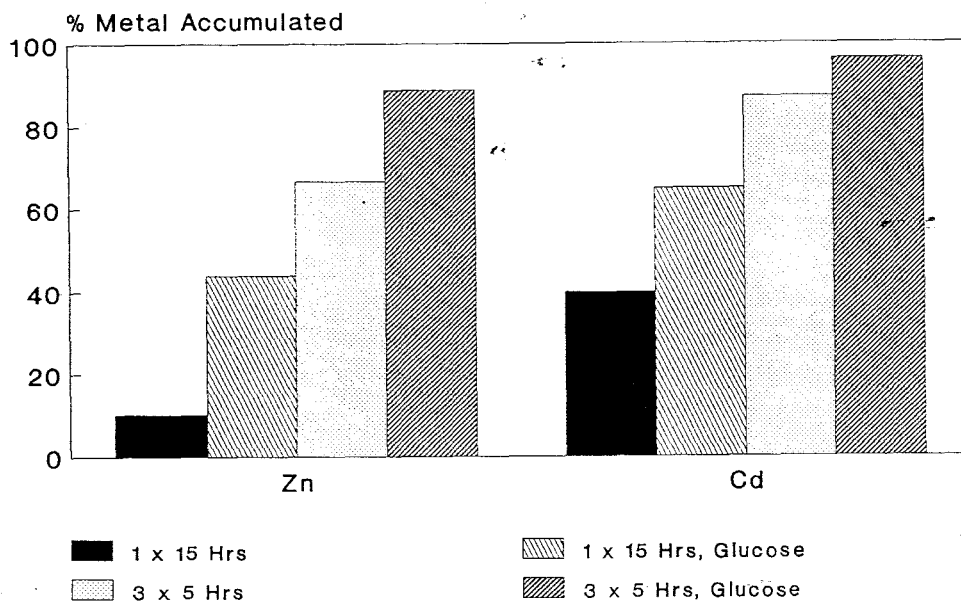


Figure 5.5. Percentage metal removed from effluent A by treated and untreated viable yeast cells in batch systems. The effect of an extended period of incubation on metal removal, was compared to multiple shorter periods of incubation. Stipulation of glucose, pH or both in the legend (Fig 5.5 - 5.7) indicates the effect of the respective treatment on metal removal.

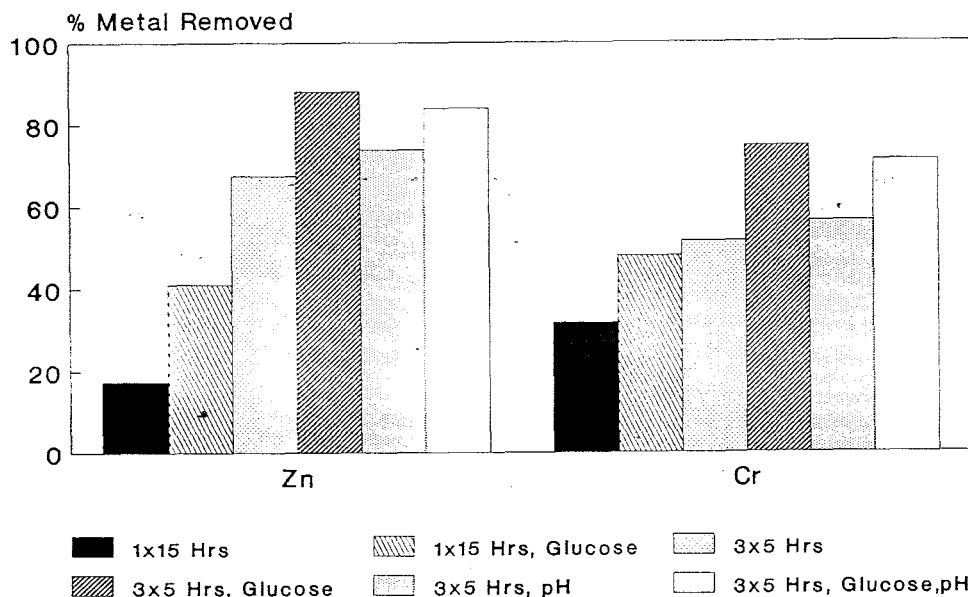


Figure 5.6. Percentage metal removed from effluent B by treated and untreated viable yeast cells in batch systems. The effect of time, pH modification and glucose treatment was observed.

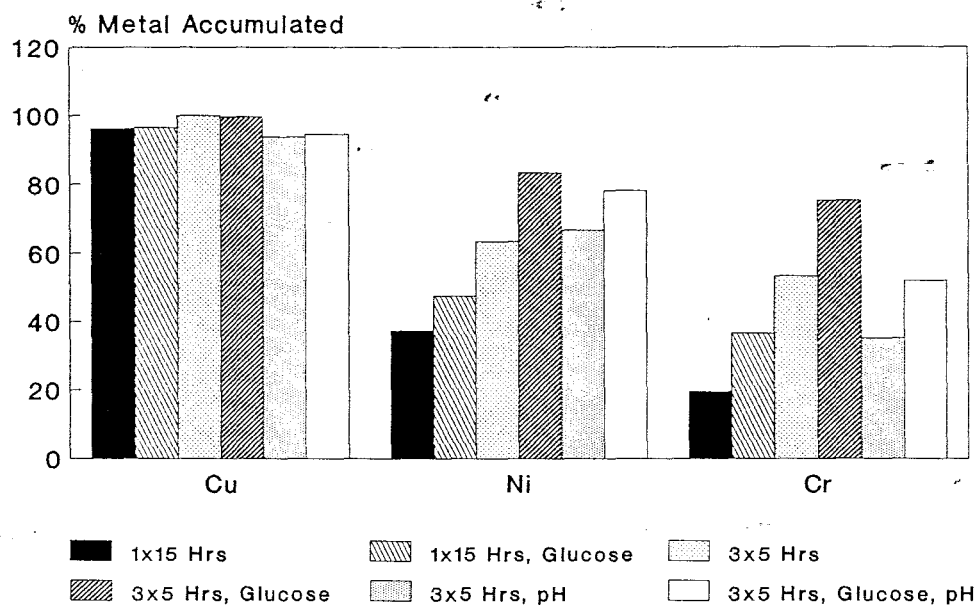


Figure 5.7. Percentage metal removed from effluent C by treated and untreated viable yeast cells in batch systems. The effect of time, pH modification and glucose pretreatment was monitored.

The amount of metal accumulated by the yeast cells from the respective electroplating effluents was quantitated (Table 5.2) and this was compared to the results obtained for metal uptake from a spiked multi-element solution (Table 5.3).

5. HEAVY METAL BIOACCUMULATION

Table 5.2. Metal accumulation ( $\mu\text{g}$  metal/g (wet wt)) by yeast from electroplating effluents: A, B and C. The effect of time, pH modification and glucose pretreatment on metal uptake is compared. The effect of the respective treatments are indicated as Hrs, glucose, pH or a combination of the three.

	Effluent A		Effluent B		Effluent C		
	Zn ( $\mu\text{g/g}$ )	Cd ( $\mu\text{g/g}$ )	Zn ( $\mu\text{g/g}$ )	Cr ( $\mu\text{g/g}$ )	Ni ( $\mu\text{g/g}$ )	Cr ( $\mu\text{g/g}$ )	Cu ( $\mu\text{g/g}$ )
1 x 15 Hrs	90	12.2	160	45	11.6	1.5	56.4
1 x 15 Hrs, glucose	230	19.9	380	68	14.8	2.8	56.6
3 x 5 Hrs	360	27.1	620	73	19.8	4.1	58.6
3 x 5 Hrs, glucose	480	29.9	810	106	26	5.8	58.5
3 x 5 Hrs, pH			680	80	20.8	2.7	55.5
3 x 5 Hrs, glucose, pH			770	101	24.4	4.0	55.4

Table 5.3. Metal accumulation ( $\mu\text{g}$  metal/g (wet wt) yeast) from a spiked solution containing  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Zn}^{2+}$  and  $\text{Ni}^{2+}$  in 50 mg/l concentrations. The initial pH (pH 1.1) was adjusted to pH 4.5 except during the first experiment (metal precipitation occurred at higher pH's). The effect of time pH modification and glucose pretreatment on metal uptake is compared. The effect of the respective treatments are indicated as Hrs, glucose, pH or a combination of the three.

	Cd ( $\mu\text{g/g}$ )	Cr ( $\mu\text{g/g}$ )	Cu ( $\mu\text{g/g}$ )	Ni ( $\mu\text{g/g}$ )	Zn ( $\mu\text{g/g}$ )
1 x 15 Hrs	25.6	8.7	45.7	22.8	9.0
1 x 15 Hrs, pH	57.4	13.6	76.8	38.4	42.4
1 x 15 Hrs, glucose, pH	60.4	31.0	83.3	45.2	48.0
3 x 5 Hrs, pH	72.8	29.0	87.6	59.8	72.0
3 x 5 Hrs, glucose, pH	76.6	42.0	88.0	59.8	74.0

An apparent uptake series ( $\mu\text{g/g}$ ) of  $\text{Cu}^{2+} > \text{Cd}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+} > \text{Cr}^{3+}$  by the yeast biomass was established. The amount of metal accumulated from the 50 mg/l spiked solution was not indicative of yeast saturation. For example, 74  $\mu\text{g/g}$  of  $\text{Zn}^{2+}$  was accumulated from the spiked solution

compared to the 770  $\mu\text{g/g}$  removed from effluent B under similar conditions. Similarly 88  $\mu\text{g/g}$   $\text{Cu}^{2+}$  was accumulated from the spiked solution (Table 5.3) compared to 55.4  $\mu\text{g/g}$  from effluent C (Table 5.2). These results (Tables 5.2 and 5.3) indicate the dependency of accumulation on the ratio of external free metal ions to the available biomass. The external metal concentration affects both the metal binding equilibrium and the concentration gradient across the cell membranes. The amount of bioaccumulation is subjected to change in response to variations in the external concentrations.

### 5.4. DISCUSSION

#### 5.4.1. EFFECT OF pH AND GLUCOSE TREATMENT ON METAL BIOACCUMULATION

The results obtained from the present study substantiate the reports that a low external pH reduces metal accumulation by viable yeast cells (10, 25, 38, 185, 189). This effect is particularly noticeable regarding the amount of metal accumulated from spiked laboratory solutions (Table 5.3). Adjustment of the pH from pH 1.1 to pH 4.5 resulted in a 124% increase of  $\text{Cd}^{2+}$ -accumulation and an increase of 371% in  $\text{Zn}^{2+}$  accumulation.  $\text{Cu}^{2+}$ ,  $\text{Cr}^{3+}$  and  $\text{Ni}^{2+}$  uptake also increased in the presence of favourable pH conditions. The pH of a solution affects the chemical speciation of metals thereby regulating their biological availability. At pH 4.5 the majority of the metals exist in their ionic form facilitating enhanced accumulation (150). The uptake of divalent cations by *S. cerevisiae* has been found to be significantly reduced below pH 5.0 (145). Optimal pH ranges exist for the uptake of specific metals into microbial cells.  $\text{Ni}^{2+}$  uptake into *S. cerevisiae* increases with increasing medium pH until pH 5.0, and pH 4.0 in *N. crassa* (16, 135), whilst the greatest accumulation of  $\text{Mn}^{2+}$  occurs between pH 5.0 - 7.0 and  $\text{Ca}^{2+}$  until pH 8.0 (16). Maximum bioaccumulation is not only species specific, but also depends on the type of biomass, for example  $\text{Zn}^{2+}$  accumulation by *C. utilis* occurs at pH 4.8, but at pH 6.5 for *N. vasinfecta*.

Pretreatment of yeast cells with glucose resulted in elevated levels of metals being accumulated. Accumulation of  $\text{Zn}^{2+}$  from effluent A is increased by 2.5 times and that of  $\text{Cd}^{2+}$  by 1.6 times by pretreating the cells with glucose (Fig 5.4). These and other results confirm the energy dependent nature of divalent metal cation uptake into yeast cells, though direct addition of glucose to the suspension failed to result in an increased metal uptake, suggesting the inability of the yeast cells to internalize the glucose. In the presence of high metal ion concentrations, glucose may be unable to enter into the cells due to the utilization or inhibition of the glucose uptake system by metal ions.

In some instances the direct addition of glucose to yeast-metal suspensions has however been able to stimulate metal accumulation, eg.  $\text{Mn}^{2+}$  (38) and  $\text{Ca}^{2+}$  (190). Substitution of glucose by analogues, fails to enhance metal accumulation, eg. in the presence of sorbose no elevated levels of  $\text{Mn}^{2+}$  were accumulated (38).

#### 5.4.2. THE RELATIONSHIP BETWEEN BIOACCUMULATION AND AMBIENT METAL CONCENTRATION IN A MULTI-ION ENVIRONMENT

A relationship exists between intracellular metal ion accumulation and the ambient metal concentration (Table 5.2 and 5.3). For example, in the presence of excessive concentrations of  $\text{Zn}^{2+}$  (effluent B) greater amounts of  $\text{Zn}^{2+}$  were sequestered by the yeast biomass than from effluent A, which had a lower initial concentration of  $\text{Zn}^{2+}$ . Similarly, in the presence of low quantities of metals, eg.  $\text{Cr}^{3+}$ ,  $\text{Ni}^{2+}$  (effluent C) or  $\text{Cd}^{2+}$  (effluent A) less of that metal was accumulated by the yeast cells, suggesting that the amount of metal accumulated is partially determined by the ratio of free external metal concentration to the available cell biomass. This trait has been noted previously in *S. cerevisiae* cells (181), viz. the dependency of  $\text{UO}_2^{2+}$  uptake on ambient uranyl ion concentration. Moreover this type of bioaccumulation is not limited to *S. cerevisiae*, similar uptake patterns have been noted in other microorganisms, eg. the bacteria *S. longwoodensis* (123), *Pseudomonas sp* (191) and *C. vulgaris* (185).

The presence of multiple metal species in solution affects the quantity and pattern of uptake of a specific species of metal ion. The apparent affinity series of  $\text{Cu}^{2+} > \text{Cd}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+} > \text{Cr}^{3+}$  (Table 5.3) verifies the presence of mutual interactions between metal cations. Both bioaccumulation and biosorption (concept expanded upon in chapter 6) can be of a competitive nature. The bioaccumulation of specific metal ions is often inhibited in the presence of a second or third metal species. The inhibition may arise due to one or more metal species complexing with the same surface ligand, or alternatively, utilizing the same uptake mechanism. The interaction of multiple metal species is not necessarily always inhibitory, the uptake of  $\text{Zn}^{2+}$  is increased by  $\text{Cu}^{2+}$ , whereas the uptake of  $\text{Mn}^{2+}$  remains unaffected (16). In the absence of added substrate,  $\text{Co}^{2+}$  enhances  $\text{Zn}^{2+}$  uptake and *visa versa*. Inhibition of metal ion uptake due to the influence of additional metal ions may vary in specificity.  $\text{Ca}^{2+}$  inhibits both  $\text{Zn}^{2+}$  and  $\text{Co}^{2+}$  uptake (25). Uptake of  $\text{Cd}^{2+}$  by *A. nodosum* from a Cd-Cu solution is sensitive to the presence of  $\text{Cu}^{2+}$ . An approximate 20% decrease in  $\text{Cd}^{2+}$  equilibrium uptake occurs due to the presence of  $\text{Cu}^{2+}$  yet the reverse is not true.  $\text{Cu}^{2+}$  and

$Zn^{2+}$  each inhibit the uptake of the other by approximately 80% (110).  $Cd^{2+}$  and  $Zn^{2+}$  also have a similar effect on the uptake of each other. A decrease in excess of 20% is noted in the equilibrium sorption of both  $Cd^{2+}$  and  $Zn^{2+}$  (110). The decrease in  $Cd^{2+}$  accumulation is due to inhibition of its internalization by  $Zn^{2+}$  as previous reports state the absence of effect of  $Zn^{2+}$  on surface binding of  $Cd^{2+}$  ions (185). The extent of inhibition of uptake of a specific metal ion varies, depending on the type of metal present.  $Co^{2+}$  uptake in *S. cerevisiae* is inhibited in the decreasing order by  $Ca^{2+} > Cd^{2+} > Mn^{2+} > Ni^{2+} > Zn^{2+}$ , whilst a  $Cd^{2+}$  uptake inhibition series exists of  $Ca^{2+} > Ni^{2+} > Mg^{2+} > Zn^{2+} > Mn^{2+} > Ca^{2+}$  (4).

### 5.5. CONCLUSIONS

Viable yeast biomass has proven to be capable of accumulating heavy metal cations from aqueous solutions. This ability has many potential applications regarding the removal of metals from either simulated or metal containing waste water solutions. The mechanisms of accumulation and quantities of metal taken up are affected by the ratio of ambient metal concentration to biomass quantity, external pH and the presence of multiple elements in solution. Due to the energy dependent nature of bioaccumulation, the amount of metal sequestered by glucose-pretreated cells far exceeds that taken up by cells in the absence of an energy source. Direct addition of glucose to the biomass-metal environment, does not appear to have any effect on metal accumulation.

Due to the energy-dependent nature of bioaccumulation, the successful implementation of such a system in an industrial bioremediation process when compared to a non-viable biomass system, would be limited. As an alternative, the substitution of viable cells by non-viable cells may enhance the potential of such a process on a large scale and will consequently be dealt with in subsequent chapters (6 and 7).

## 6. BIOSORPTION OF HEAVY METAL CATIONS BY IMMOBILIZED NON-VIABLE YEAST BIOMASS

### 6.1. INTRODUCTION

Recent waste water bioremediation techniques for heavy metal removal have focussed on biosorptive mechanisms rather than bioaccumulation by microbial cells for the removal of metal cations from solution. Biosorption, is a property of non-viable biomass to retain and/or concentrate metal ions from solutions via adsorption or a non-active absorption processes (138), and acts as a potential bioremediation tool which can be implemented using several types of microbial biomass (see chapter 1).

The advantages of non-viable microbial cells are numerous. Inactive biomass has the advantage of being independent of a supply of nutrients for cell growth and maintenance, nor does it involve any time loss due to culture propagation or contamination (97, 139). Moreover, all living cells are prone to the toxic effects of effluents, and in fact the concentration of metals in non-viable biomass often exceeds that of viable biomass due to the inactivation of the resistance mechanisms of the viable yeast cells (97,192). Killed cells can also be stored or used for extended periods of time at room temperature without the onset of putrefication (139).

The chemical composition of microbial cells, and particularly the cell wall or envelope largely determines the biosorbent property of non-viable microbial cells (107, 192). The cell walls and envelopes of microbial biomass are mainly composed of polysaccharides, proteins and lipids and offer abundant ligands for metal interaction, eg. carboxylate, hydroxyl, sulphhydryl, phosphate and amino groups, which differ in their affinity and specificity for metal ions (185). Surface biosorption, a physico-chemical phenomenon based on ion-exchange, coordination, complexation, chelation, adsorption and microprecipitation, occurs relatively rapidly and can be reversible (185, 192). In addition to the external physico-chemical factors, eg. pH and temperature, the solution chemistry of the metal largely influences biosorption (192). The equilibrium amount of metal bound onto the cell surface would therefore be determined by the relative affinities of the binding sites for the toxic metals and other cations present as well as the residual concentrations of these metals remaining in solution (185).

The surfaces of yeast cells have been associated with ion exchange and complexation reactions as well as the formation of stable complexes (192). Strandberg (193) postulated that the polyphosphate and carboxyl groups on the cell surface of *S. cerevisiae* are active in the complexation of uranium. The phosphoryl groups appear to form stable complexes with uranium, whilst the carboxyl groups only become involved at the onset of saturation of the phosphoryl groups. Non-viable strains of bakers and brewers yeast have also been shown to remove greater amounts of cadmium and zinc from solution than live cultures (180, 182). Similarly *S. cerevisiae* has demonstrated a preference to accumulate cobalt and lead via biosorptive mechanisms in preference to bioaccumulative pathways.

Biosorption is not limited to a specific microbial species, but extends to a wide range of biomass types, eg. yeasts, fungi, algae, bacteria and moss (see chapter 1). Volesky and Holan (192) reviewed the metal accumulating abilities of all documented biomass types. The biosorptive capacities vary depending on the microbial strain and metal species present. Some biosorbents are involved in the adsorption of a range of metals with no specific binding priority whilst others are specific for selected metals (138, 185, 192, 194). Conclusive evidence proved that whilst bacterial and fungal biomass types prove to be adequate biosorbents, marine algae (111, 138) and sphagnum peat moss (138, 143, 144, 195) are superior metal chelators. The utilization of biomass species as bioremediation tools does not depend solely on their metal sorption capacity, since the availability of the biomass and cost factors are also important criteria regarding their implementation. Whilst non-viable biomass of the yeast *S. cerevisiae* may only exhibit a moderate level of biosorbent activity when compared to other microbial species (138), it remains the most widely used yeast and waste product in the fermentation industry in South Africa and subsequently is a freely available source of biomass suited as a bioremediation tool.

The cost effective nature of bioremediation remains its primary drawcard (192). Conventional methods for removing metals from industrial waste solutions, eg. chemical precipitation, chemical oxidation or reduction, filtration, electrochemical treatment, membrane technology and evaporation recovery may be ineffective or extremely expensive especially when treating high-volume, low-concentration effluent. The use of ion exchange resin for purification of waste waters has also been excluded due to the high cost of the materials required. In comparison non-viable biosorbents can easily be used for remediation of waste water and particularly in cases where combined purification or recovery are required. The importance of biomass in metal recovery has been reviewed in the light, that not only are the metal uptake capacities high, but selective metal recovery can also occur

(192). The successful industrial application of this method does however require a high capacity biosorbent, with suitable physical properties, and a thorough knowledge of the system under operating conditions (196).

Some methods of killing cells may improve the biosorptive properties of the biomass. For example, *S. cerevisiae* cells when killed by heat drying and grinding increase their ability to accumulate a range of metals (139). Heat-killed *Chlorella* cells also accumulate greater amounts of metals than live *Chlorella* cells (108). Alternative mechanisms of producing non-viable biomass include chemical modification, and in some instances immobilization. Immersion in formaldehyde both kills and cross-links cells, thereby immobilizing them. Non-viable *R. arrhizus* biomass, killed by 1% formaldehyde solutions has proven to be extremely durable and is therefore able to endure greater chemical and physical stress conditions than living biomass (192).

To facilitate the separation of biomass from solution and to improve biomass performance and its physical characteristics, non-viable biomass is commonly immobilized either in a granular or polymeric matrix (144). Many methods of immobilizing cells have been developed. Immobilized non-viable biomass killed and cross-linked using extreme chemical and physical conditions may possess very different metal accumulating parameters compared to the original living biomass (188). For bioremediation purposes the microbial biomass need to be immobilized in a particulate form that preserves its biosorptive properties (197). Immobilization can also improve performance in column reactors and enhance reusability (192), eg. immobilized *Candida tropicalis* cells used in the degradation of phenol, exhibited a half-life between 20 - 40 days, whereas free cells could only be used for 20 hrs (cited by 197).

Evaluation of immobilized biosorbents depends on a number of criteria (192). Both the metal uptake and desorption properties are important, though ultimately the maximum metal loading capacity remains one of the most important features characterizing its performance, especially at high metal concentrations. In addition, the rate of metal uptake and release, selectivity of biosorption and desorption, mechanical and physical properties, efficiency of implementation and operation and economic feasibility of production and implementation of the biomass remain important selection criteria (192).

Immobilization of biomass with the intent to use it for the treatment of industrial waste water has been investigated by a number of researchers (cited by 198). Immobilizing polymers tested have included agar, agarose,  $\kappa$  - carrageenan, collagen, polyacrylamide, polyurethane and cellulose, though not all of these polymers have proven to be suitable for industrial purposes, often due to poor mechanical strength and durability, toxicity or high production costs (198).

Two immobilizing polymers, viz. polyvinylalcohol (PVA) and polyethylenimine (PEI) appear to possess the required criteria for this type of work. PVA, is a non-toxic polymer, which can be produced cheaply on industrial scale as durable beads, whilst the embedding and cross-linking procedure involving PEI is a simple process, also producing durable beads.

In the present study the production of three types of immobilized non-viable yeast biomass was undertaken and their metal accumulating capacity determined. Two immobilized systems employing PVA, viz. PVA Na-alginate and PVA Na-orthophosphate beads were compared to alkali-treated PEI:glutaraldehyde (GA) pellets with respect to their mechanical properties and metal adsorption properties. Bearing in mind that the ultimate purpose of the development of these biomass types was for their utilization in the bioremediation of industrial waste water, the metal affinities of the respective biomass types were determined. A Cu-solution was used as a standard for this work. Additional experiments using the selected biomass type focussed on adsorption isotherms using batch reactions, and the optimization of fixed bed reactor columns, with respect to pH and breakthrough volumes using single and multi- element solutions. The multi-element solutions contained Cu, Cd, Cr, Ni and Zn.

## 6.2. METHODS AND MATERIALS

### 6.2.1. BIOMASS PREPARATION

#### 6.2.1.1. POLYVINYLALCOHOL (PVA) Na-ORTHOPHOSPHATE IMMOBILIZATION

The standard washing procedure was applied to commercial *S. cerevisiae* cells. 70 g (wet weight) of the washed yeast was suspended in Milli-Q water to obtain a 51% (w/v) solution.

The immobilization procedure followed was an adapted version of the method outlined by Chen *et al.* (199). A 20% (w/v) aqueous solution of PVA (Av MW 70 000 - 100 000, Sigma Chemical Co) was prepared by adding 33 g of PVA to 165 ml of Milli-Q water. The solution was heated to 70°C to enable the PVA to dissolve and was subsequently cooled down to 55°C prior to being mixed with the yeast suspension. The final suspension contained 23% (w/v) cells (wet wt) and 10.8% (w/v) PVA.

The immobilized yeast was further cooled down to 45°C and the PVA-cell suspension extruded via a peristaltic pump through a needle (Terumo needle: gauge 1.2 mm x 38 mm) into 2 l of saturated boric acid (Sigma Chemical Co) and stirred for 2 hrs. Spherical bead formation (3 mm diameter) resulted from cross-linking the PVA with the saturated boric acid.

To initiate the gelling and hardening, the beads were transferred from the boric acid solution, washed using Milli-Q water and placed in 2 l of 1.0 M Na-dihydrogen orthophosphate for 2 hrs. On removal from the phosphate solution the PVA beads were washed using Milli-Q water and freeze-dried for 36 hrs.

#### 6.2.1.2. PVA Na-ALGINATE IMMOBILIZATION

Following the method of Shindo and Kakimura (200), washed yeast cells were immobilized as hollow PVA beads.

Using 21 g (wet wt) a 10% (w/v) suspension of washed yeast in Milli-Q water was prepared. 13.2 g of PVA was suspended in 165 ml of Milli-Q water. To this 3.3 g of alginic acid (low viscosity, Na-salt, Sigma Chemical Co) was added with stirring.

The PVA Na-alginate suspension was heated with stirring and maintained at 95°C for 20 min to dissolve the components. On cooling (45°C), the suspension was added to the yeast and mixed thoroughly. Bead formation was attained by dropping the PVA-yeast solution into 2 l of 2% (w/v) CaCl<sub>2</sub>·2H<sub>2</sub>O through a thin needle (Terumo needle: gauge 1.2 mm x 38 mm). Spherical beads with an approximate diameter of 3 mm were formed. These were gently stirred for 2 hrs, the CaCl<sub>2</sub> drained and the beads rinsed with Milli-Q water. Lyophilization for 24 hrs yielded dried hollow PVA beads.

### 6.2.1.3. POLYETHYLENIMINE (PEI) AND GLUTARALDEHYDE (GA) IMMOBILIZATION

#### 6.2.1.3.1. IMMOBILIZATION

200 g (wet wt) of washed yeast and 20 ml Milli-Q water were blended to a smooth paste (Waring commercial blender) before being treated with embedding and cross-linking agents. 25% (v/v) GA (50% aqueous glutaraldehyde, Sigma Chemical Co) was added to the biomass-water paste and blended until smooth. 33% (v/v) PEI (50% aqueous PEI, Aldrich Chemical Co) was added and mixing initiated until a moist dough was obtained. Similar to the method used by Brierley and Brierley (140), the ratios of PEI:GA:yeast were varied to obtain a stable complex. Initial ratios of PEI:GA:yeast (ml:ml:g) of 1.2:1:40, 1.2:1:50 and 1.2:1:65 were tested. The moist dough was crumbled to form granules which were oven-dried for 12 hrs at 80°C.

#### 6.2.1.3.2. HOT ALKALI TREATMENT

Dried, immobilized yeast biomass pellets were suspended in 3% (w/v) KOH solutions (0.1 g/ml) and heated to 70°C. The granulated material was separated from the caustic solution by settling, the latter retained whilst the pellets of granulated material were washed thoroughly with Milli-Q water. The washing procedure was repeated twice and the rinsing water added to the caustic solution.

Reconstitution of the alkali-soluble components was achieved through acidification. 32% HCl (AECI Ltd) was slowly added with stirring until pH 6.0. The decrease in pH resulted in the reconstitution and precipitation of the alkali-soluble fractions. Subsequent centrifugation (5000 g x 10 min) pelleted out the reconstituted biomass.

The washed, reconstituted biomass was added to the alkali-insoluble fraction. Both fractions were heat dried (80°C) overnight.

### 6.2.2. DETERMINATION OF STRUCTURAL PROPERTIES

Scanning electron microscopy (JOEL JSM 180 SEM) of gold coated biomass samples provided information regarding the porosity of the pellets and the surface area available for metal binding.

Subjective stability tests were performed on the PVA and PEI:GA biomass types ( untreated and 3% KOH treated 1.2:1:40, 1.2:1:50 and 1.2:1:65 PEI:GA biomass preparations were tested). Dried biomass granules were soaked overnight in Milli-Q water at room temperature (20°C). The moist biomass granules were handled manually to determine their physical stability. Poor stability was reflected by disintegration of the pellets, whilst the stable preparations retained their integrity under handling pressure.

### 6.2.3. DETERMINATION OF THE BIOMASS BIOSORPTIVE PROPERTIES

#### 6.2.3.1. METAL ACCUMULATION IN PACKED-BED COLUMNS

5 g (dry wt) of PVA Na-alginate and PVA Na-orthophosphate pellets were rehydrated (2% (w/v) CaCl<sub>2</sub> solution and Milli-Q water respectively) and rinsed prior to being packed into columns. LKB glass chromatography columns (internal diameter 16 mm) were used for this purpose. Both packed columns measured column volumes of 40 ml. 10 g (dry wt) of 3% KOH treated 1.2:1:40 PEI:GA biomass was soaked for 1 hr in Milli-Q water followed by two water rinses. The wet biomass was packed into similar glass columns as a slurry. Excess water was drained and the biomass evenly compacted (column volume: 40 ml). All columns were packed in duplicate.

To determine the metal biosorptive capacity of the pellets, 300 ml of 100 mg/l Cu-solution (pH 1.4 and pH 5.5 respectively) was pumped through the relevant columns. The influent Cu-solution was pumped upwards (to prevent compacting of the column) through the column at a flow rate of 55 ml/hr. 10 ml aliquots of the column were collected (LKB 2112 fraction collector) and analysed with respect to pH and Cu<sup>2+</sup> accumulation (1275 Varian Atomic Absorption Spectrophotometer).

#### 6.2.3.2. BIOMASS REGENERATION AND METAL REACCUMULATION

One column volume (40 ml) of Milli-Q water was flushed through the columns exposed to the pH 1.4 Cu-solution. Desorption of the bound metal was achieved by pumping 2 column volumes of 1 mM EDTA upwards through the column (flow rate 55 ml/hr). A final rinse of 2 column volumes of Milli-Q water completed the desorption procedure.

On removal from the columns the biomass types were rinsed in Milli-Q water. The PVA-biomass

preparations were lyophilized (section 6.2.1.1. and 6.2.1.2.), whilst the PEI:GA biomass was dried overnight, reconditioned with an alkali solution (section 6.2.1.3.2.) and oven-dried. Any change in biomass weight due to desorption and reconditioning procedure was ascertained by comparing the dry weight of the regenerated pellets to their initial dry weight.

The rehydration, packing and metal accumulation procedures outlined in section 6.2.3.1. were repeated for each of the biomass columns and the Cu removal determined using the standard atomic absorption procedure.

#### 6.2.4. METAL BIOSORPTION TESTS

The effect of metal ion concentration on the PEI:GA biomass biosorption performance was evaluated by contacting the biomass pellets with respective  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Ni}^{2+}$  and  $\text{Zn}^{2+}$  metal solutions of increasing concentrations (0.5, 1.0, 5.0, 10.0, 25.0, 50.0 and 100.0 mg/l solutions).

4 g (wet wt) of hydrated biomass was placed in acid washed glass flasks and suspended in 25 ml of the relevant concentration of metal solution and the pH adjusted to pH 5.5 - 5.6. After gentle shaking for 2 hrs at room temperature the biomass was separated from the metal solution. Metal biosorption was determined indirectly by measuring the concentration of the metal remaining in the solution. To minimize any error due to adsorption of the metal to the flask walls, the experimental criteria of the control flasks remained identical to those of the test flasks. The control flask contained either no biomass (*ie.* only the relevant concentration of the metal solution adjusted to the correct pH), or alternatively aliquots of yeast suspended in ultra-pure Milli-Q water.

#### 6.2.5. FIXED-BED BIOSORPTION USING PEI:GA BIOMASS

##### 6.2.5.1. BREAKTHROUGH DETERMINATION OF INDIVIDUAL CATION SPECIES

PEI:GA biomass fractions (10 g dry wt) were rehydrated and packed into columns as for section 6.2.3.1. Stock solutions (1000 mg/l) of  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Ni}^{2+}$  and  $\text{Zn}^{2+}$  (SARchem) were diluted to 100 mg/l. Using 3% KOH the pH of each of these solutions was adjusted to pH 5.5. 1.5 l of the respective metal cation solutions were pumped upwards through separate columns (column volume: 40 ml, column height: 20 cm). A flow rate of 55 ml/hr was maintained throughout the duration of

the experiment.

Aliquots (10 ml) of the eluants were collected and quantitatively analysed using a GBC 909 Atomic Absorption Spectrophotometer to determine metal accumulation and breakthrough. pH profiles for each of the column eluents were established.

#### **6.2.5.2. MULTI-ELEMENT COMPETITION AND EFFECT OF SOLUTION pH ON BREAKTHROUGH DETERMINATION**

Due to differences in the atomic weights of the metal ion species, competition and affinity studies were conducted using equimolar metal solutions, containing the 5 metal species. In an attempt to remain within the same concentration range of the individual metal cation solutions (except for Cd) the eventual concentration of the metal cations in the equimolar solutions were established as 1.5 mM (mg/l concentrations for the respective metals of 77.99 mg/l Cr; 88.05 mg/l Ni; 95.32 mg/l Cu; 98.07 mg/l Zn and 168.62 mg/l Cd ).

To determine the effect of pH, three equimolar solutions of pH 1.5, 2.5 and 4.5 (at pH 5.5 the metals precipitated out of solution) were prepared. Adjustments to the pH were made using 3% KOH. The respective multi-cation solution (0.5 l) was pumped upwards through the column at a flow rate of 55 ml/hr. The experiment was conducted in triplicate, exposing fresh biomass to different pH solutions. Sample collection and analysis proceeded as outlined in section 6.2.5.1.

### **6.3. RESULTS**

#### **6.3.1. IMMOBILIZED AND STRUCTURAL PROPERTIES OF THE BIOMASS**

Multiple criteria established the basis for successful immobilization. Though % yield of the final product remains important, evaluation is also gauged by the simplicity of the procedure. The % yield (dry wt of end product expressed as a % of the initial wet wt) of the PVA immobilized biomass fractions, viz 54.2% for PVA Na-alginate and 38.6% for PVA Na-orthophosphate, compared favourably to that of the alkali PEI:GA biomass (15 - 17%). The low % yield of PEI:GA biomass can be ascribed to the alkaline treatment of the immobilized biomass. Hot alkali solubilizes the mannan and some of the  $\beta$  1-6 glucan fractions of the cell wall, although some of the material may

be reclaimed using acid. Alkali treatment was limited to immobilized biomass. Brierley *et al.* (140), reported that the alkaline treatment of native yeast biomass resulted in the unacceptable loss of biomass. Embedding and cross-linking enhanced the stability of the product, thereby limiting biomass loss. Initial PEI:GA immobilization resulted in a 29% yield which mirrors the dry wt : wet wt ratio for yeast suggesting no initial loss of biomass during immobilization.

The simplicity of the cross-linking and embedding immobilization of the PEI:GA biomass exceeded that of the PVA bead formation, with respect to both the number of solutions and time required for the immobilization procedure. Additional drawbacks related to the PVA beads included limited quantities of yeast immobilized per batch. Attempts to increase the batch size resulted in numerous complications. In comparison, the batch size of PEI:GA biomass could be increased successfully, whilst still maintaining the integrity of the pellets.

Electron micrographs (Fig 6.1 - 6.6) of the three biomass types reveal enlarged surface areas available for metal-biomass interactions. Noticeably porous were the two PVA based bead types (Fig 6.1 - 6.4), whilst PEI:GA pellets appeared more compact and dense (Fig 6.5 and 6.6). Although similar in shape, colour and structure to the naked eye, micrographs revealed differences in the microstructure of the PVA beads. PVA Na-alginate beads exhibited a lattice structure (Fig 6.1 and 6.2) whilst PVA Na-orthophosphate beads were more sponge-like in appearance (Fig 6.3 and 6.4). Although porous, both bead types possessed sufficient integrity to withstand the elevated pressures of column conditions. Attrition tests (grinding and manual handling) appeared not to affect either their physical properties or metal accumulating capacities.

At low magnification (Fig 6.5) PEI:GA immobilized yeast cells appeared as solid compact pellets with limited available access to the interior of the pellet. The rough, undulating nature of the pellet surface increased the external binding area. Under high magnification (Fig 6.6) pores in the exterior of the pellet become apparent. Although the latter would increase the accessibility of the pellet interior to metal cations, the general structure of the pellet suggested the majority of metal cation interaction to occur between surface ligands. In order to attain maximum metal biosorption, the ratio of surface : metal solution should be kept high. This necessitates the production of the smallest possible pellets without compromising on the physical integrity of the granules. Pellets containing 1.2:1:40 (ml:ml:g) PEI:GA yeast exhibited the highest level of stability, whilst an increase in the amount of yeast added to the preparation (1.2:1:50 and 1.2:1:65 ratios) resulted in the formation of unstable pellets prone

to disintegration and flaking on application of pressure.

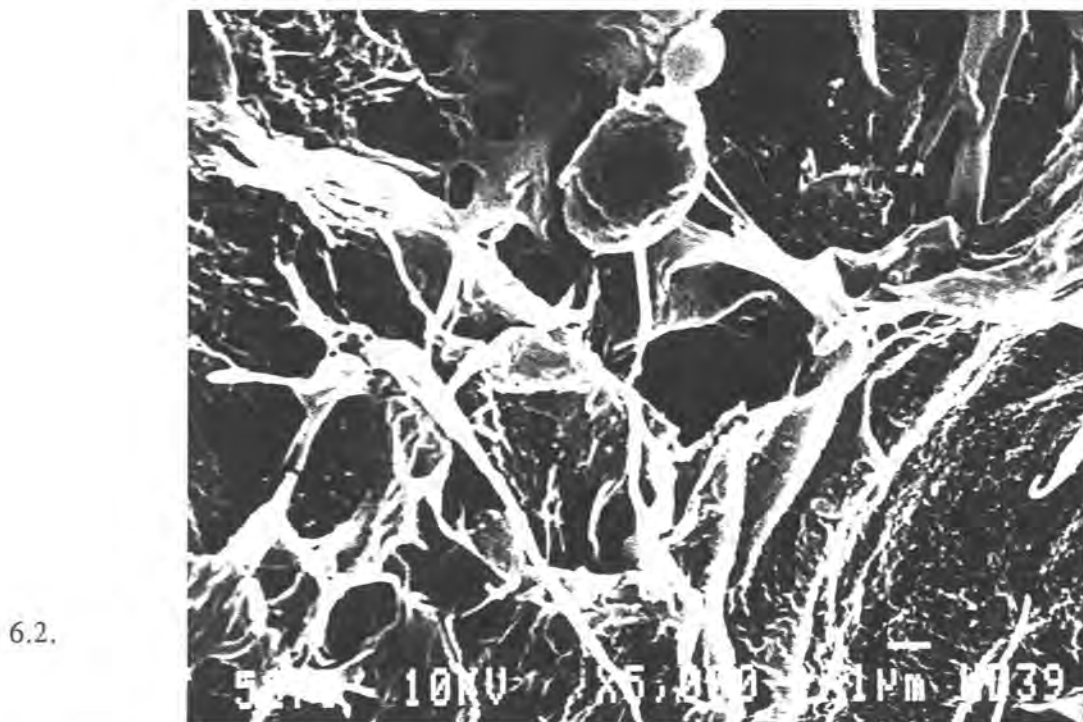
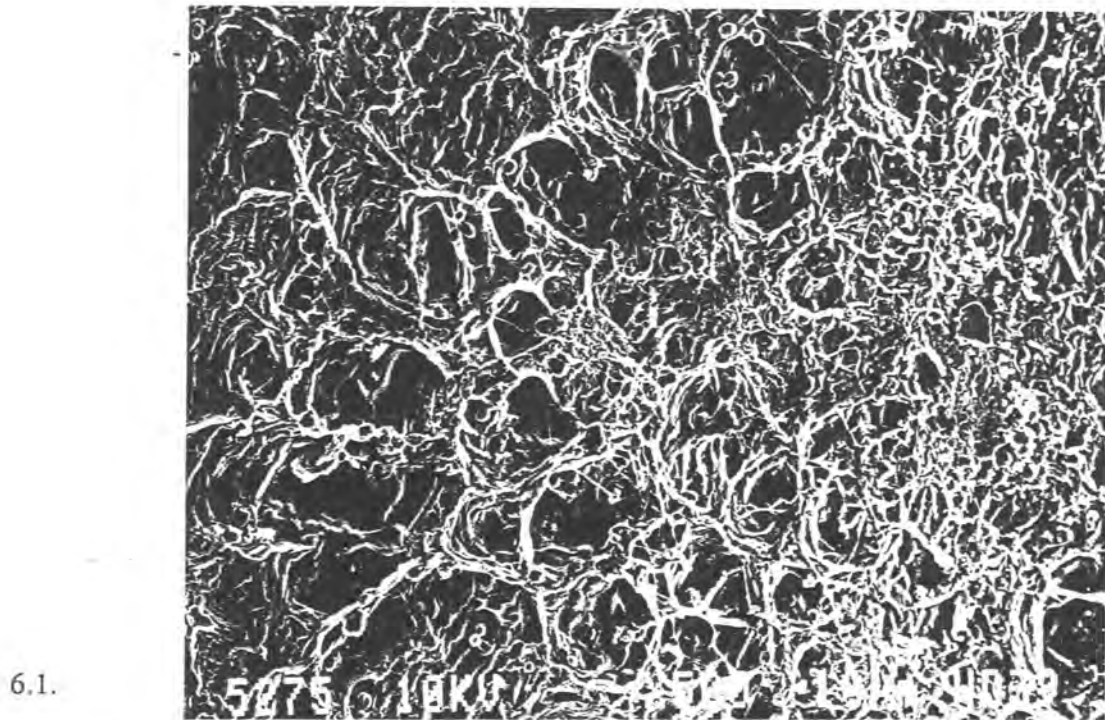
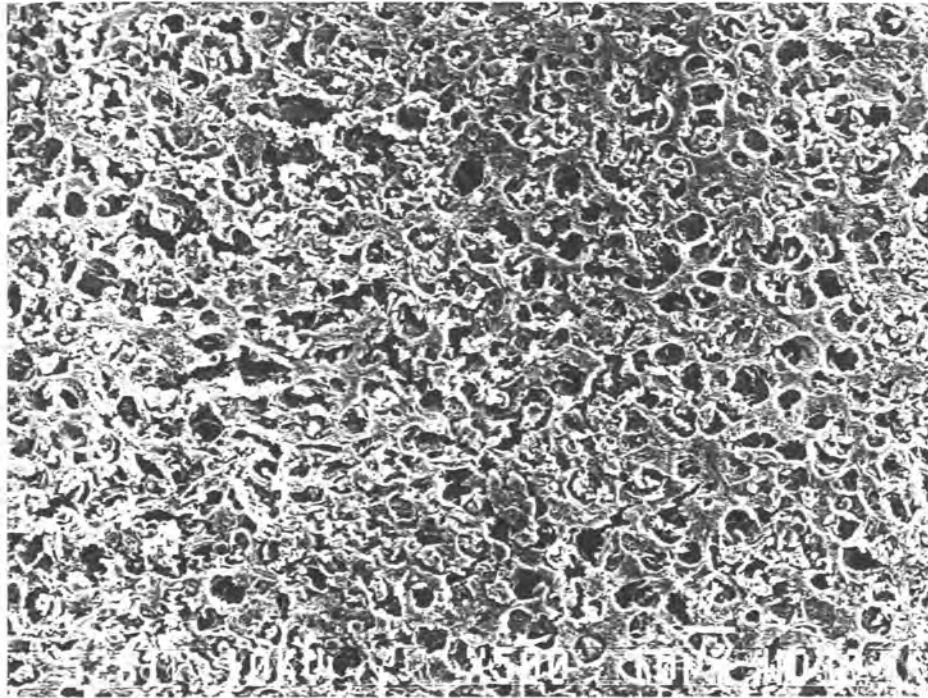


Figure 6.1 - 6.2. Scanning electron micrographs of the surface of yeast containing PVA Na-alginate pellets at 6.1) 500x and 6.2) 5 000x magnification. Note the lattice structure of the pellet due to cross-linking.

6.3.



6.4.

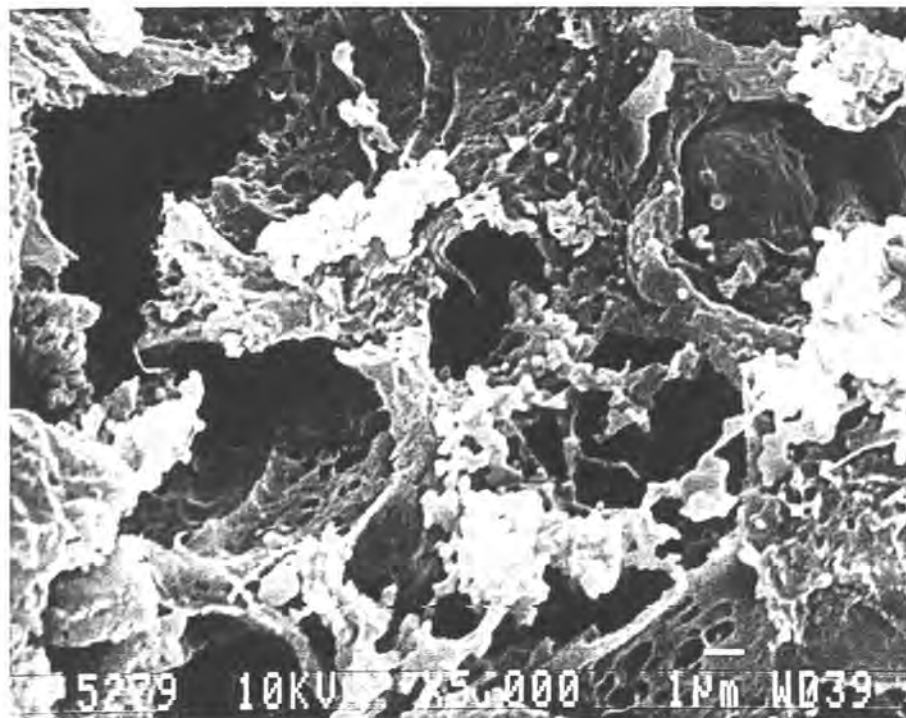
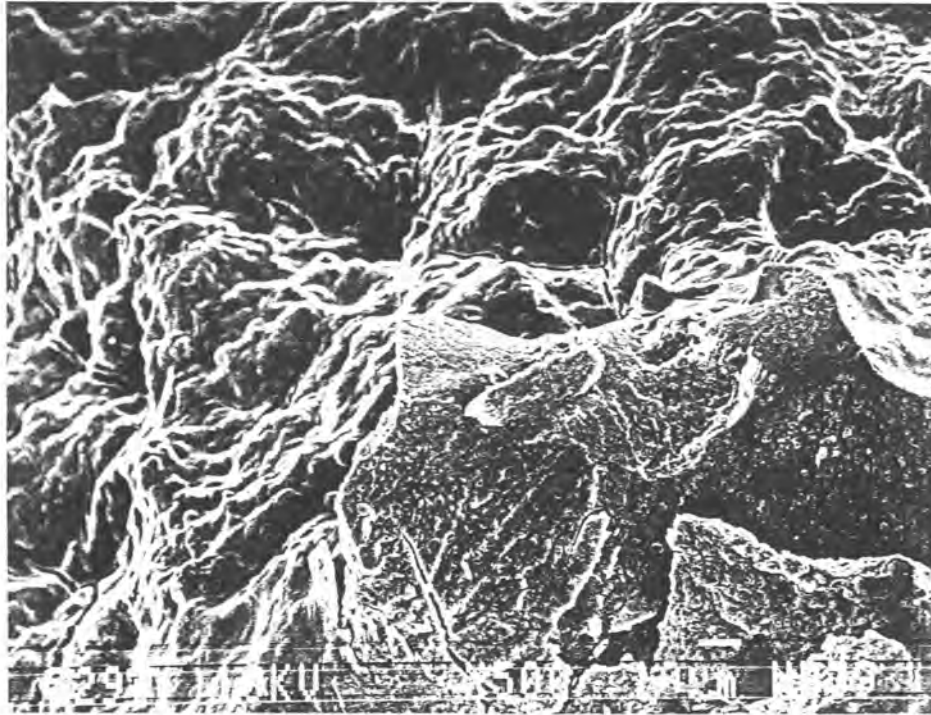


Figure 6.3 - 6.4. Scanning electron micrographs of the surface of yeast containing PVA Na-orthophosphate pellets at 6.3) 500x and 6.4) 5 000x magnification. The external appearance is sponge-like.

6.5.



6.6.

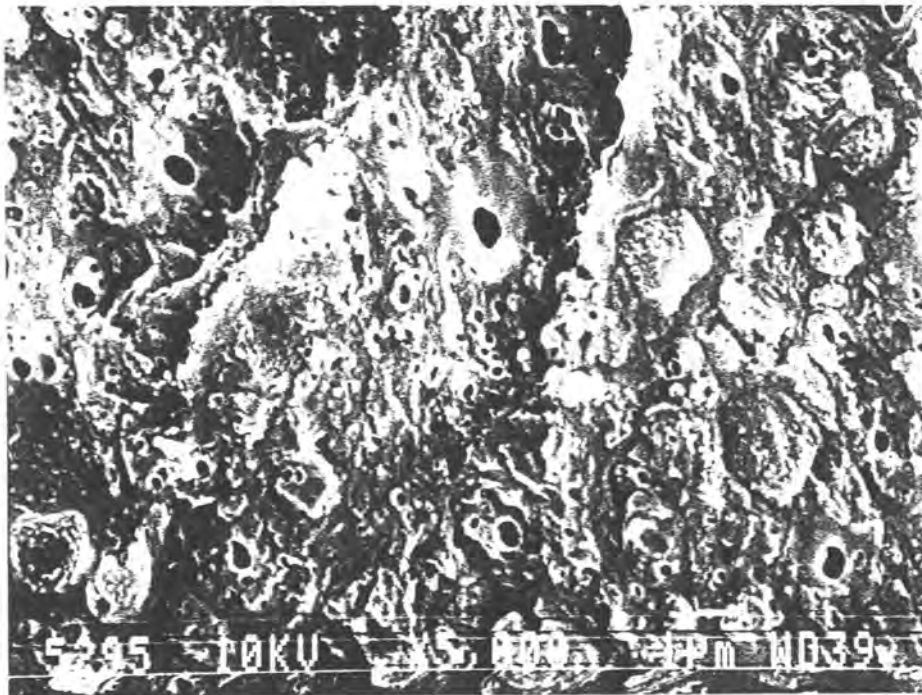


Fig 6.5 - 6.6. Scanning electron micrographs of KOH treated PEI:GA biomass pellets at 6.5) 500x and 6.6) 5 000x magnification. Note the initial compact appearance of the granules, though pores do become apparent at higher magnifications.

## 6.3.2. METAL ACCUMULATION

The biosorbent capacities of the three types of biomass pellets can be compared in Fig 6.7 - 6.9. The polymers form a very small portion of the immobilized biomass. Subsequently the contribution thereof to biosorption can be regarded as minimal. Immediately apparent is the effect of the influent pH on metal accumulation and its resultant effect on the onset of the breakthrough volumes (80% metal accumulation) of the columns, most noticeably so for KOH PEI:GA (Fig 6.7) and PVA Na-alginate (Fig 6.8) pellets. The pH profile of the PEI:GA column was initially influenced by the residual alkalinity of the PEI:GA biomass. An influent acidic solution (pH 1.4) results in low breakthrough volumes for all three biomass types, viz. 10 ml for PVA Na-alginate and Na-orthophosphate and 100 ml for PEI:GA pellets respectively. An increase in the pH of the influent solution viz. pH 1.4 to pH 2.5 had a dramatic effect on the breakthrough volume of the PEI:GA biomass though the onset of saturation still elicited an almost immediate decline in biosorption by the column biomass.

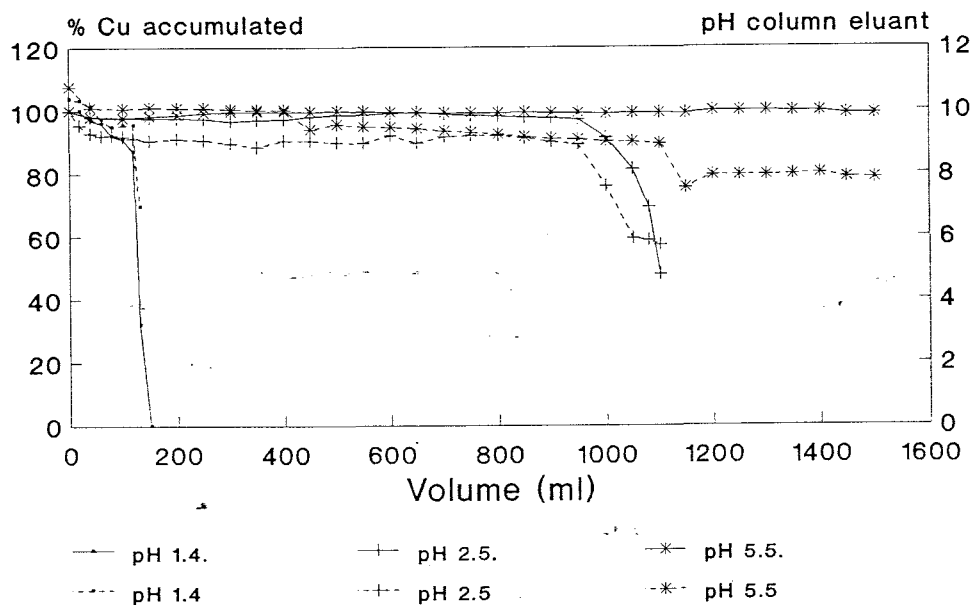


Figure 6.7. The effect of pH of the influent solution on Cu-accumulation and breakthrough volume of KOH treated PEI:GA biomass within a fixed-bed reactor. The corresponding dotted lines represent the pH profile of the column at that influent pH.

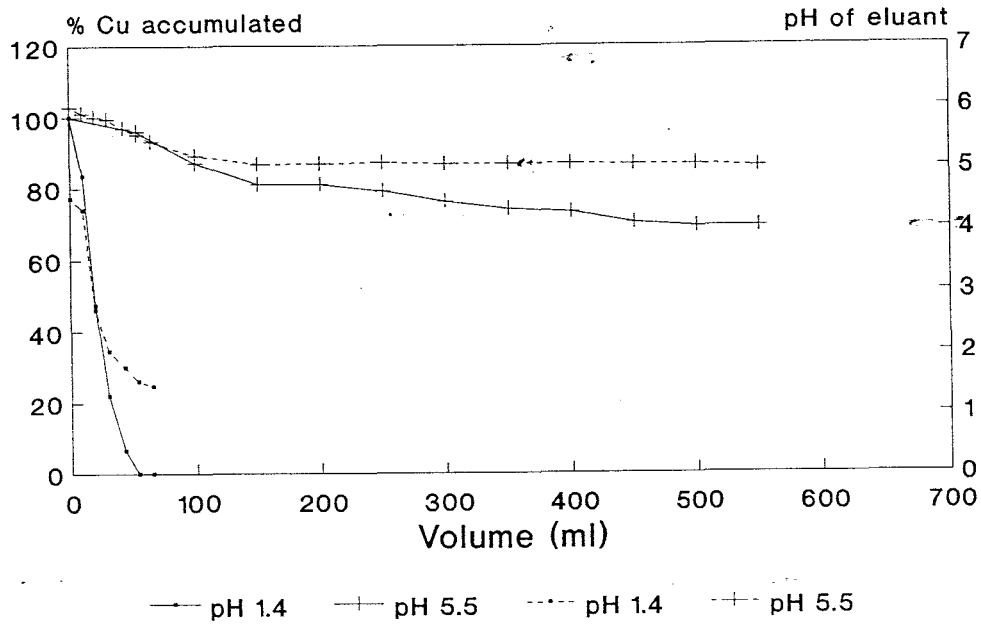


Fig 6.8. The effect of pH of the influent metal solution on Cu-accumulation and breakthrough volume of PVA Na-alginate pellets in a fixed-bed reactor. The corresponding dotted lines represent the pH profile of the column at that influent pH.

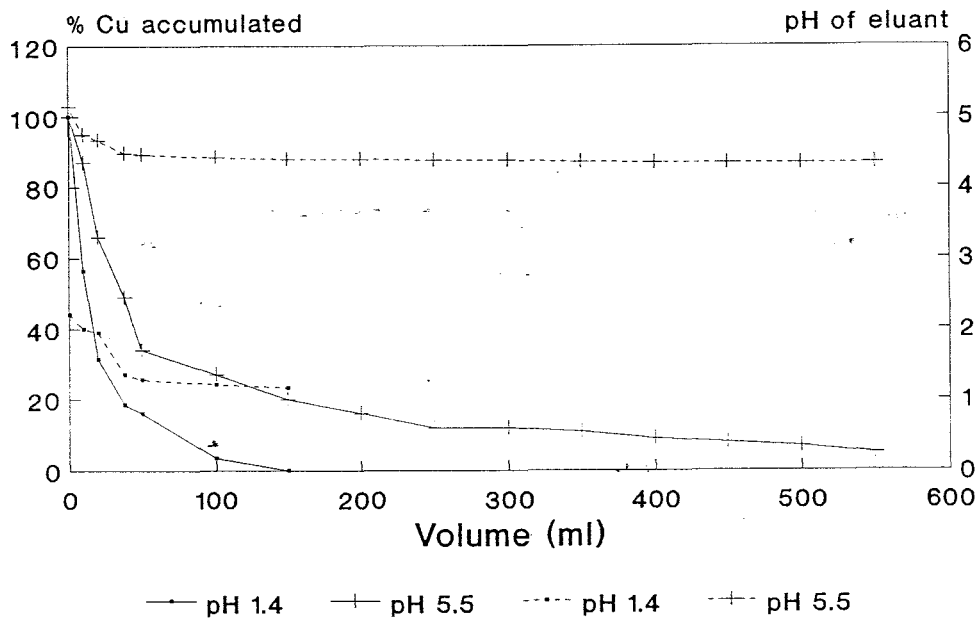


Fig 6.9. The effect of pH of the influent metal solution on Cu-accumulation and breakthrough volume of PVA Na-orthophosphate pellets in a fixed bed reactor. The corresponding dotted lines represents the pH profile of the column at that influent pH.

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Cu biosorption appeared to favour an influent pH of pH 5.5. At higher pH values precipitation of the solution occurred within the columns. After processing 1.5 l of the influent Cu-solution (pH 5.5), breakthrough had not yet occurred in the PEI:GA column. A gradual decline in the amount of  $\text{Cu}^{2+}$  accumulated by the PVA Na-alginate pellets at this pH occurred over time with the onset of saturation. In contrast, PVA Na-orthophosphate pellets accumulated only slightly more  $\text{Cu}^{2+}$  from the alkaline solution than from the acidic one (Fig 6.9).

The breakthrough volumes of the respective biomass types in the columns were determined by the pH of the column. This phenomenon occurred at the interchange between alkali or neutral pH to mildly acidic conditions within the column. For example, breakthrough (1014 ml) of the PEI:GA column (influent pH 2.5) occurred as a result in the decline of pH from pH 7.6 to pH 6.15. The column eluant of the two PVA columns remained acidic (pH 5.90 and pH 5.14 respectively) despite an influent pH of 5.5, which explains the low metal biosorption in these columns.

Biomass saturation and subsequent breakthrough of native and regenerated biomass columns (Fig 6.10 - 6.12) mirrored pH activity. Accumulation and reaccumulation of metals for each of the respective biomass types exhibited similar trends, though regenerated PVA beads appeared to be slightly better metal accumulators than the native pellets (Fig 6.11 and 6.12). Although the onset of Cu-saturation (80% metal accumulation) of PVA beads occurred at low influent volumes, the metal bound to the pellet was not eluted during breakthrough. In comparison, Cu-saturation of the PEI:GA biomass (Fig 6.10) resulted in the release of all complexed  $\text{Cu}^{2+}$  (represented by the negative accumulation values). The majority of the metal was released as a result of biomass saturation, though any residual metal was eluted by the remaining influent Cu-solution. The pH-induced breakthrough affected the binding capacity of the PEI:GA pellets. Subsequent to breakthrough, no  $\text{Cu}^{2+}$  was accumulated.

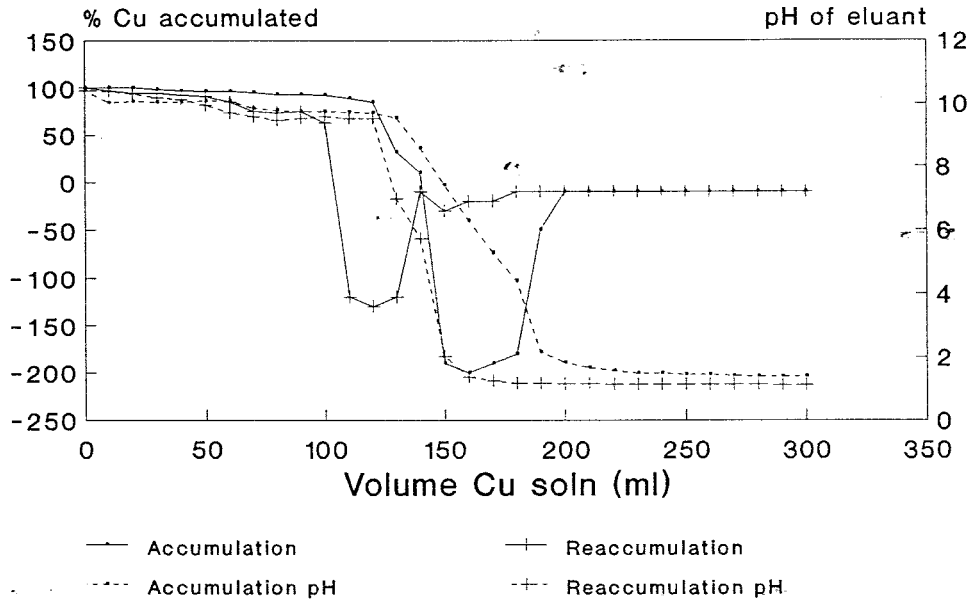


Fig 6.10. A comparison between  $\text{Cu}^{2+}$  biosorption by fresh and regenerated KOH treated PEI:GA biomass pellets. The influent metal solution was at pH 1.4, whilst the dotted lines represent the respective pH profiles.

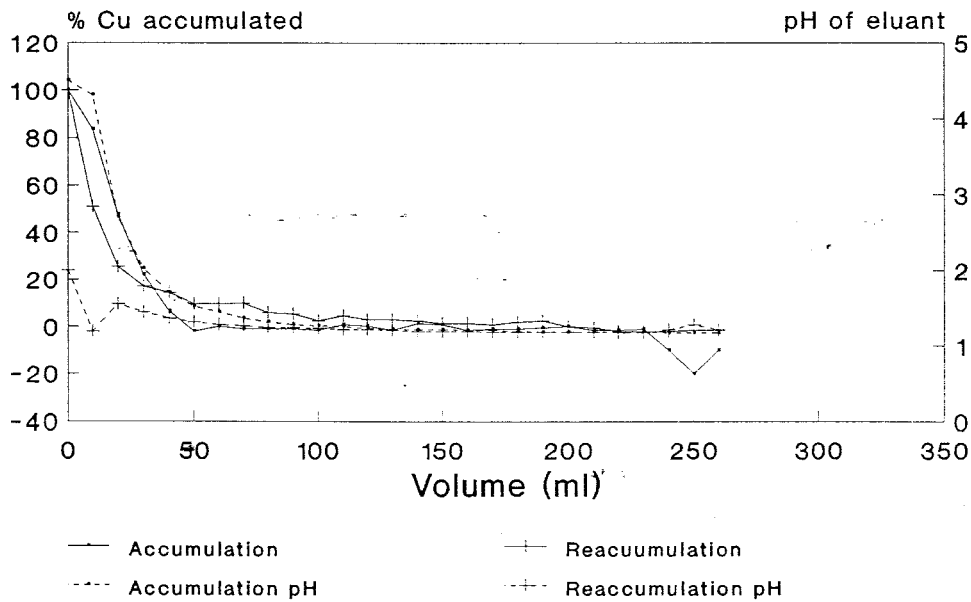


Fig 6.11. A comparison between  $\text{Cu}^{2+}$  biosorption by fresh and regenerated PVA Na-alginate pellets. The influent solution was maintained at pH 1.4, whilst the dotted lines represent the respective column pH profiles.

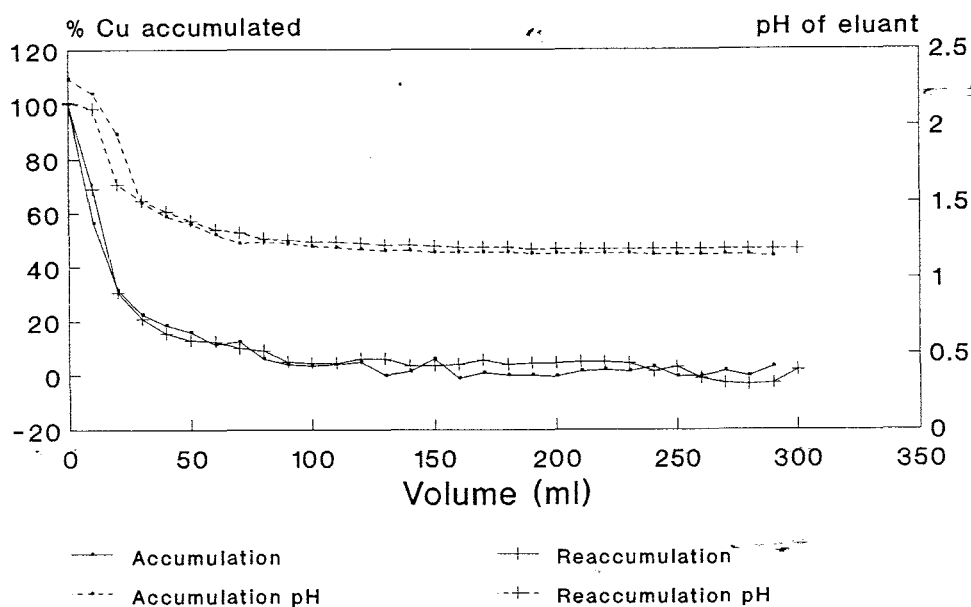


Fig 6.12. A comparison between  $\text{Cu}^{2+}$  biosorption by fresh and regenerated PVA Na-orthophosphate. The influent metal solution was maintained at pH 1.4, whilst the dotted lines represent the pH profiles of the respective columns.

### 6.3.3. COLUMN BIOSORPTION

Fixed column reactors behave in accordance to the theoretical plate theory, and due to the multiple equilibrium plates are surmised to remove metals more efficiently than batch reactors which permit only a single equilibration. Breakthrough should thus occur gradually at the onset of saturation of the majority of the theoretical plates. On passing the respective metal-solutions through columns containing the granular biosorbent (PEI:GA biomass) complete removal of the Cd and Cu from 1.5l of the respective metal solution was attained (Fig 6.13). Less efficient was the removal of Zn, Ni and Cr, though subsequent to the onset of breakthrough of these elements, biosorption of the metals still occurred, though at lower efficiencies, until the onset of complete saturation. Initial breakthrough of Cr occurred after 550 ml of the influent solution had been processed, a further

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350 ml was processed less efficiently, until the onset of total saturation. Similar trends regarding the biosorption of Ni and Zn were apparent at greater volumes of the influent solutions. The ability of the biosorbent in columns to remove a single metal from solution decreased in the order of Cu, Cd, Zn > Ni > Cr, with the onset of the respective breakthrough volumes at 750 ml for Cr, 1200 ml for Ni, and those for Cd, Cu and Zn undetermined.

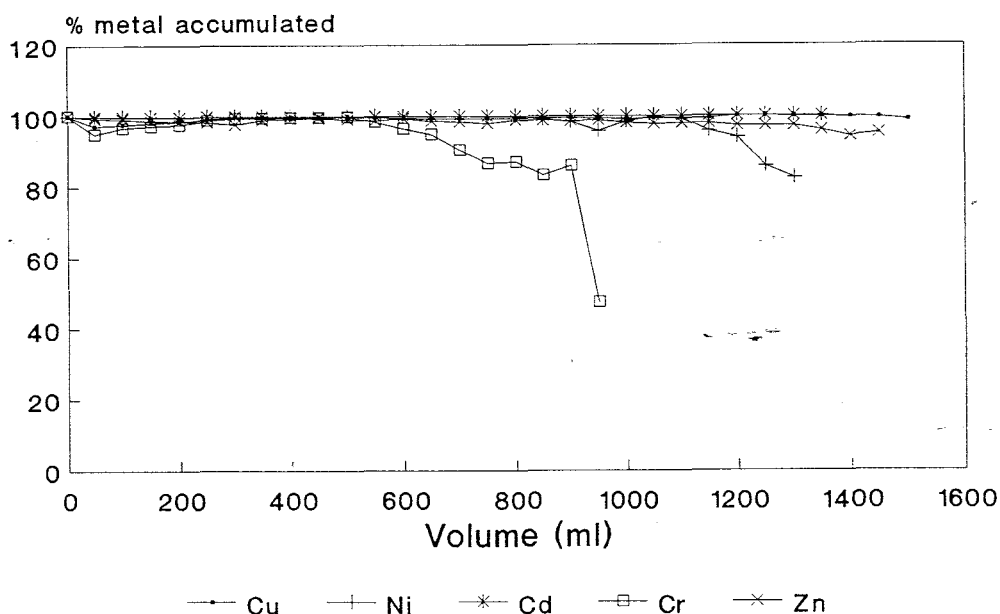


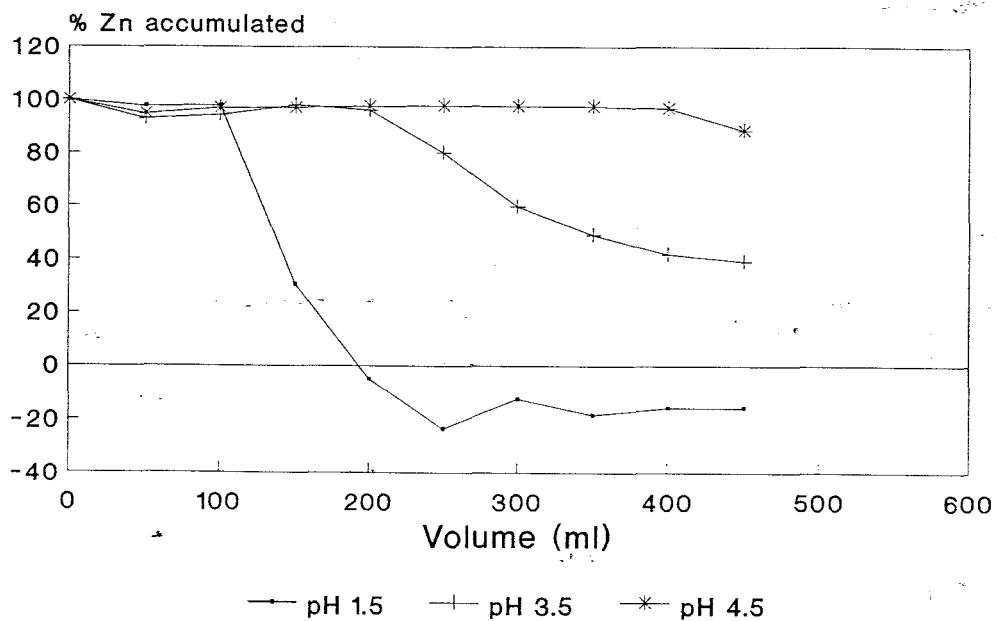
Fig 6.13. Comparison of metal biosorption by KOH treated PEI:GA biomass in fixed bed reactors from respective single element solutions (100 mg/l, pH 5.5).

The introduction of multiple metal cations in solution greatly affected the metal accumulating ability of the PEI:GA biomass (Fig 6.14a - e). Due to the superior metal binding abilities of PEI:GA biomass compared to those of the PVA pellets, the former was preferentially used as the biosorbent. Competition between the metal ions for binding sites on this biomass resulted in the reduction of the breakthrough volume of the columns. Due to the nature of the influent solution (*ie.* the number of different ion species) the pH thereof was unable to be raised above pH 4.5 without precipitation occurring. However, the metal accumulating ability of the biomass was limited at extremely low pH values. An increase in the pH of the influent solution from pH 1.5 to pH 2.5 markedly increased the amount of metal accumulated, especially Zn, Cu and Cr (Fig 6.14a - c). At the onset of breakthrough

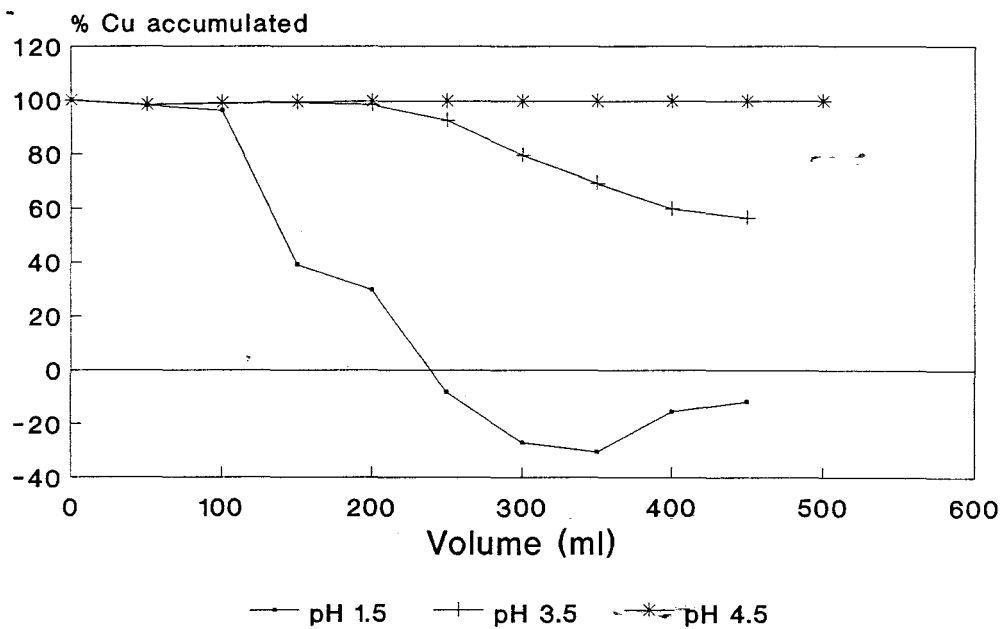
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at pH 1.5, biosorption of these metals rapidly ceased, and at higher influent volumes of the metal solution, elution of previously bound metals from the columns occurred. The elution of these bound metals from solution confirms the pH sensitivity of the biosorption of these metals, viz. Cu, Zn and Cr.

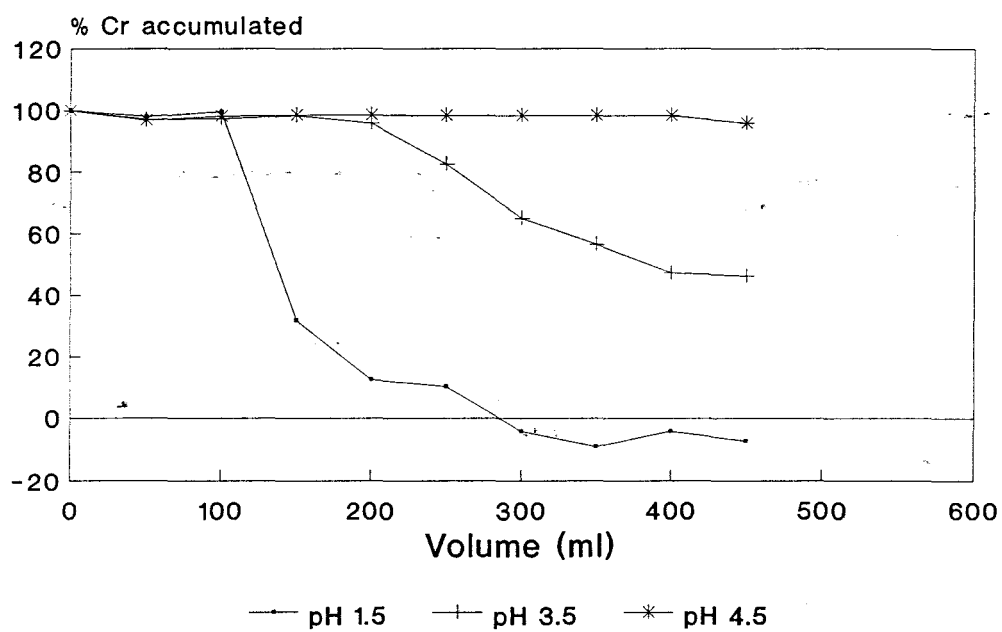
The valency of the metal is largely determined by pH of the influent solution. An increase in the pH of this solution results in a change of speciation occurring and at pH 4.5 the majority of the metals exist as divalent cations, thereby enabling maximum biosorption to occur in the columns (150). Under these conditions (pH 4.5) an affinity series for the biosorption from a multi-element solution of  $\text{Cu} > \text{Zn}, \text{Cr} > \text{Ni} > \text{Cd}$  exists. The preferential biosorption of individual metal ions from a mixed metal solution, suggests the partial specificity of the available binding sites for each metal.



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b)



c)

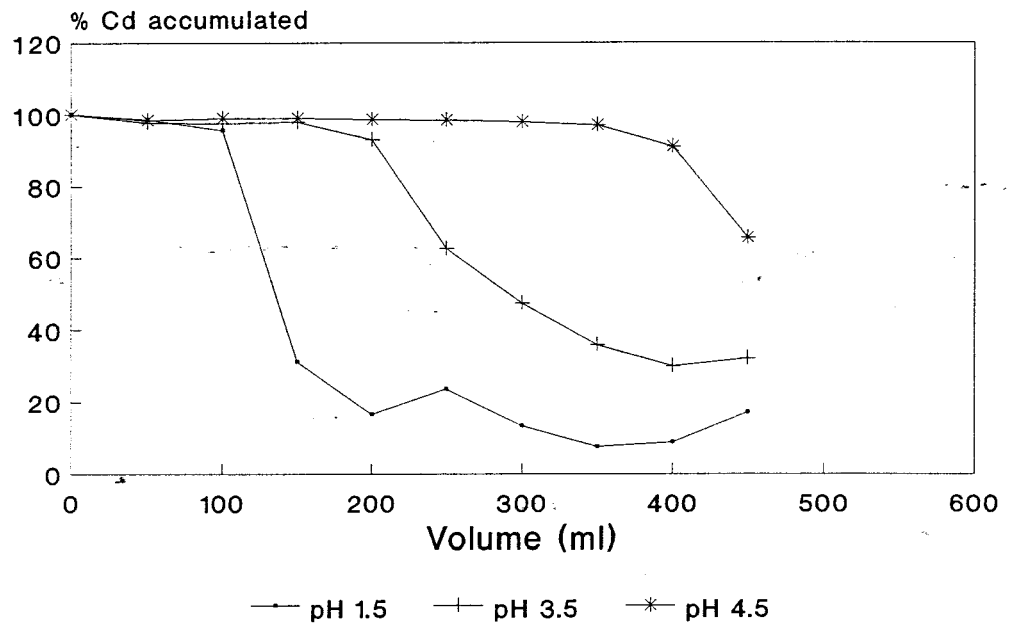
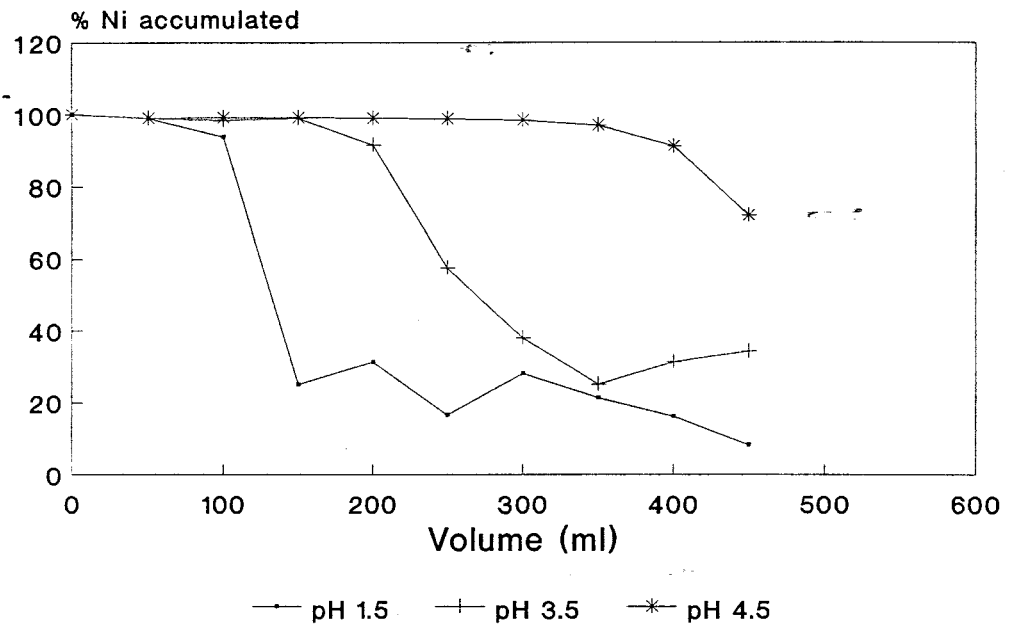


Figure 6.14. Effect of pH on the % metal accumulated by PEI:GA biomass in columns and the respective breakthrough volumes of multi-element equimolar solutions, a) Zn, b) Cu, c) Cr, d) Ni and e) Cd.

## 6.3.4. PEI:GA BIOMASS SORPTION CAPACITY

The results presented in Fig 6.15 indicate the correlation between biosorption of metal cations and the ratio of external free metal ion concentration to the available biomass. Biosorption of metal cations is dependent on this external metal concentration. The linear nature of the graphs indicates continued biosorption, whilst the levelling off reflects the onset of saturation. The biomass accumulated metal cations at varying concentrations, but did not appear to be specific for a single metal species, though Cd saturation was reached before that of other metals. In comparison, at ambient concentrations of 100 mg/l continued biosorption of Ni and Zn occurred. The affinity of the biomass for different metal cations varied at different external concentrations of the metals.

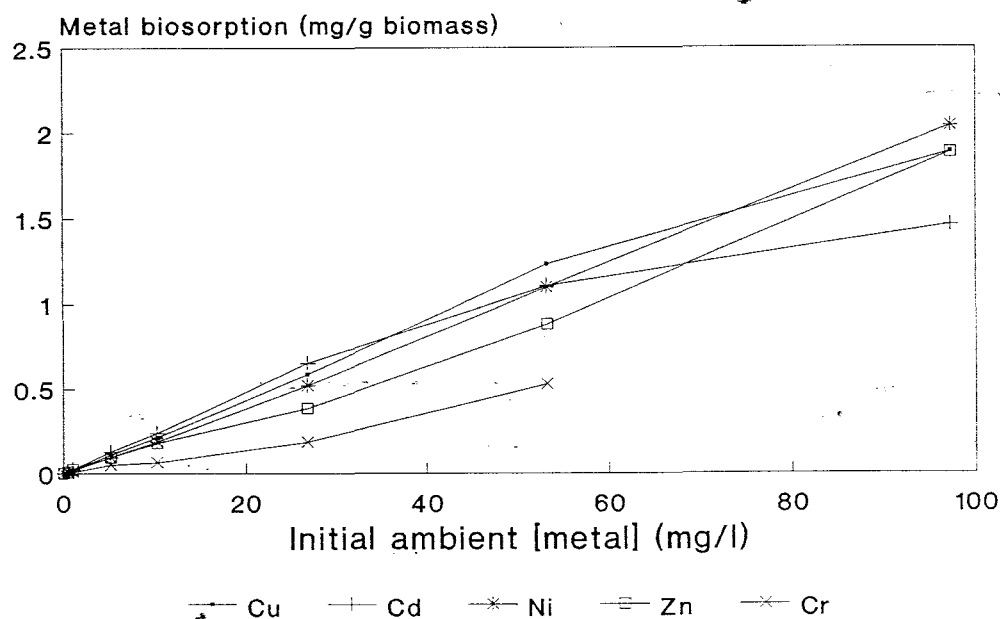


Fig 6.15. Adsorption isotherms indicating the relationship between initial ambient metal concentration and metal biosorption by PEI:GA biomass in batch reaction studies.

#### 6.4. DISCUSSION

The three granular biosorbents produced in this study, *viz.* PVA Na-alginate, PVA Na-orthophosphate and PEI:GA biomass fulfilled the necessary physical requirements, *viz.* that of mechanical strength, rigidity and porosity. From the micrographs, the microstructure and surface area available for metal binding of both types of PVA pellets can be seen to exceed that of the PEI:GA pellets, and can be ascribed to the gel lattice formation initiated during cross-linking. The porosity of the PEI:GA biomass pellets improved on treatment with hot alkali, though this also results in a loss of biomass due to the solubilization of predominantly the mannan and some of the  $\beta$  1-6 glucan cell wall fractions in the alkali. Due to the role these components play in metal adsorption, the loss of these fractions needs to be minimized. Brierley *et al.* (140), favoured alkali treatment of immobilized biomass to that of raw material, due to a diminished biomass loss.

The metal accumulating capacity of the fixed-bed reactor containing PEI:GA biomass exceeds that of the PVA pellets under identical conditions. Arguably the amount of yeast (wet wt) contained per column (column volume: 40 ml) for both PVA productions was less than that of the alkali preparation, though the efficiency of metal accumulation of the PEI:GA pellets still exceeds that of the other two pellet types. Due to the spatial limitations, the feasibility of utilizing the PVA types of biomass in fixed-bed reactors is limited.

Hot alkali-treatment enhances the metal loading capacity of the PEI:GA biomass (results not shown), through increasing the porosity of the biomass and increasing the alkalinity of the granules themselves. Though washed extensively, residual alkali may remain behind in the biomass pores, thereby influencing the biomass metal accumulating pattern. As noted by Brierley *et al.* (cited by 139), not all caustic treated biomass has the same capacity or affinity for metals. For example, the pretreatment of *Penicillium* biomass with NaOH greatly reduces its uptake capacity and in a previous study, alkali-treated native *S. cerevisiae* cells were found to bind less metal than the untreated cells (139).

PEI:GA biomass is capable of binding individual metal ion species from both single and mixed metal solutions. Regenerated biomass, though not as efficient as fresh KOH treated PEI:GA pellets mirror the binding pattern of the latter whilst managing to attain 65% Cu removal after processing 100 ml of influent solution. Metal accumulation in fixed bed columns is largely dependent on the pH of the

influent solution; the greater the acidity of the influent solution, the less metal accumulated. Similarly, from batch studies (data not represented) at very high influent pH values very little metal accumulation by PEI:GA biomass occurs. The majority of metals occur as divalent cation species between pH 4.0 - 6.0 thereby facilitating easy accumulation.

As a general biosorbent the PEI:GA biomass has extensive potential regarding metal ion removal from multi-element solutions. Although the affinity of the biomass for the respective metal cations varies depending on the metal ion species and ambient concentration thereof (Fig 6.15), the mechanism of biosorption appears to be similar. Comparison of the breakthrough volumes of single element solutions to those containing multiple metals (Fig 6.13 and 6.14) confirms the competitive nature of metal biosorption. If the cation-ligand interactions were metal species specific, the breakthrough volumes for the respective metals in a multi-element solution would remain relatively similar to those in a single element solution. Yet due to the competitive nature of the biosorption and limited number of binding sites, the breakthrough volumes of the multi-cation solutions are greatly reduced in comparison to single element breakthrough volumes. Further reductions in the amount of metal accumulated can result from steric hinderance by surface-bound metal ions.

Biosorption of the individual metals by the PEI:GA biomass is largely dependent on the ratio of external free metal ion concentration to the available biomass. The biological availability of metals is determined by their chemical speciation (charge). Shifts in pH affect the metal biosorption, by changing their solution chemistry, the nature of functional groups in the biomass as well as the competition of metallic ions with hydrogen and other monovalent ions for the binding sites (139, 150). Similar to the results obtained from the present study, Kuyucak and Volesky (150), noted that almost all the metallic species being studied, viz. U, Zn, Cd, Co and Cu, with the exception of Au were ionized as various cationic species between pH 4.0 - 5.0. Due to the nature of the metal biosorption, subsequent desorption of the metal ions is readily achieved by changing the pH of the influent solution. Mild solutions of acids, eg. hydrochloric, nitric and sulphuric are often used as desorption agents (197) due to the high solubility of metals in these solutions.

## 6.5. CONCLUSIONS

The three preparations of non-viable biomass, viz. PVA Na-alginate, PVA Na-orthophosphate and alkaline treated PEI:GA granules, produced during the present study all exhibit excellent mechanical stability. However, the metal biosorption capacity of the PEI:GA biomass exceeds that of the other two biomass types. PEI:GA biomass has proven to be an efficient metal accumulator, with the amount of metal accumulated largely dependent on the ratio of free metal ion concentration to available biomass. The availability of the metals is influenced by the solution pH. At low pH the affinity of the biomass for metals decreases and subsequently breakthrough occurs at low volumes of the influent solutions. Enhanced metal biosorption by the biomass in columns occurs at higher pH values, eg. pH 4.5 - 6.0. Biosorption is of a competitive nature. In the presence of multiple elements a decline occurs in the amount of complexed metal and the resultant breakthrough volumes of the individual metal ions occurs at lower influent volumes.

The data from this aspect of the study indicated that the immobilized non-viable yeast biomass could be successfully used for the bioremediation of effluents such as those from electroplating industries. An attempt at such an application of bioremediation is described in the following chapter.

## 7. APPLICATION OF METAL BIOSORPTION BY NON-VIABLE YEAST BIOMASS TO AN ELECTROPLATING EFFLUENT

### 7.1. INTRODUCTION

The most extensive, though not the sole use of bioremediation was possibly that which involved the clean up efforts in Alaska resulting from the Exxon Valdez oil spill in the 1980's (201). The publicity generated by this event sparked off world wide interest in bioremediation, though awareness of numerous other applications has remained limited. The development of bioremediation processes for water is one of the main applications of biological treatment systems, including the clean up of ground water, soils, lagoons, sludges and process waste streams. In the last decade the application of biological based technology has branched out to the treatment of metal containing water with areas of potential application covering detoxification of metal-bearing waste water, decontamination of radioactive waste water, recovery of metals from ore processing solutions and the concentration and recovery of rare and strategic metals from sea water (192). The biosorption processes are based on solid-liquid contact, not dissimilar to those used for ion-exchange or activated carbon application (192). However, biosorbents have most successfully been applied in the treatment of low-concentration, high-volume waste streams, eg. mine waste water streams (202), with very few studies having been conducted using high-concentration metal effluent, eg. industrial effluent. Several methods already exist for the treatment of this type of waste, such as electroplating effluent. Reverse osmosis, electrodialysis, diffusion dialysis, electrolytic metal recovery, evaporation and ion-exchange have all been implemented as treatment systems (203). An alternative approach employed an integrated waste treatment system (204), yet its sorbent material was rather inefficient and non-regenerative. Biosorptive treatment of this effluent using bioreactor tanks would therefore offer a novel and cost effective approach to a difficult problem.

When designing bioreactors, numerous options exist depending on the required criteria, though the types of reactors used in industry for carrying out heterogenous solid-liquid contact reactions can be classified in two broad categories (192, 205, 206). There are reactors in which the solid state particles remain in a fixed position relative to one another, eg. fixed bed, trickle bed and moving bed reactors or secondly reactors in which the solid state is suspended in a fluid and constantly moving about, eg. fluidized bed and slurry reactors (206). For the purposes of bioremediation of

## 7. APPLICATION OF BIOSORPTION BY NON-VIABLE YEAST BIOMASS

metal containing waste water, variations to both of these categories accomplish appropriate contact between the solution and solid biosorbent phase (192). For example, fixed packed bed reactors in their most basic form consist of cylindrical tubes or columns packed with biosorbent pellets (206), through which the metal-bearing liquid percolates (192). The principle of metal biosorption within these columns is based on theoretical plate or multi-equilibrium theory. The line of saturation of the biomass progresses through the bed in the direction of the flow until onset of total biomass saturation at the breakthrough volume. Depending on the nature of the biosorbent an upwards or downward line of flow can be selected (192).

Stirred tanks in the form of batch or continuous flow reactors can be used, though the latter is preferred by chemical and bioremediation industries (192, 205). Different multi-step modifications can be developed for the basic continuous flow stirred tank scheme. Multi-step or phase modification such as the introduction of multiple bioreactors, or recycling of the metal solution in combination with biomass regeneration can increase the operating efficiency of the system, but also its operating costs (192).

Several multi-component fixed bed adsorption models (204, 207 - 209) have been applied to metal removal from metal-contaminated waste water (141, 144, 210, 211). Treatment of acid mine drainage water by a three-column circuit, containing a lead-load column, scavenger column and an elution column has successfully removed Ni, Co, Cu and Zn from acid mine drainage water to within the stipulated water criteria limits (144, 210, 211). Elsewhere, p-chlorophenol and mercuric ions were removed from waste water using fixed bed columns (211).

Equally successful has been the removal of metal ions from acid mine drainage water using low maintenance circuits (144, 210). These low maintenance circuits, *viz.* a trough and bucket system are based on continuous flow principles, yet have been designed for field application in remote areas. Both trough and bucket systems utilize porous bags containing the biomass and in the absence of stirrers, a hydraulic gradient is used to circulate the water.

Biosorbent technology especially on an industrial scale is still in its infancy. Though examples of successful application have been cited, the scope for bioremediation extends far beyond the treatment of relatively dilute mine waste water. Industrially produced waste water, eg. tannery or electroplating effluent, due to the nature of the processes often contain several metals in excess of those found in

mine water, though in smaller volumes. Ultimately a system equipped to handle low-volume, high metal content waste water is required.

In the present study the application of a bioremediation process for the treatment of electroplating waste water was investigated. The systems studied and the effluents used are based on the results of the small scale investigations with electroplating effluent as described in chapter 6. The PEI:GA immobilized biomass developed previously (see chapter 6) was used in preference to the PVA type biomass pellets for the duration of this study, due to its superior metal accumulating properties and mechanical strength. The primary objectives of this investigation was to evaluate the biomass performance during the treatment of the industrial effluent and to compare the applicability of two types of bioremediation systems. Samples of water were obtained from several factories, but the particular waste water used in the present study (effluent A in chapter 6) was selected due to the number and levels of the metal species contained therein. Efficiency of metal biosorption and subsequent metal removal by the biomass was investigated using continuous-flow fixed bed and stirred bioreactors as single or multiple reactors which operated either independently or as part of a biphasic process. Comparative estimations of the efficiency of metal removal and the feasibility of industrial applicability were drawn between the two systems.

## 7.2. MATERIALS AND METHODS

### 7.2.1. ELECTROPLATING EFFLUENT

The electroplating effluent (effluent A) was obtained from a factory operating three plating lines, viz. acid/zinc, zinc/cyanide and cadmium/cyanide lines. Run-off and discharge water from all three systems were combined prior to disposal, resulting in the discharge of an extremely toxic waste product. The levels of the respective metal ions varied depending on which process was being flushed out. Corresponding fluctuations in the pH of the Raw effluent occurred. A pH range between pH 1.0 - 7.0 was noted.

The present waste disposal system is relatively primitive consisting of two compartmented tanks in series (Fig 7.1). These tanks are fed directly from the factory floor and serve as settling tanks for the precipitation of excess metal ions. The effluent undergoes no further treatment and the remaining liquor is flushed directly into the sewage disposal system. Sampling was conducted at two points

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along the waste disposal line. Initial samples were provided from the site where the raw effluent entered into the settling tanks (subsequently referred to as Raw effluent) whilst the second samples were collected at the point where the waste water left the settling tanks prior to disposal in the sewage system (subsequently referred to as post settling tank (PST) effluent). The pH of the PST effluent ranged between pH 1.5 - 6.0. Depending on the nature of the Raw effluent, varying concentrations of metal ions were precipitated in the slurry within the tanks, yet the level of the metal ions in the liquor frequently exceeded the permitted criteria (Table 7.1). Raw effluent was examined in the initial studies in order to determine whether bioremediation of this effluent would eliminate the need for settling tanks.

Table 7.1. Levels of metal (mg/l) present in Raw and PST electroplating effluent compared to stipulated national drinking water and aquatic ecosystems criteria (median levels).

	Cu	Cd	Cr	Ni	Zn
Drinking water criteria	1.0	0.01	0.05	0.05	5.0
Dam/River water (aquatic life) criteria	0.005	0.003	0.05	0.05	1.0
Raw effluent	2.0 - 2.5	20 - 80	60 - 70	2.0 - 3.0	1000 - 3500
PST effluent	0.2 - 1.0	20 - 80	5 - 50	0.2 - 1.2	300 - 1500

### 7.2.2. CONTINUOUS-FLOW FIXED BED REACTORS

#### 7.2.2.1. PROCESSING OF RAW EFFLUENT

Using 2M KOH the pH of Raw effluent (initial pH 1.5) was adjusted to pH 2.5, 3.5, 5.0 and 6.0 respectively to determine the pH optimum for the biomass under experimental conditions (pH optimum for metal biosorption from spiked aqueous solutions was previously established between pH 4.5 - 6.0). Precipitated matter (brought about as a result of increased solution pH) in the pH 5.0 and 6.0 solutions was allowed to settle out and subsequently discarded prior to use. The pH modification of the Raw effluent served a dual purpose, not only to ascertain an optimum pH range for metal removal, but also to remove some of the metal ions present in solution.

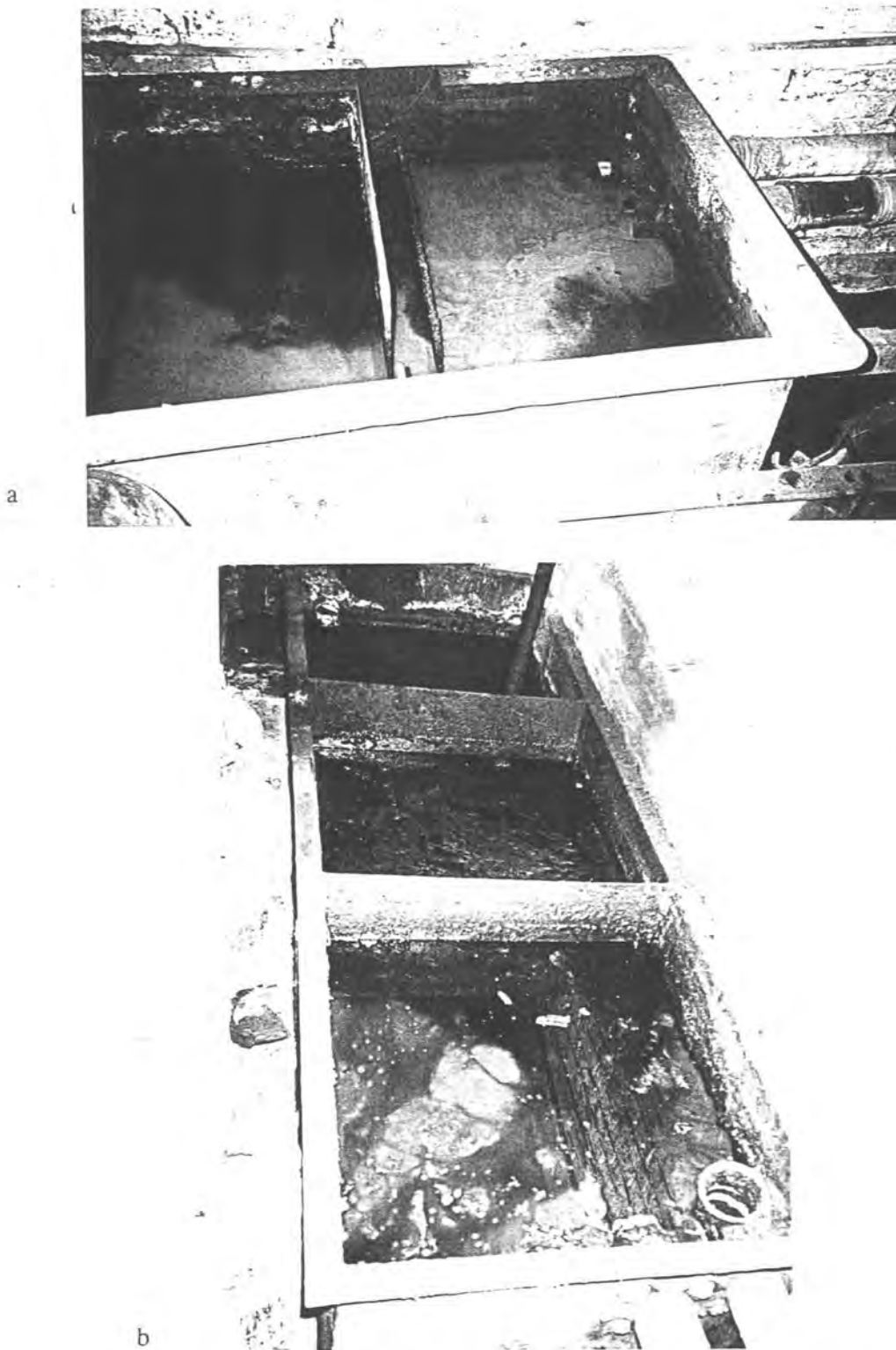


Fig 7.1. The waste water treatment tanks operated in the electroplating factory. Two sets of tanks were linked in series allowing precipitation of metals to occur (a-b). The difference in colour of the effluent is due to pH change. Note the precipitated sludge in the first tank.

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The respective Raw effluent solutions were pumped upwards through the PEI:GA biomass packed glass columns. Untreated Raw effluent (pH 1.5) was used as the control. All experimental criteria, viz. dry wt (g) biomass used, biomass preparation and column volume and height remained identical to that described in section 6.2.3.1. The flow rate was maintained at 55 ml/hr, permitting a contact time in excess of 1 hr between the biomass and metal solution. The pH and metal content of the column eluant fractions were compared to those of the pre-column effluent.

### **7.2.2.2. DUAL COLUMNS IN SERIES**

1.4 l of pH 5.0 Raw effluent (initial pH 2.23) was pumped through columns linked together in series with the eluant from column 1 fed directly to column 2. Both columns were identical with respect to dimensions and biomass load, and a flow rate of 55 ml/hr was maintained. From column 2, 10 ml fractions were collected and the metal content and column pH profile analysed. The success of the metal removal in this and following systems was often gauged by the breakthrough volumes for Zn and Cd (based on the high levels of these metals within this solution and the extreme toxicity of Cd in solution).

### **7.2.2.3. SINGLE AND DUAL REACTOR TREATMENT OF PST EFFLUENT**

pH modification of the PST effluent used in this particular experiment was not required (influent pH 5.58). 1.4 l volumes of PST effluent were pumped through respective single and dual column systems (flow rate 55 ml/hr). Column parameters and experimental criteria remained unchanged to previous experiments. Fractions of column eluant (10 ml) were collected and analysed accordingly.

### **7.2.2.4. PROCESS SCALE UP: EXPERIMENTAL PARAMETERS**

Perspex LKB Chromatography columns were adapted (silicon sealed and water-proofed) to fulfill the necessary requirements. The columns had an internal diameter (ID) of 3.7 cm, column height of 30 cm and total volume of 325 ml.

Three columns were run, each differing with respect to the biomass load, viz. 85, 90 and 95 g (dry wt). Prior to packing, the biomass was rehydrated and rinsed three times using Milli-Q water. The wet pellets were contained within the column by two fine mesh sieves to prevent fouling of the inlet

## **7. APPLICATION OF BIOSORPTION BY NON-VIABLE YEAST BIOMASS**

and outlet ports. The influent pH values of each of the columns ranged from pH 5.3, 5.26 to 5.05. PST effluent (5.0 l) was pumped upwards through the fixed beds at a flow rate of 4.5 l/hr, and 10 ml fractions were collected every 200 ml and analysed for metal content and pH.

### **7.2.3. CONTINUOUS-FLOW STIRRED BIOREACTORS**

#### **7.2.3.1. BIOREACTOR DESIGN**

Cylindrical bioreactors made from perspex served as reactor tanks. All three bioreactors were of identical dimensions, each containing a single inlet port at the base of the tanks and three outlet ports at calibrated 2.5 l volumes. The height of the bioreactors measured 430 mm, ID 172 mm and contained a total volume of 10.0 l. The design of the tanks enabled operation at 2.5, 5.0 and 7.5 l capacity thereby facilitating scale-up. The reactor tanks were not sealed entities allowing easy access to the biomass and enabling constant top-stirring thereof (60 - 100 Upm).

#### **7.2.3.2. SINGLE BIOREACTOR SYSTEM**

7.0 l of PST (initial pH 1.98, final KOH-modified PST pH 5.0) was pumped through the bioreactor containing 100 g (dry wt) of washed biomass. A flow rate of 5.0 l/hr was established and entry into the tank was facilitated via the bottom port. The tank was operated at 2.5 l capacity with continuous stirring. 10 ml fractions were collected from every 0.1 l of eluant for the first 3 l and subsequently from every 0.5 l. These fractions were analysed with respect to pH change and metal content using the standard protocol in order to determine the metal loading capacity and breakthrough volumes of the bioreactors.

To determine the effect of scale up on the operating efficiency of the biomass, the experiment was repeated using 200 g (dry wt) of biomass and 5.0 l capacity of the bioreactor. 9.5 l of PST, pH 6.0 (initial pH 1.93) was pumped through the system at the set flow rate of 5.0 l/hr. The collection and analysis procedure of the fractions proceeded as before. Due to the decline in efficiency of metal biosorption brought about by increasing the bioreactor volume, the bioreactor was not operated at its full capacity (7.5 l).

### 7.2.3.3. DUAL BIOREACTORS LINKED IN SERIES

The development of a dual bioreactor system required the eluant from tank 1 to be pumped directly into tank 2. Sampling points were established at the outlet ports of both tanks where 10 ml fractions were collected at 0.5 l intervals and analysed accordingly. The influent pH of the PST effluent was maintained at pH 6.0 (initial pH 2.02 - 2.43) and the operating capacity of tank 1 at 5.0 l, whilst that of tank 2 was increased from 2.5 l to 5.0 l after the initial experiment in order to establish its metal loading capacity. This operating capacity was maintained for subsequent experiments.

The breakthrough volume of tank 1 and effect of biomass replacement therein on the total metal removal required large influent volumes of PST to be pumped through the system. 10.0 l was sampled from both tanks with sampling occurring every 0.5 l. To determine the effect of biomass substitution on the total metal removal, the effluent in tank 1 was drained and the biomass replaced subsequent to collecting every 4.5 l of eluant. A total of 20.0 l was pumped through the system, necessitating 4 biomass changes.

### 7.2.3.4. DETERMINATION OF MAXIMUM LOADING CAPACITY

The evaluation of the performance of the bioreactor system necessitated the introduction of a third tank to ascertain its efficiency as a scavenger tank in removing the remaining metals from solution. Two variations of the system were tested, each containing triple bioreactors linked in series (Fig 7.2).

In system 1 each of the tanks was operated at 5.0 l (200 g biomass (dry wt)) capacity. 55 l of influent PST was prepared to pH 5.4, yet due to the residual volumes remaining within each of the tanks only 40 l of effluent was sampled from each bioreactor. The biomass in tank 1 was replaced with fresh material subsequent to every 5.0 l of eluant collected. Biomass replacement in tank 2 occurred at the onset of saturation (approximately 22.0 l), with the biomass from the second bioreactor being replaced with that from tank 3. Tank 3 was supplied with freshly prepared biomass.

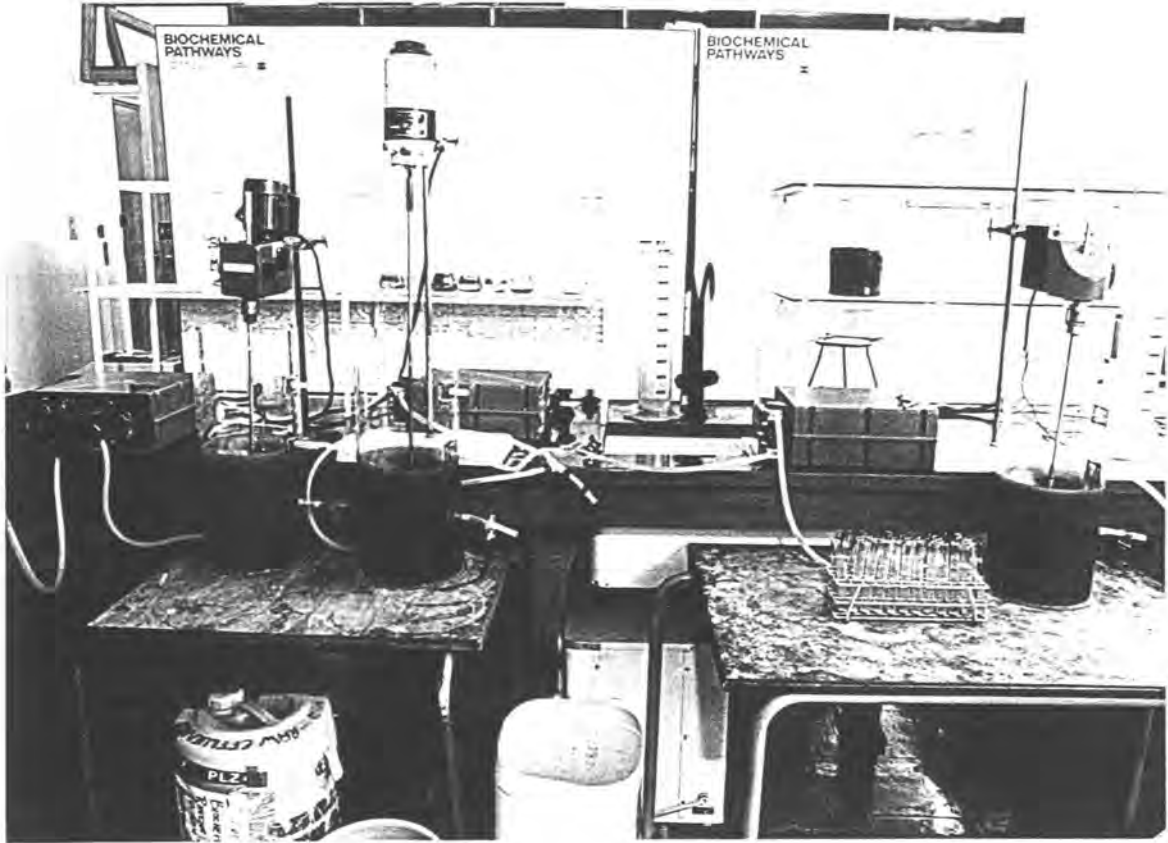


Fig 7.2. The continuous-flow stirred tank bioreactor system. Three bioreactors operating at 5.0 l capacity were linked together in series, with the eluant from one tank being fed directly into the next. Sampling points were established at the outlet of each of the bioreactors.

The experimental criteria for system 2 remained unchanged though changes to the influent pH (pH 4.5) and biomass substitution in this reactor were implemented. At the onset of saturation in tank 2 (22.0 l), the biomass was replaced with fresh material whilst that in tank 3 remained unchanged. 75.0 l of PST effluent was prepared with 40 l being sampled from each of the tanks. The remainder of the effluent was lost either due to pH induced precipitation or as residual fluid in the bioreactors. A crucial step in the success of both bioreactor systems was the washing procedure of the biomass prior to bioaccumulation. Extensive washing and rinsing of the biomass in Milli-Q water was required to remove the residual alkali from the PEI:GA pellets. In the presence of very high alkalinity flocculation and precipitation occurred within the tanks.

### 7.3. RESULTS

#### 7.3.1. CONTINUOUS-FLOW FIXED BED REACTORS

##### 7.3.1.1. SINGLE AND MULTI-PHASED RAW AND PST EFFLUENT TREATMENT

###### 7.3.1.1.1. RAW EFFLUENT

Fixed bed bioreactors can be operated either independently as in this study or as the second phase of a biphasic process, where the biological phase was preceded by the initial chemical phase involving pH modification of the Raw effluent. Chemical modification served a dual purpose, viz. priming of the effluent for further use and the precipitation of excess metals. The increase in the pH of the effluent resulted in the decrease in solubility of the metal ions in solution and caused the subsequent precipitation of the metals predominantly as insoluble hydroxides (181), eg. at pH 5.0 and 6.0 (Table 7.2). The increase of influent pH decreased the influent-metal concentration and thereby correspondingly extended the lifespan of the column biomass prior to breakthrough occurring.

Table 7.2. The effect of pH on the metal concentration of the influent Raw effluent and the resultant breakthrough volumes (BV) in each of the small-scale single columns. Due to variations in the initial metal ion concentrations the metal levels are expressed as % of the initial concentrations. The breakthrough volumes (l) reflects the carrying capacity of the columns. The effluent was run through single columns.

	Cu		Cd		Cr		Ni		Zn	
	%	BV (l)	%	BV (l)	%	BV (l)	%	BV (l)	%	BV (l)
Raw effluent (pH 1.45)	100	0.16	100	0.13	100	0.45	100	0.14	100	0.12
pH 2.5	99	>0.3	99.8	0.21	100	0.27	98.2	0.25	99.8	0.19
pH 3.5	79.7	>0.3	94.5	0.22	100	0.30	87.2	0.28	99.8	0.19
pH 5.0	63.2	>1.1	95.9	0.5	20.5	>1.1	96.3	>1.1	89.2	0.46
pH 6.0	25.6	>1.1	98	0.48	0.0	>1.1	87	>1.1	82	0.46

## 7. APPLICATION OF BIOSORPTION BY NON-VIABLE YEAST BIOMASS

From Table 7.2 it is apparent that pH and metal concentration are closely related to breakthrough volumes. Accumulation increased with increasing pH until a adsorption plateau was reached and subsequently the breakthrough volume of the columns remained unchanged over the volumes measured, regardless of fluctuations in pH, eg. pH 5.0 and 6.0. From preliminary batch reaction studies (results not reported) it was determined that the plateau was not unlimited, but for most metals probably extended between pH 5.0 - 7.0, after which a decline occurred in the amount of metal accumulated. The results presented in Table 7.2 were obtained from separate experiments. Initial experiments were run using smaller influent volumes of effluent and based on the results obtained from these experiments, larger influent volumes were used for subsequent experiments. Consequently instances where breakthrough volume has not yet been attained, (eg. Cu, Cr and Ni) indicate that breakthrough had not been reached for the total volume of effluent used.

Since optimal operating conditions for the treatment of Raw effluent were achieved between pH 5.0 and 6.0, the feasibility of using influent Raw effluent instead of PST effluent in a biphasic system had to be considered. The precipitation of metals from Raw effluent due to alkali treatment was therefore compared to the effectiveness of metal removal in the settling tanks (PST effluent) (Fig 7.3).

Though the initial concentration of metals in both solutions varied (Table 7.1), the data in Fig 7.3 represents the general trend of metal removal by pH adjustment or settling. Although the PST effluent used in Fig 7.3 had an influent pH of 4.6, the pH values for PST effluent ranged between pH 1.5 - 6.0. However, the data shown in Fig 7.3. are representative of all the PST effluents. With the exception of Cd, the amount of metal precipitated out from the Raw effluent by the settling tanks exceeded the metal removal by the chemical treatment. 99.2% of Cr was removed from the Raw effluent by settling, thereby almost totally eliminating it as a problem metal whilst Cu, Ni and Zn removal in the settling tanks varied between 60 - 85%. The amount of Cd in the PST solution actually exceeded the initial levels from the Raw effluent by 1.5 times due to conditions (eg. low Ph) within the settling tanks enabling the mobilization of sludge Cd into solution, and thereby compounding the problem of removing this metal.

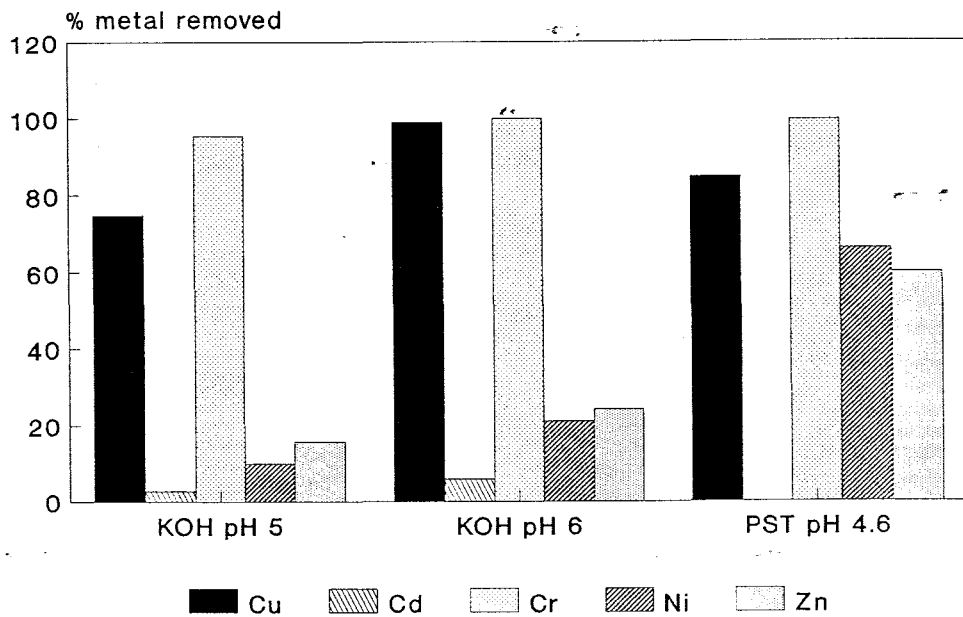


Fig 7.3. Comparison between alkali (KOH) treatment and settling tanks as a means of precipitating out excess metal from Raw effluent. Levels of Cd in PST were 1.5 times higher than those in the Raw effluent.

The introduction of a second column into the system had a minimal effect on the breakthrough volume or the amount of metal removed from the Raw effluent (Fig 7.4). The onset of breakthrough (*ie.* 80% removal of metals) of Zn occurred at 400 ml and that of Cd at 520 ml compared to 460 ml for Zn and 500 ml for Cd in a single column (Table 7.2). This suggests a change in the metal chemistry in the solution, which lowers the affinity of the biomass in the second column for the metals.

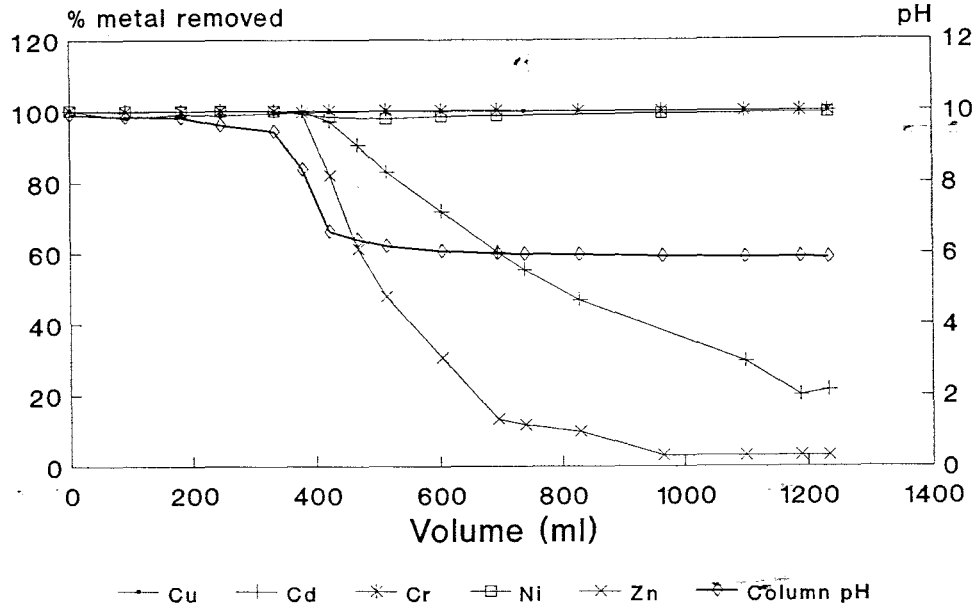


Fig 7.4. Metal removal from Raw effluent (pH 5.0) using dual bioreactor columns linked in series.

#### 7.3.1.1.2. PST EFFLUENT

Appropriate treatment of the PST effluent was determined by the influent pH. High acidity ( $\text{pH} < 4.5$ ) necessitated chemical priming using KOH, whilst an influent  $\text{pH} > 4.5$  enabled the implementation of a single phase system (*ie.* no chemical treatment).  $\text{pH} 4.5$  was selected as the cut-off pH for chemical treatment, since even mild alkali modification at this influent pH resulted in a large increase in the alkalinity of the system. The onset of breakthrough occurred at 400 ml for Cd and Zn from untreated PST effluent ( $\text{pH} 4.5$ ) pumped through a single column (Fig 7.5). The amount of metal removed was increased by the introduction of a second column into the system (Fig 7.6). Constant operating efficiency of the first column was assumed to occur in the dual column system, thereby implying a lower operating efficiency for the second column. The cumulative effect of both columns resulted in 80% removal of Cd and Zn occurring for 630 and 550 ml effluent respectively. However, the residual level of these two metals in the processed solutions, *viz.* 9.76 mg/l of Cd and 106 mg/l for Zn still remained in excess of the stipulated water quality criteria.

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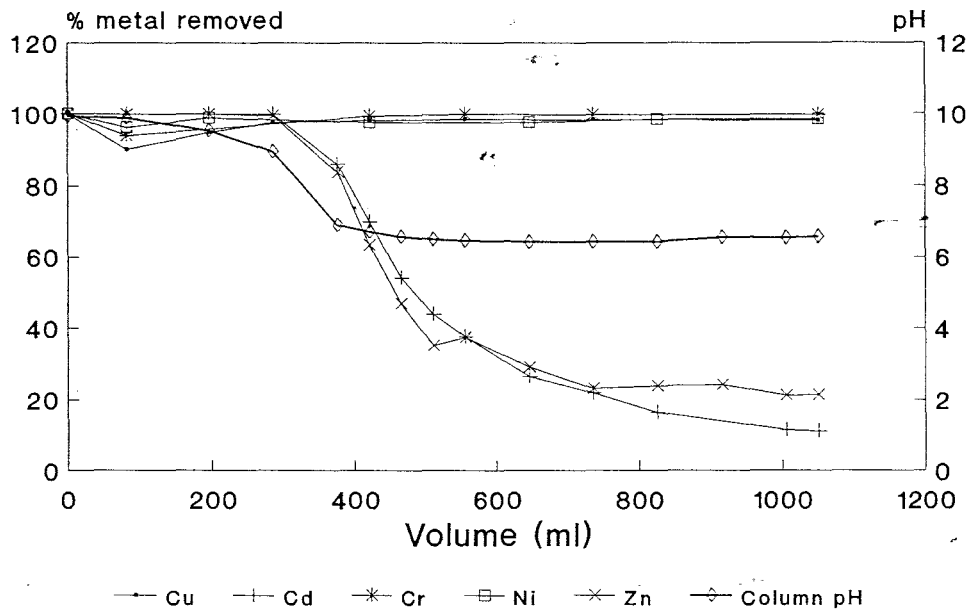


Fig. 7.5. Metal removal from PST effluent (pH 4.5) using a single column system. No prior chemical modification of the effluent was necessary.

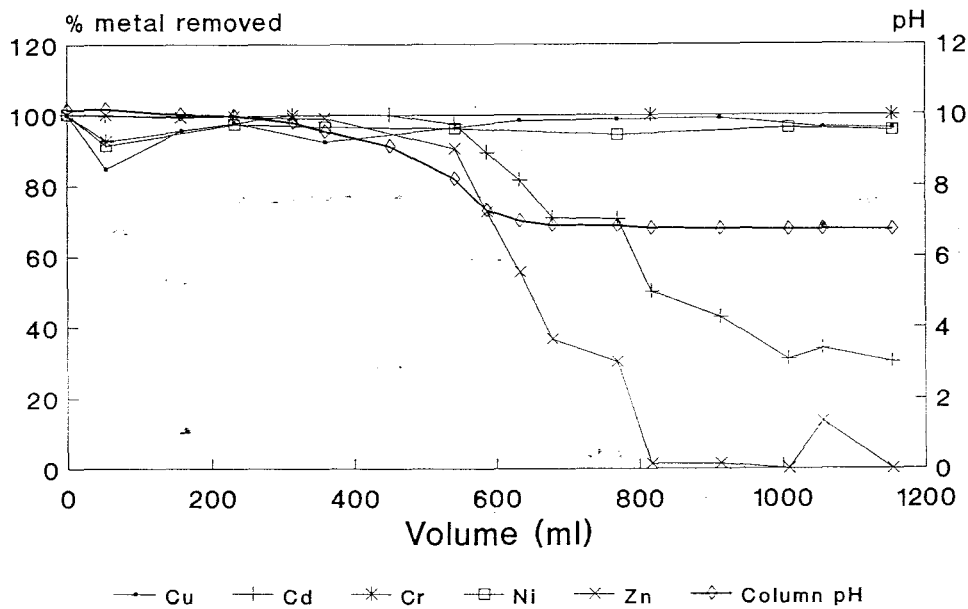


Fig 7.6. Metal removal from PST effluent (pH 4.5) using dual columns in series. No prior chemical modification of the effluent was necessary.

**7.3.1.2. PROCESS SCALE UP**

Using PST effluent, scale-up of fixed bed columns from a 40 ml (10 g (dry wt)) capacity to a column capacity of 325 ml (85 - 95 g (dry wt)) failed to produce any corresponding increase in metal removal (Cd used as a standard) (Table 7.3). Similar to smaller scale columns, the decline in metal accumulation was influenced by the pH profile within the columns with breakthrough occurring due to a drop in the pH of the column effluent. Furthermore, total biomass contained within the columns, column performance and biomass characteristics under operating conditions also influenced the breakthrough volumes.

Table 7.3. The effect of process scale-up on the breakthrough volumes and final concentrations of Cd in solution. The breakthrough volumes (BV) were predominantly affected by the biomass capacity of the column.

Column No.	Biomass (g (dry wt))	BV (l)	Final [Cd] (mg/l)
1	10	1.1	45.6
2	85	4.3	46.0
3	90	3.5	48.5
4	95	4.0	46.7

Several problems were experienced during process scale-up. The column-contained immobilized biomass was unable to maintain its integrity under high pressure (created by the flow rate of 4.5 l/hr), resulting in compacting and channelling of the biomass. Compacting was not alleviated by the upward flow of the effluent or by increasing the amount of biomass contained within the column. A cut-off point for the biomass loading capacity and flow rate of the columns existed, beyond which counter pressure developed, resulting in leakage of the columns.

**7.3.2. CONTINUOUS-FLOW STIRRED BIOREACTORS**

Pretreatment of the PST effluent proved to be an integral part of the process in these experiments, since PST effluent in these studies was pH 1.98. Not only did this prime the effluent for further treatment, but also resulted in the removal of metal, eg. 67% of Cu, 5% of Cd, 99.5% of Cr, 15%

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of Ni and 26% of Zn removal was achieved through KOH precipitation (typical results from 6 experiments). The minor metals, viz. Cu, Cd and Ni responded well to this treatment, but due to the excessive levels of Zn in the effluent, the residual amount of Zn in the influent solution averaged 850 - 1000 mg/l and Cd 40 - 60 mg/l.

### 7.3.2.1. BIOREACTOR EFFICIENCY

The reactor volume and proportional amount of biomass contained therein, influenced the amount of metal removed from solution (Table 7.4). Though the total amount of metal removal increased, the efficiency of metal removal in the 5.0 l tank depended on the initial concentration and the metal species in question. For example, Cu and Zn removal increased threefold, yet Cd accumulation was limited to approximately 61% of the potential removal capacity and no increase in Ni removal occurred.

Table 7.4. Comparison between the efficiency of metal removal by 2.5 l and 5.0 l tanks. The amount of metal removed is expressed as % of modified PST effluent, and was determined subsequent to 4.0 l of effluent being processed by each of the bioreactors.

	Cu	Cd	Cr	Ni	Zn
2.5 l tank	22.0	25.7	93.3	87.3	22.4
5.0 l tank	77.2	41.5	99.9	83.2	74.1

By increasing the bioreactor volume, yet maintaining the flow rate as in the above experiment, the contact time between the biomass and effluent was extended to ensure maximum metal removal. Batch studies (data not shown) have however indicated that the majority of metal accumulation occurred within the first 10 min. For example, in batch studies 82% of Cd was removed within 10 minutes, whilst after an hour only 85% of removal had been achieved. Thus in larger volume bioreactors, the flow rate can be increased, without negatively affecting the amount of metal removed, thereby increasing the operational efficiency of such a system.

### 7.3.2.2. DUAL BIOREACTORS IN SERIES

The decline in efficiency of removal of certain metals, eg. Cd and Ni with increased bioreactor

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capacity, reduced the feasibility of operating the bioreactor to at its full capacity (7.5 l). Operating a second bioreactor coupled in series provided a more efficient alternative. Though the majority of the metal removal occurred in tank 1 (Table 7.5), optimum metal removal was achieved by operating both tanks at 5.0 l capacities.

Table 7.5. Comparison of efficiency of metal removal from PST effluents between two dual bioreactor systems, viz. 5.0 l and 2.5 l or 2 x 5.0 l tanks. Total metal removal from PST effluent and the metal concentrations (mg/l) of the influent and eluant solutions was measured. % metal removal and metal concentration were determined after 5.0 l of effluent had been processed.

	Cu	Cd	Cr	Ni	Zn
(%) of metal removal by Tank 1	52.9	39.9	62.2	74.6	47.1
(%) of metal removal by Tank 2 (2.5 l)	-	22.9	12.5	8	22
(%) of metal removal by Tank 2 (5.0 l)	-	34	33.7	13	28
Total removal (%) by 5.0 + 2.5 l system	94	69.2	99.9	83.5	76.1
Total removal (%) by 2 x 5.0 l system	94.8	72.2	99.9	90.6	85.6
[Metal] in influent PST (mg/l)	0.078	58.9	0.066	0.361	809
[Metal] in eluant of 5.0 + 2.5 l system (mg/l)	0.039	19.2	0.006	0.07	279
[Metal] in eluant of 2 x 5.0 l system (mg/l)	0.04	17.2	0.005	0.037	171

With the exception of Cd and Zn, the metal levels in the eluants of both systems fulfilled the required drinking water criteria. Removal of Cd and Zn from PST effluent ranged between 69 - 72% for Cd and 76 - 86% for Zn.

7.3.2.3. BREAKTHROUGH DETERMINATION

The onset of breakthrough for Cd and Zn in tank 1 occurred between 4.5 - 5.0 l, depending on the influent concentrations of these metals (Fig 7.7). At this stage approximately 60% of Cd and 70% of Zn are removed from the effluent. The estimated breakthrough volume of tank 2 was 22 - 25 l depending on the influent Cd and Zn levels. The implementation of biomass substitution in tank 1 extended the lifespan of the second bioreactor. (Data relevant to this is shown in the following section) Subsequent to processing 20.0 l, 99.5% Cd and 98% Zn removal was still being achieved in the second reactor.

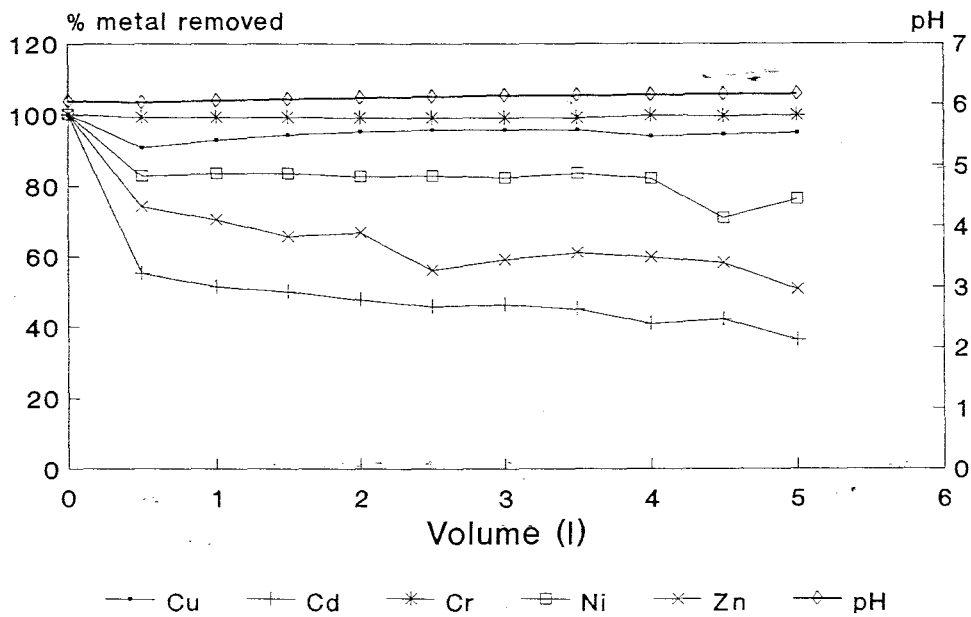


Fig 7.7. Biomass saturation and resultant breakthrough volume of tank 1 in a dual tank system. Depending on the influent concentrations the breakthrough volume of this tank was established between 4.5 - 5.0 l.

### 7.3.2.3. PROCESS SCALE UP

Process scale up involved the introduction of a third bioreactor into the bioremediation system. The first bioreactor was retained as the primary site for metal removal from the PST effluent, whilst the second tank removed the majority of the remaining cations from solution. The aim of a third tank was to provide a scavenging unit to remove the metal still in the water, thereby producing an eluant which complied with the water quality criteria.

The implementation of the third bioreactor within the system however had a minimal effect on the efficiency of metal removal and subsequently the total amount of metal removed (predominantly Cd and Zn) from the effluent (Fig 7.8 - 7.10). The majority of metal removal occurred in tank 1, whilst metal removal in tank 2 remained very similar to that attained by the second reactor in a dual reactor series. Fluctuations in the levels of Zn and Cd and to a lesser extent of Ni and Cu in tank 1 indicated the pattern of biomass saturation. The replacement with fresh biomass in tank 1 after every 5.0 l of influent solution resulted in an immediate increase in the amount of metal removal (Fig 7.8). A similar effect was noted in tank 2, where the replacement of the saturated biomass with fresh biomass after 22.0 l resulted in a 22% increase in Zn and 16% increase in Cd accumulation (Fig 7.9). Substitution of the biomass in tank 3 (result obtained from system 1, section 7.2.3.4.- not shown) had no effect on the metal removal in this tank. The trend of metal removal in tank 3 remained similar to that of the unsubstituted tank (Fig 7.10).

The efficiency of Zn and Cd accumulation by the first two tanks complemented each other (Fig 7.11 and 7.12). Zn and Cd accumulation in tank 2 was low when the biomass in tank 1 had been replaced with fresh material. Saturation of this biomass resulted in the biomass of tank 2 accumulating greater quantities of Cd and Zn. This phenomenon was more apparent for Cd accumulation (Fig 7.11) than for Zn removal (Fig 7.12). Replacement of the saturated biomass of tank 2 with that from tank 3 had only a marginal effect on the amount of Zn accumulated (the biomass in tank 3 was replaced with fresh biomass - results not shown). Ni removal within tank 2 was low, yet still remained within the stipulated criteria for drinking water (Fig 7.13). The nominal amount of metal accumulated in tank 3 reduced the feasibility of replacing the biomass in this tank. The most feasible option may be to utilize the biomass in tank 3 until the onset of saturation, whilst replacing the saturated biomass in tank 2 with fresh biomass.

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The implementation of a third tank in the process failed to produce waste water containing Cd and Zn within the stipulated drinking water criteria, though on average only 18% of the initial Zn and 17% of the initial Cd remained in solution after 40.0 l of the effluent had been processed. Although only 2 - 5% removal of Cu, Cr and Ni was achieved by tank 3, this was adequate for the concentration of these metals to fall within the drinking water standards. This decreased accumulation in tank 3 was thought to be a consequence of the change in the solution chemistry of the effluent. Exposure of the effluent to the biomass in the first two bioreactors resulted in an increase of eluant solution pH (>pH 7.5) and subsequently a change in metal chemistry and a decline in metal sorption. Extensive washing of the biomass contained in tank 2 and 3 with Milli-Q water prior to implementation in the system appeared to reduce the pH induced effect.

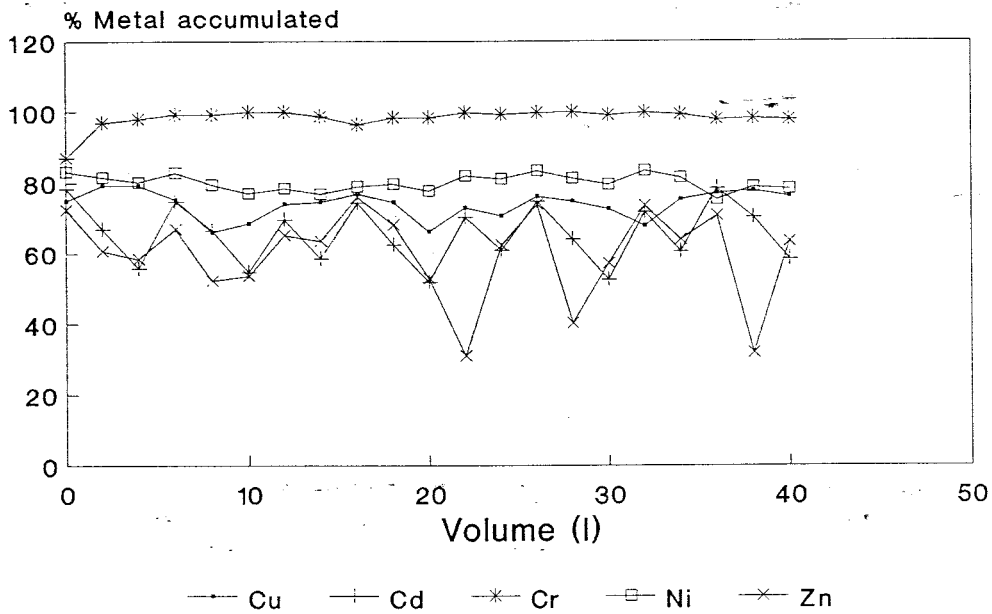


Fig 7.8. Metal accumulation from PST effluent by tank 1 (of a set of triple tanks linked in series). The % metal removed from solution fluctuated depending on the extent of biomass saturation and subsequent replacement with fresh biomass (after every 5.0 l of effluent processed).

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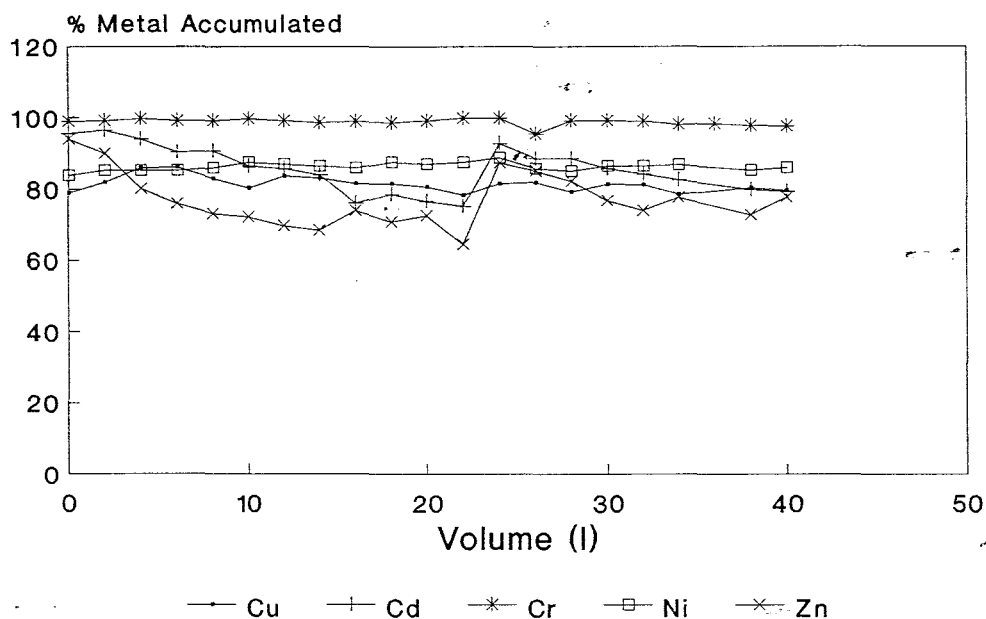


Fig 7.9. Metal removal from PST effluent in tank 2 (triple tanks linked in series). Note the increase in Zn and Cd removal in the presence of fresh biomass (22.0 l).

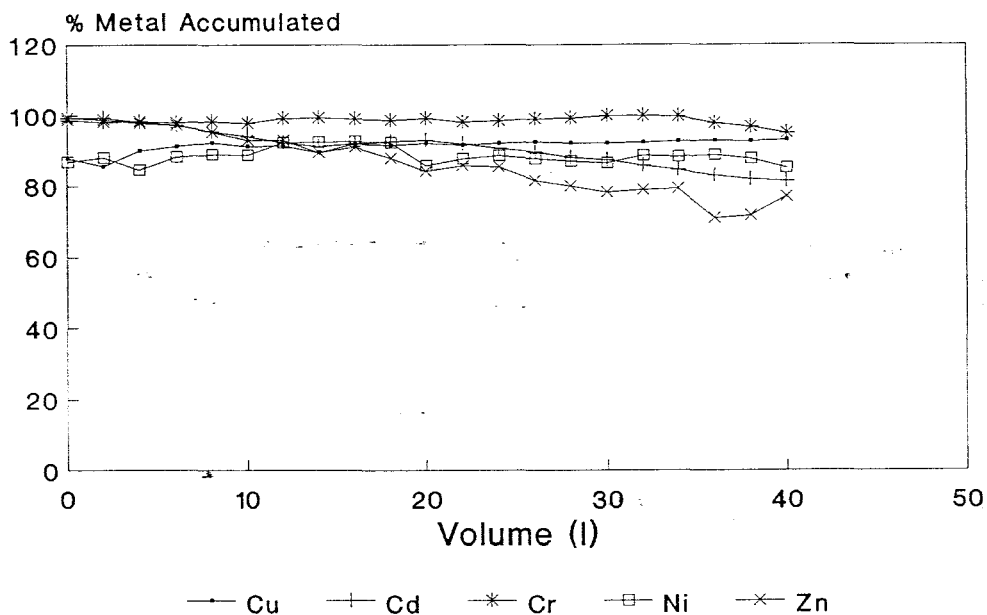


Fig 7.10. Metal removal from PST effluent in tank 3 (triple tanks linked in series). No biomass substitution occurred in the tank.

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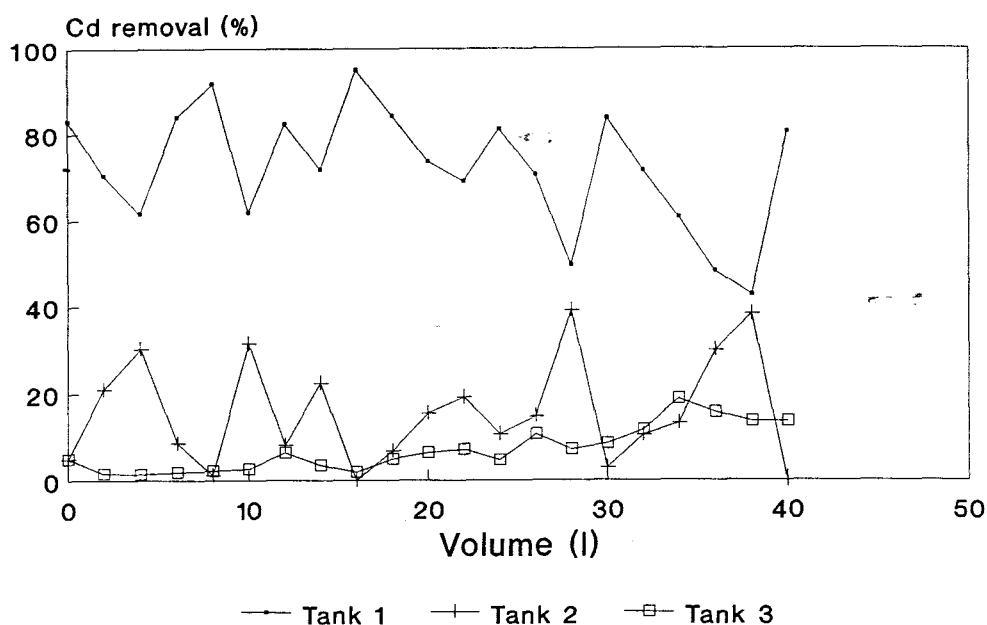


Fig 7. 11. Comparison of the efficiency of Cd removal from the initial influent PST solution by each of the bioreactor tanks. The concentration of Cd in the eluant of the tanks 1,2 and 3 after 40.0 l of PST had been processed was: 19.21 mg/l (tank 1), 9.62 mg/l (tank 2) and 8.64 mg/l (tank 3).

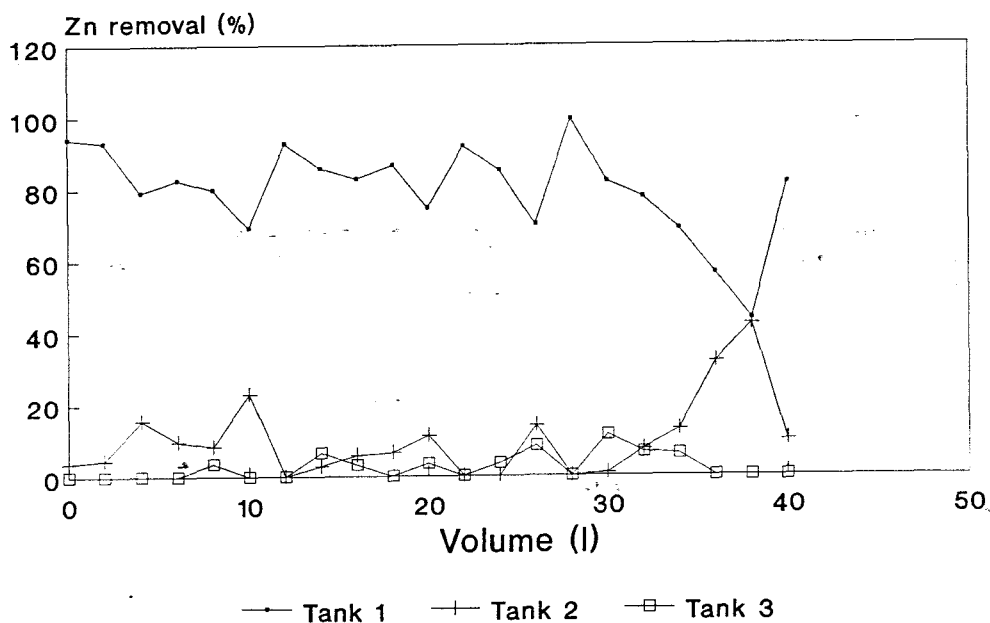


Fig 7.12. Comparison of the efficiency of Zn removal from the initial influent PST solution by each of the bioreactor tanks. The concentration of Zn in the eluant from tanks 1,2 and 3 after 40.0 l of PST had been processed was: 279 mg/l (tank 1), 170 mg/l (tank 2) and 164 mg/l (tank 3).

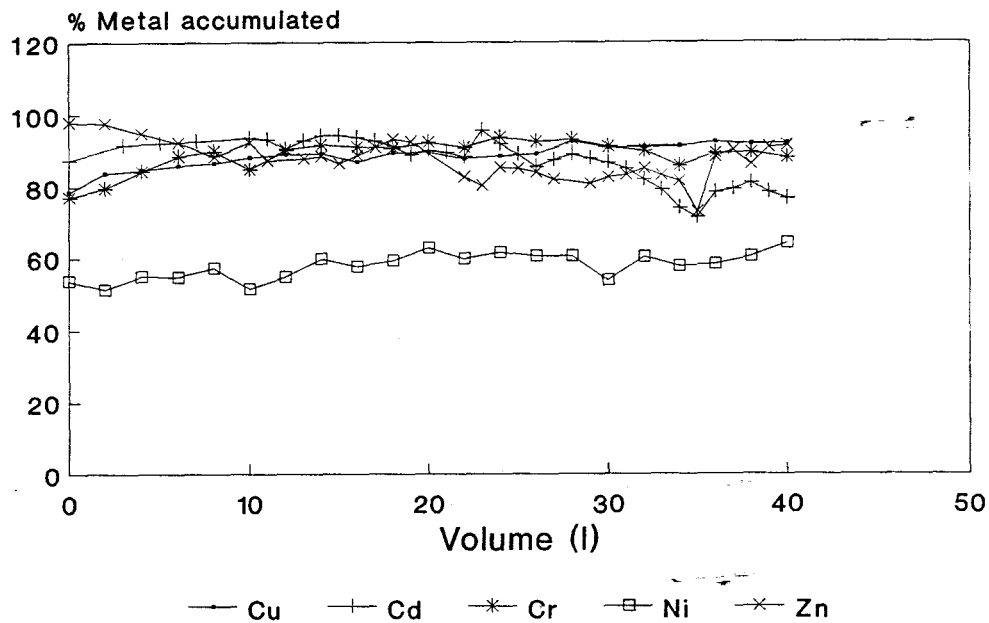


Fig 7.13. Comparative metal accumulation from PST effluent in tank 2 (triple tanks linked in series). The biomass in tank 2 was replaced by that from tank 3 after 22.0 l.

#### 7.4. DISCUSSION

Though chemical precipitation has remained one of the most widely used processes for the removal of metal contaminants from waste waters, depending on the initial metal concentration it is not always capable of achieving sufficiently low levels of metal in the effluent (144). Similarly most biosorbents have only been effectively applied in the treatment of low-concentration, high-volume metal containing waste water (202). Subsequently when faced with high-concentration, low-volume metal containing waste water, eg electroplating effluent the problem is compounded. The implementation of a combined biphasic metal removal system, consisting of primary phase chemical modification followed by a secondary phase bioremediation process offers advantages to both systems.

The results from this study indicate that the primary phase involving the chemical modification of

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The results from this study indicate that the primary phase involving the chemical modification of the effluent serves a dual purpose. The addition of KOH to the solution results not only in the precipitation of excess metal ions, but as a result of the low initial pH of the effluent, it serves to prime the effluent for the secondary bioremediation phase. A favourable pH for metal biosorption by PEI:GA biomass occurs between pH 4.5 - 6.0 (see chapter 6). At these pH levels the solubility of the metals decreases and they precipitate out predominantly as insoluble hydroxides, thereby presenting a more dilute solution for treatment. Though both PST and Raw effluent can be treated with alkali to decrease levels of the metal in solution, greater amounts of metal are removed from the Raw effluent by the settling tanks than by KOH. Biphasic treatment of PST in an industrial scheme therefore remains preferable to using modified Raw-effluent.

Two alternatives can be considered in the implementation of the secondary stages of the bioremediation process, *viz.* continuous-flow fixed bed reactors or continuous-flow stirred tank reactors. Both are application orientated systems, capable of accumulating metal ions from solution, yet differ with respect to mode of action and performance. Small-scale fixed bed reactors allow efficient utilization of the sorbent, due to the improved flow of the effluent through the columns. Using these reactors, the efficiency of metal removal in this study largely depended on the chemistry of the influent solutions and the fixed-bed characteristics. Metal removal from acidic effluent solutions was poor with breakthrough occurring at low volumes, whilst low efficiency can also be attributed to loosely packed columns. Correct packing of the column ensures even dispersion of the effluent solution throughout the column. To increase the amount of metal removed from aqueous systems two possibilities can be considered, *ie.* the introduction of a second column into the circuit or secondly, process scale-up. Depending on the nature of the influent solution the introduction of a dual column system can increase the total amount of metal removed from the effluent, though the operational efficiency of the second column may be less than the original column, or in some instances may have no additional effect on removal of a particular metal.

Process scale-up of fixed bed reactor systems did not provide the most effective solution to waste water treatment. Increasing the bed depth of the column by increasing the amount of biomass contained within the column extended the breakthrough volume of the column. Although the breakthrough volume is dependent on the amount of biomass contained therein, the operating efficiency of these columns was extremely low, and was not proportionally increased to that of the smaller scale columns. Compacting and channelling of the biomass occurred within the larger

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bioreactor columns. Both of these conditions decrease the available surface area of the biosorbent, thereby affecting the ratio of binding ligands : metal ions, which negatively affects the sorption kinetics. In addition, compacting of the biomass causes build-up of counter pressure within the column. To minimize the effect of compacting and channelling, columns of short bed depths with lowered contact time could be utilized.

The alternative to fixed-bed continuous-flow systems, *ie.* continuous-flow stirred tank reactors were more suited to larger scale processes due to the simplicity of the system and the ease with which scale up was achieved. Although scale-up within this system resulted in a slight decline in the overall efficiency of metal removal occurring, this decline is negligible when compared to the efficiency of scale-up of the previous system. Greater efficiency of metal removal is achieved through operating the multiple bioreactors in series at lower operating capacities (5.0 l) in preference to a single bioreactor at maximum capacity (7.5 l). A circuit containing dual bioreactors, operating at 5.0 l capacity and linked in series is more efficient with regard to metal removal than a circuit containing triple bioreactors operating at the same volume. Sorption of the majority of the metals occurs in the first two bioreactors with the third tank appearing to be incapable of removing the remainder of the residual Zn and Cd. A second tank, operating at maximum capacity (7.5 l, 300 g biomass (dry wt)) remains a possibility instead of coupling a third bioreactor to the system.

The majority of metal sorption occurs in the first bioreactor though the onset of biomass saturation takes place at low eluant volumes (4.5 - 5.0 l). Immediate replacement of the saturated biomass with fresh material extends the lifespan of the second bioreactor, resulting in the 200 g (dry wt) of PEI:GA biomass in the bioreactor being capable of treating 22 - 25 l of effluent prior to breakthrough occurring. Though the eluant produced by a dual reactor circuit contained levels of Cu, Cr and Ni within the required levels, the amounts of Cd and Zn remained in excess of required standards for both dual and triple bioreactor systems *ie.* 171 and 174 mg/l for Zn and 8.65 and 17.2 mg/l for Cd. The inability of the unsaturated biomass in the second and third bioreactors to remove significant amounts of metals once again appears to originate from a pH change and change in the solution chemistry of the effluent. Overcoming these problems requires the pH of the reactor eluants to be maintained within the optimum adsorption range of the PEI:GA biomass (*viz.* pH 4.5 - 6.0), or alternatively the implementation of a third type of phase within the circuit such as the use of another type of biosorbent in the system.

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Though both of the secondary phase bioremediation systems are capable of removing metal ions from electroplating waste solutions, several factors define their applicability on an industrial scale. Prior to implementation of either of these systems, the economic feasibility of the initial outlay and subsequent maintenance costs has to be assessed. The cost of implementing these systems will have to be compared to the operating efficiency and percentage metal removal attained. The efficiency of metal removal by small scale fixed-bed continuous flow reactor columns exceeds that of stirred continuous flow tanks, yet several limitations regarding the biomass characteristics (eg. compacting and channelling within the columns) and column parameters (low flow rate, long contact periods) negatively influenced the metal removal in columns from larger volumes. In contrast continuous-flow stirred bioreactor tanks can operate efficiently under a wide range of conditions. Due to the short contact time required for maximum metal-biomass interaction, the flow rate of these systems can be altered without negatively influencing the biomass sorption characteristics. Scale-up of the bioreactor tanks is easily facilitated with a minimal loss of efficiency of metal removal. An additional advantage of stirred reactor tanks is the short contact time required for metal sorption to occur, with the majority of metals being removed within the first 10 min. Moreover constant exposure of the biomass to the effluent solution and resultant increased biosorption is achieved by constant stirring of the biomass-effluent solution, although the cost of providing stirrers and their operation will need to be taken into account.

Metal ion removal from PEI:GA biomass can be achieved through effective desorption methods, eg. EDTA (see chapter 6), though due to environmental considerations mild acidic solutions, eg. inorganic acids (HCl) or organic acids are predominantly used. The desorbed biomass can be readily regenerated and utilized effectively in bioreactor tanks. Both packed bed and stirred continuous flow reactor tanks would be able to utilize this regenerated biomass, especially in scavenger bioreactors.

### 7.5. CONCLUSIONS

While the metal accumulating ability of PEI:GA biomass had already been proven, its efficiency as an industrial applicant remained unknown. Successful metal biosorption was achieved from high-concentration solutions in the present study, though the efficiency of metal removal improved when PEI:GA biosorption was implemented as part of a biphasic and not a single phase remediation system. The initial phase requires chemical treatment in order to precipitate excess metals out of solution and

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to prime the effluent for bioremediation. Although alkaline solutions (KOH) have been used in this study, low- or metal free industrial waste solutions may also be used as priming agents in larger scale pilot plants, thereby minimizing costs.

The industrial applicability of the two secondary phase biological systems, *viz.* continuous-flow fixed bed and continuous-flow stirred tank reactors, vary. Due to the long contact time and deep bed depths of the fixed beds, column implementation on a large scale appears to be non-viable. Though the efficiency of the system may be improved by using several shorter columns with a low contact time in series, this will increase the labour intensity of the system.

The implementation of continuous-flow stirred tanks in a large scale system has several advantages. Due to the largely unsophisticated nature of waste treatment systems in most factories the design of the stirred tanks were kept as simplistic as possible, whilst striving to attain efficient metal removal. Single or multiple reactors can be used depending on the metal content and nature of the effluent. In the presence of high-concentration effluents, dual reactors are required for efficient biosorption, with the first bioreactor serving as the prime metal removal site and the subsequent bioreactor(s) scavenging the remainder of the metals.

The implementation of such a system, *ie.* biphasic continuous-flow stirred bioreactors on an industrial scale shows potential especially for the treatment of low volume effluents. Such a system would involve the production of smaller, more cost effective bioreactors, which could be installed and operated on-site. In addition desorption, reconditioning and reuse of the biomass would limit the operational costs of such a plant.

Though only operated on a laboratory scale at this stage, the results obtained from the present study confirm the potential of using microbial matter as biosorbents for bioremediation. This study is one of the first in South Africa to use bioremediation for the removal of heavy metals from high-concentration, low-volume electroplating waste water. In comparison to many of the chemical agents used for waste water purification, the PEI:GA biomass is relatively cheap to produce. The cost of immobilizing 10 kg of commercially purchased biomass varies between R 300 - 350 (approximately \$75 - 80, 1995 prices) compared to an estimated price for ion exchange resins of \$ 300 - 400/10 kg. The cost effectiveness of such a system will be further increased through desorbing the metals from the biomass and reconditioning and reusing the biomass.

## 8. GENERAL DISCUSSION AND CONCLUSIONS

### 8.1. BIOACCUMULATION

Accumulation of heavy metals is an attribute shared by yeast and many other microbial cells. This concept was explored in the present study where metal binding experiments were performed using *S. cerevisiae* in an attempt to establish the cellular responses to heavy metal ions and the mechanisms of interaction between the metal cations and various components of yeast cells. Waste water often contains several different metal ions and the development and optimization of a bioaccumulation process for the treatment of metal containing effluent requires a comprehensive understanding of the mechanisms of metal accumulation.

From this study yeast cells have been shown to be capable of accumulating heavy metals under a wide range of ambient conditions. This uptake of heavy metals by yeast cells appears to be largely influenced by external factors, though it is also influenced by the inherent cellular characteristics. A relationship was established between intracellular metal ion accumulation and the ambient metal ion concentrations. The higher the concentration of the metal ions in solution, the greater the amount of metal accumulated by the yeast. The accumulation of heavy metals by yeast cells appears to be partially non-specific. Although the affinity of yeast-metal interactions varied depending on the respective species of metal ions, the affinity for, and the levels of individual heavy metal cations accumulated from multi-element solutions decreased, compared to the amount of metal accumulated from single ion solutions. Furthermore, the amount of accumulated metal is negatively influenced in the presence of unfavourable pH conditions. Accumulation from very acidic solutions (eg. pH 1.5) was negligible, whilst the amount of metal taken up increased with an increase in the alkalinity of the solution (approximately pH 6.0 - 7.0).

After an initial, rapid phase of accumulation, the rate of accumulation of metals declines over time. In viable cells the predominant mechanism of internalization is energy dependent. The addition and pretreatment of cells with an energy source, *viz.* glucose, results in enhanced metal uptake, whilst in the absence of an external energy source metal accumulation decreases after the utilization of the available energy source by the yeast cell.

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Initial yeast-metal contact occurs at the cell wall, followed by internalization and subsequent sequestration. Intracellular metal ion deposition was shown to occur in the soluble cytosol and insoluble vacuolar fractions. Though the primary site for intracellular deposition appears to be the vacuole, granular metal ion deposits did form within the cytosol, eg.  $\text{Cd}^{2+}$  and  $\text{Co}^{2+}$  depositions. The accumulation of heavy metal ions by *S. cerevisiae* cells elicited certain cellular responses. Exposure of the cells to, and the subsequent accumulation of  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$  by the cells triggered an ion-exchange phenomenon, whereby  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Mg}^{2+}$  (but not  $\text{Ca}^{2+}$ ) were eluted from the yeast cell interior (as shown by EDAX spectra). Not all heavy metal cations bring about this effect. For example,  $\text{Co}^{2+}$  uptake did not result in any change in the intracellular ion-profile of yeast cells. Moreover in the presence of all of these heavy metal cations, permeabilization of the plasma cell membrane occurs.

Two primary sites for cellular heavy metal ion deposition are the yeast cell wall and the vacuole. Based on the results from this study it appears that all of the cell wall components are responsible for metal accumulation, and that isolated cell wall components are better metal accumulators than intact cell walls. Accumulation appears to reflect the spatial arrangement of the cell wall. Mannan, the outermost cell wall polymer exhibited the highest metal binding capacity, whilst in contrast glucan binds relatively low amounts of metals. An apparent affinity sequence for metal accumulation exists of mannan > chitin > glucan > intact cell walls, though not all metals are accumulated to the same extent. The limited amounts of metal accumulated by intact yeast cell walls can be ascribed to spatial limitations, and masking of available binding sites by other cell wall components.

The storage capabilities of the second major metal accumulating organelle; viz. the yeast vacuole warrants further research regarding its accumulating and concentration capabilities of heavy metals from solution. While results from the present study have indicated that metal ions are concentrated within this organelle, the vacuole appears to have a limited, yet indefinite capacity for heavy metal storage. The amount of metal concentrated therein is dependent on the metal species. Metals were accumulated in the order of decreasing ionic size, ie.  $\text{Cu}^{2+} > \text{Co}^{2+} > \text{Cd}^{2+}$ . The majority of vacuolar metal ion accumulation occurred during the initial contact phase (within the first 30 min). Although the uptake mechanism for heavy metals into the vacuole is not clearly understood, according to several other reports the energy dependent  $\text{H}^+$ -ATPase translocation pathway involved in the uptake of amino acids and several other cations, appears to play an important role. The vacuolar ATPase differs from that of the plasma cell membrane and plant V-ATPase systems due to the fact

that some ATPase uncouplers, eg. DNP, are ineffective in inhibiting vacuolar metal uptake and translocation.

## 8.2. BIOSORPTION AND EFFLUENT TREATMENT

Though prior knowledge regarding the mechanisms of metal bioaccumulation by viable yeast cells is essential for bioremediation, it is not always feasible to use viable yeast cells in these bioremediation processes. The advantages of using non-viable yeast biomass for bioremediation are numerous, and the ability to recondition and reuse the biomass must be one of the most important.

One of the most effective methods of utilizing non-viable yeast biomass involves immobilization of the yeast cell, though it has remained essential to develop a method of immobilization which is both inexpensive compared to conventional metal removal techniques, and yet does not diminish the biosorptive capacity of the biomass. In addition such a system has to allow for complete metal recovery, as well as the recovery and reuse of the biomass.

In the present study three immobilized non-viable yeast systems were tested. While the three preparations *viz.* PVA Na-orthophosphate, PVA Na-alginate and hot alkali treated PEI:GA produced biomass, fulfilled the required mechanical and physical criteria, the superior metal accumulating ability of the alkali treated PEI:GA pellets determined their selection as metal biosorbent material.

Due to the nature of the PEI:GA pellets, this biomass appeared to preferentially accumulate metal from influent effluent solutions between pH 4.5 - 6.0. In the treatment of metal-containing waste water (using the PEI:GA biomass pellets) two types of bioremediation systems were implemented, *viz.* continuous-flow fixed bed reactors and continuous-flow stirred tank reactor systems. The designs of both of these systems have successfully been used for the treatment of waste water by other groups, yet due to the different nature of the effluent used in this study, *ie.* low-volume, high-concentration of metals, several adaptations to both systems were necessary. Depending on the metal concentration of the influent waste water effluent the treatment required thereof varied. Industrial waste water containing extremely high levels of heavy metals cannot be adequately treated by bioremediation processes alone. This type of effluent requires initial primary phase chemical treatment, not only to prime the effluent (eg. regarding pH optimum) for further treatment, but also to precipitate out excess metals.

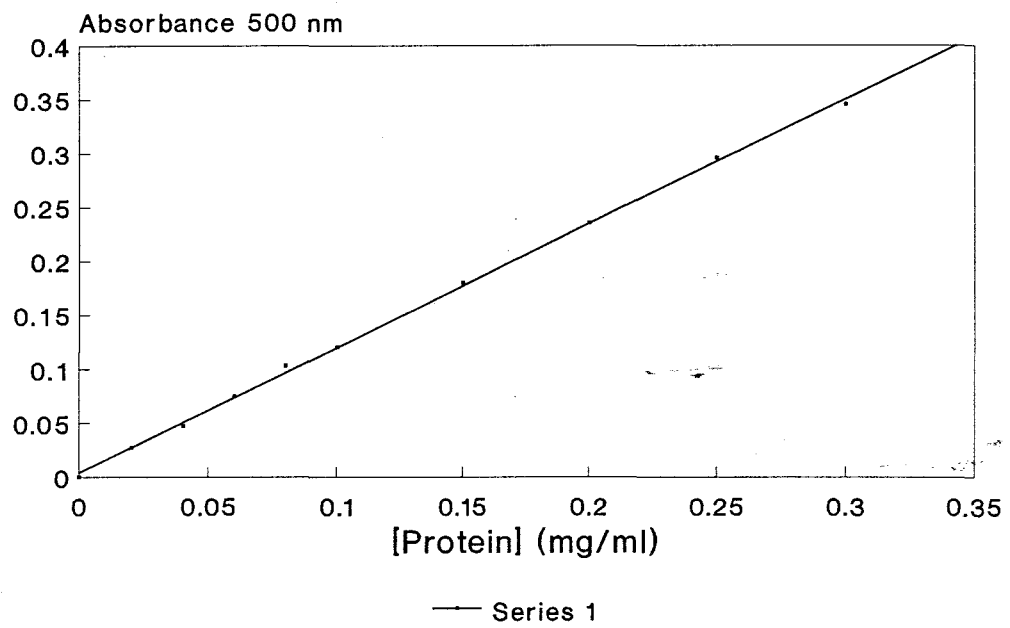
The potential applicability of the two types of secondary bioremediation systems in industry varies. The ideal system should be cost effective and simplistic, yet effective regarding metal removal and should be easily implemented on-site or at larger waste disposal collection sites. Based on these criteria it becomes apparent from the present study that the continuous-flow stirred bioreactor tanks possess greater industrial application potential than continuous-flow fixed bed reactors. Though fixed bed systems can be implemented in industrial systems, due to the nature of the columns several problems may be encountered. When using columns with a deep bed depth, the time required for the bioremediation process is extended. Columns containing short bed depths can be used, yet to achieve effective metal removal several of these will have to be implemented in series.

In contrast, efficient metal biosorption in stirred bioreactors occurs rapidly, thereby enabling a high flow rate to be maintained and larger volumes of the effluent to be processed. An additional advantage noted from the present study is the minimal loss of efficiency of metal removal on increasing the bioreactor capacity up to a certain point. Depending on the type of effluent being treated, the number and operational volume of the tanks used in such a system can vary.

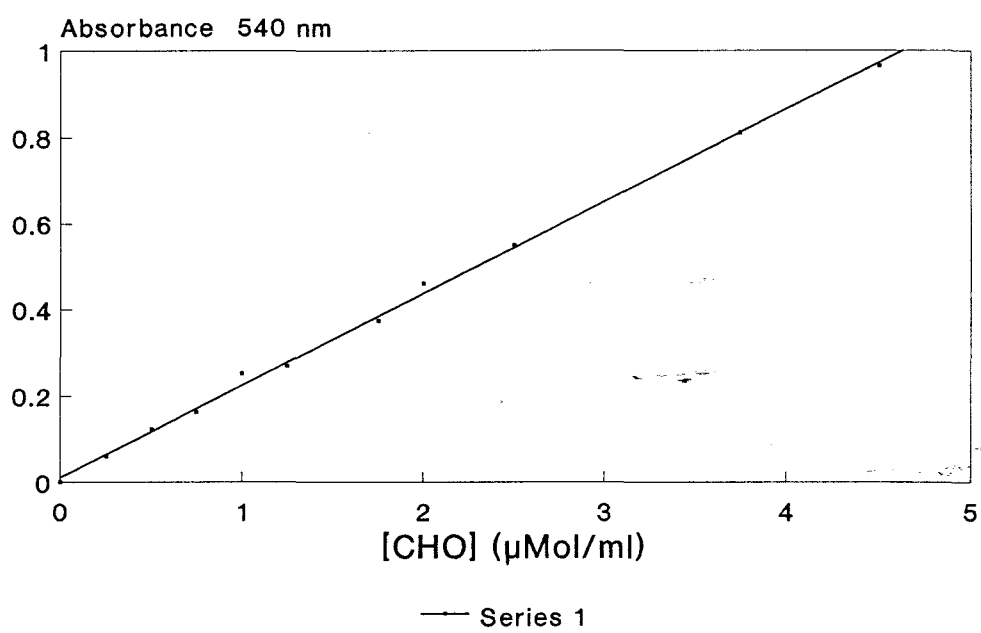
### 8.3. GENERAL COMMENTS

While the knowledge regarding the mechanisms of biosorption and accumulation by the yeast *S. cerevisiae* may not be complete, sufficient information exists to realize the potential of these cells as biosorbent agents. The implementation of biological systems in the place of conventional-waste water treatment processes requires the former to be a highly efficient, yet cost effective system. These biological systems must contain biosorbent material which has similar or improved metal accumulating abilities compared to the native biomass and must simultaneously allow for the effective recovery of the metal and regeneration and reuse of the biomass. As of yet the application of biosorbent technology is still in the trial stages with very few systems fully operational under pilot scale or industrial conditions, though several options are available for implementation eg. cross-flow microfiltration units or alternatively immobilized preparations. The bioremediation processes need not operate as complete systems, and a particular process can be designed for a specific application and need not be used as a general bioremediation tool.

## APPENDICES



APPENDIX 1. Protein standard curve



APPENDIX 2. Carbohydrate standard curve

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